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Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Submission to Microchemical Journal - manuscript number

1 message

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Manuscript Number: MICROC-D-22-02727
Application of validated and simple spectrophotometric method to quantify metformin in in vitro and ex vivo studies in hyperglycemic-mimicked conditions from glucose response microparticles loaded dissolving microneedles

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BUKTI REVIEW DARI REVIEWERS

Universitas Hasanuddin

Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Decision on submission to Microchemical Journal

1 message

Microchemical Journal <em@editorialmanager.com> Mon, Sep 26, 2022 at 1:42 PM
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Manuscript Number: MICROC-D-22-02727
Application of validated and simple spectrophotometric method to quantify metformin in in vitro and ex vivo studies in hyperglycemic-mimicked conditions from glucose response microparticles loaded dissolving microneedles

Dear Dr. Permana,

Thank you for submitting your manuscript to Microchemical Journal.

I have completed my evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Nov 25, 2022.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

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Microchemical Journal values your contribution and I look forward to receiving your revised manuscript.

Kind regards,

Miguel de la Guardia
Editor
Microchemical Journal

Editor and Reviewer comments:

Although reviewer 2 suggests separating Figure 5 into three figures to magnify them, a maximum number of five figures is mandatory for authors, as requested before. Therefore, I recommend the authors delete some figures or move them to supplementary materials.

Reviewer 1: The following issues should be addressed before accepting the manuscript:
1. I recommend shortening the title of the manuscript to be more concise and informative. In the paper, I have understood that you developed glucose-responsive microparticles containing MTF to be delivered by dissolving microneedles, followed by spectrophotometric analysis of MTF in this developed preparation. You must clarify this point.
2. There are different developed methods for the synthesis of metformin microparticles using different polymers such as poly(lactic acid). What is the superiority of these developed microparticles over the previously developed ones?
3. Chitosan microparticles/nanoparticles loaded with metformin were also reported. Please

4. Another thing else, you did not perform in vivo study. Do you think that spectrophotometric technique will be the best technique for pharmacokinetic studies? What about the possible interferences from plasma? In my opinion, HPLC will be more appropriate in such case.
5. Abstract: line 3: "resulting in the low.". There is a missed information here (low what?), therefore, I recommend rewriting this sentence for better understanding.
6. Correlation coefficient has the abbreviation (R) while determination coefficient has the abbreviation (R^2) which is R-square. Correct.
7. The manuscript should be revised carefully for language and grammatical errors. I found many language mistakes; therefore, I recommend revising it by English native speaker or a professional editing service.
8. References: The references should be revised according to the journal guidelines. The page numbers in many references are missed.

Reviewer 2: The paper describe both an interesting glucose-responsive formulation of metformin and a simple UV analytical method to study metformin release in PBS medium, therefore a lot of data (maybe too much) are provided.
The title and abstract suggest that the aim of paper is the validation of an analytical method, but at least half the content of paper focuses on microneedles preparation and in vitro and ex vivo studies.
The large amount of data makes it difficult to organize the results in tables and figures, and indeed figure 5 is too "crowded", since the results of different experiments are put it all together and release profile graphs are too small to be seen clearly.
I suggest to focus the paper on description, characterization and testing of glucose-responsive formulations, and to move a large part of UV analytical method validation in the supplementary material.

Further issues:
- Revise some sentences that don't make sense and proofread typing errors (i.e. "glucose responsive" and not "glucose response", "specificity" and not "specification", etc.).
- Check the right citation of tables and figures (i.e. figure 7 and tables 3, 4, 5 and 6 are cited in the text but are not provided).
- Split figure 5 in three different figures and enlarge the size of graphs.
- Revise carefully the references: ref 23 and 38 is the same, ref 26 and 29 is the same.

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Universitas Hasanuddin

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Confirming submission to Microchemical Journal

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Microchemical Journal <em@editorialmanager.com> Tue, Oct 4, 2022 at 6:28 PM
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Manuscript Number: MICROC-D-22-02727R1

Application of validated spectrophotometric method to quantify metformin in the development of glucose-responsive microparticles loaded dissolving microneedles

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Microchemical Journal

Application of validated spectrophotometric method to quantify metformin in the development of glucose-responsive microparticles loaded dissolving microneedles

--Manuscript Draft--

Manuscript Number:	MICROC-D-22-02727R1
Article Type:	Research Paper
Section/Category:	Molecular spectroscopy
Keywords:	Metformin; UV-Vis spectrophotometric; validation; Glucose-Responsive Microparticles; Dissolving Microneedles
Corresponding Author:	Andi Dian Permana INDONESIA
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Order of Authors:	Sumayya Binti Abd Azis
	Nur Syafika
	Hanin Azka Qonita
	Tiara Resky Anugrah Mahmud
	Ahmad Abizart
	Andi Dian Permana
Abstract:	Metformin (MTF) is a first-line drug in the treatment of type 2 diabetes mellitus. Delivered through the oral route, MTF has several limitations, mainly due to the side effects in gastrointestinal, non-specific release and low intestinal permeability, resulting in the low bioavailability of MTF in the body. Here, we developed glucose-responsive microparticles (GR-MP) containing MTF delivered via dissolving microneedles (DMNs) to overcome these limitations. To support the development of the formulation, in this study, a simple analytical method was developed using a UV-visible spectrophotometer. The method was validated in four different media, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose, to mimic the normal and hyperglycemic condition. The method was further validated as per International Conference Harmonization (ICH). This analytical method was applied to quantify the amount of MTF in the GR-MP preparation, in vitro release, drug content in DMNs and, importantly, ex vivo permeation study in in vitro hyperglycemic conditions. The results exhibited that the calibration curves in all media showed a correlation coefficient (R) of 0.998, indicating the linearity of the method. Moreover, LLOQ values in the four different media were 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL, and 2.88 µg/mL, respectively. Importantly, the method was precise and accurate with desired dilution integrity according to ICH, implying the validity of the methods. Finally, the method was successfully applied in the development of DMNs containing GR-MP of MTF, showing that the incorporation of MTF into this combination approach could selectively control the release of the drug according to the glucose concentration both in in vitro release and ex vivo permeation studies. Therefore, this approach could be a favorable system to solve the oral administration of MTF. Further in vivo analytical methods should now be developed to explore the effectiveness of this system in a suitable animal model.

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The Editor

Microchemical Journal

October 4, 2022

Dear Sir/Madam,

I wish you to re-consider our manuscript entitled **"Application of validated and simple spectrophotometric method to quantify metformin in *in vitro* and *ex vivo* studies in hyperglycemic-mimicked conditions from glucose response microparticles loaded dissolving microneedles"** for publication in ***Microchemical Journal.*** As we have revised our manuscript based on comments from reviewers, we changed our title to **"Application of validated spectrophotometric method to quantify metformin in the development of glucose-responsive microparticles loaded dissolving microneedles**"

We have made some changes to the manuscript as a result of these comments. We believe that the manuscript is now substantially improved. We have addressed each of the reviewers' comments in the response to the reviewer file. Importantly, we have made a great effort to improve the English and the discussion parts of our revised manuscript.

We believe that these findings will be of interest to scientists working on the development of analytical method validation, application of UV-Visible spectrophotometric analytical methods, and the use of glucose-responsive polymeric materials. This manuscript has not been previously published in any language anywhere and that it is not under simultaneous consideration by another journal. We appreciate your attention. We hope you will now consider publishing our research in ***Microchemical Journal*** and look forward to hearing from you in due course.

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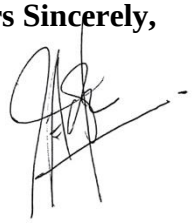
Yours Sincerely,



Andi Dian Permana (on behalf of all authors)

Faculty of Pharmacy

Hasanuddin University
Indonesia
Email: andi.dian.permana@farmasi.unhas.ac.id

Manuscript Number: MICROC-D-22-02727

Application of validated and simple spectrophotometric method to quantify metformin in in vitro and ex vivo studies in hyperglycemic-mimicked conditions from glucose response microparticles loaded dissolving microneedles

Response to reviewers:

We thank the expert reviewers who have taken the time to read and review our manuscripts, as well as provide very useful feedback in improving our manuscripts. We have made some changes to the manuscript in response to the feedback that has been given. We believe this manuscript is now substantially more developed.

We have addressed each of the reviewers' comments in detail below. Importantly, we have made a great effort to improve the English and the discussion parts of our revised manuscript.

Reviewer 1.

I recommend shortening the title of the manuscript to be more concise and informative. In the paper, I have understood that you developed glucose-responsive microparticles containing MTF to be delivered by dissolving microneedles, followed by spectrophotometric analysis of MTF in this developed preparation. You must clarify this point.

Response:

We thank the Reviewers for the suggestion. As a result, we have changed our title to be more concise and informative in the revised manuscript. The revised title is "Application of validated spectrophotometric method to quantify metformin in the development of glucose-responsive microparticles loaded dissolving microneedles".

There are different developed methods for the synthesis of metformin microparticles using different polymers such as poly(lactic acid). What is the superiority of these developed microparticles over the previously developed ones?

Response:

We thank the Reviewers for the suggestion and the very useful insight. We have included this in the revised manuscript, as follows:

In designing a controlled release form of drug delivery, the choice of polymer is one of the crucial things to consider [1]. There are many polymers that can be used in designing controlled release systems in the form of microparticles, one of which is a synthetic polymer in the form of poly(lactic) acid, which is a polymer with great potential, but controlling the particle size and drug adsorption efficiency of this polymer is quite difficult, and initial burst release may occur [2,3]. Another polymer that is usually used in the manufacture of microparticles is ethyl cellulose. However, the structure of ethyl cellulose which does not have a carboxyl group, makes PBA compounds unable to linked to gelatin polymers and form polymer complexes that are responsive to glucose [4].

Chitosan microparticles/nanoparticles loaded with metformin were also reported. Please

Response:

We thank Reviewers for the comment. Therefore, we have added more information in the revised manuscript, as follows:

Previously, several studies have been carried out in formulating metformin in the form of microparticles. One of them is research conducted by Avram et al 2017 who formulated MTF in the form of microparticles using a syringe technique using chitosan polymer [1,5]. However, the particles were not developed for selective delivery for hyperglycemic condition. Therefore, further development is required to selectively release MTF based on glucose concentration.

Another thing else, you did not perform in vivo study. Do you think that spectrophotometric technique will be the best technique for pharmacokinetic studies? What about the possible interferences from plasma? In my opinion, HPLC will be more appropriate in such case.

Response:

We thank the Reviewers for the valuable comment. It is true that in this research, we did not conduct an in vivo release study. In our opinion, in quantifying the amount of MTF from in vivo studies, HPLC instruments are currently preferred, considering the interference results from plasma. However, it is possible that MTF quantification in in vivo studies can also be carried out using a UV-Visible spectrophotometer.

The results of research conducted by Georgia et al., 2021 in quantifying irinotecan from human plasma using UV-vis spectrophotometric techniques show that this technique was still relevant and valid in analyzing drugs from blood plasma [6]. This has prompted us to carry out research in the future to ensure that the UV-vis spectrophotometry technique can also be used to quantify MTF from in vivo blood plasma assays from glucose-responsive microparticles delivered through the dissolvable microneedle system.

Abstract: line 3: "resulting in the low.". There is a missed information here (low what?), therefore, I recommend rewriting this sentence for better understanding.

Response:

We thank the reviewer for pointing this out, we have corrected that part in our revised manuscript, as follows:

Metformin (MTF) is a first-line drug in the treatment of type 2 diabetes mellitus. Delivered through the oral route, MTF has several limitations, mainly due to the side effects in gastrointestinal, non-specific release and low intestinal permeability, resulting in the low bioavailability of MTF in the body.

Correlation coefficient has the abbreviation (R) while determination coefficient has the abbreviation (R^2) which is R-square. Correct.

Response:

We thank the reviewer for pointing this out, we have made changes to the abbreviation of correlation coefficient (R) in our manuscript.

The manuscript should be revised carefully for language and grammatical errors. I found many language mistakes; therefore, I recommend revising it by English native speaker or a professional editing service.

Response:

We thank the Reviewer for the suggestion. We have re-read the manuscript and have made a great effort to improve the English throughout.

References: The references should be revised according to the journal guidelines. The page numbers in many references are missed.

Response:

We thank the reviewers for pointing this out, we have revised and completed the references section, in accordance with the journal guidelines.

Reviewer 2

The paper describe both an interesting glucose-responsive formulation of metformin and a simple UV analytical method to study metformin release in PBS medium, therefore a lot of data (maybe too much) are provided. The title and abstract suggest that the aim of paper is the validation of an analytical method, but at least half the content of paper focuses on microneedles preparation and in vitro and ex vivo studies.

Response:

We thank the reviewers for taking the time to review this manuscript, and providing very useful suggestions. As a result, we have moved several formulation development data to the supplementary materials.

The large amount of data makes it difficult to organize the results in tables and figures, and indeed figure 5 is too "crowded", since the results of different experiments are put it all together and release profile graphs are too small to be seen clearly. I suggest to focus the paper on description, characterization and testing of glucose-responsive formulations, and to move a large part of UV analytical method validation in the supplementary material.

Response:

We thank the reviewers for pointing this out. We have separated the results from Figure 5 into two Figures in our revised manuscript. We believe that the results can be seen clearly. Regarding the focus of the paper, as we aim to fulfill the scope of the journal, we validated the analytical method and applied the method in the formulation development. Several data have been moved to supplementary data to avoid too much data in our manuscript. We believe that data presented in the revised manuscript has reflected the title and the abstract.

Revise some sentences that don't make sense and proofread typing errors (i.e. "glucose responsive" and not "glucose response", "specificity" and not "specification", etc.).

Response:

We thank the reviewers for pointing this out, in our revised manuscript, we have corrected ambiguous-sounding words/sentences in our manuscript.

Check the right citation of tables and figures (i.e. figure 7 and tables 3, 4, 5 and 6 are cited in the text but are not provided).

Response:

We thank the reviewers for pointing this out, in our revised manuscript, we have corrected the table and figure citations, so that they are in accordance with what is written in the contents of the manuscript.

Split figure 5 in three different figures and enlarge the size of graphs.

Response:

We thank the reviewers for their suggestions. As we have reached the maximum number of figures, in the revised manuscript, we have separated figure 5 into two different images. We believe that the images can now be seen more clearly.

Revise carefully the references: ref 23 and 38 is the same, ref 26 and 29 is the same.

Response:

We are grateful that this has been pointed out, we have corrected the references on the manuscript according to the journal guidelines, and ensured that there are no duplicate references.

References

[1] M. Lengyel, N. Kállai-Szabó, V. Antal, A.J. Laki, I. Antal, Microparticles, microspheres, and microcapsules for advanced drug delivery, Sci Pharm. 87 (2019) 1–39. https://doi.org/10.3390/scipharm87030020.

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[6] G.E. Tsotsou, P. Gkotzamani, V. Petro, A. Argyropoulou, P. Karkalousos, A simple, rapid and low-cost spectrophotometric method for irinotecan quantification in human plasma and in pharmaceutical dosage forms, Analytical Methods. 13 (2021) 258–266. https://doi.org/10.1039/d0ay02201b.

Highlights

- Spectrophotometric method was developed to determine metformin in *in vitro* hyperglycemic-mimicked conditions
- The spectrophotometric method was validated as per ICH guidelines
- The spectrophotometric method was applied in the development of glucose response microparticles loaded dissolving microneedles

Application of validated and simple spectrophotometric method to quantify metformin in *in vitro* and *ex vivo* studies in hyperglycemic-mimicked conditions from glucose response microparticles loaded dissolving microneedles

Sumayya Binti Abd Azis[1], Nur Syafika[1], Hanin Azka Qonita[1], Tiara Resky Anugrah Mahmud[2], Ahmad Abizart[2], Andi Dian Permana[1*]

1. Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia
2. Faculty of Medicine, Hasanuddin University, Makassar, 90245, Indonesia

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Highlights

- Spectrophotometric method was developed to determine metformin in *in vitro* hyperglycemic-mimicked conditions
- The spectrophotometric method was validated as per ICH guidelines
- The Spectrophotometric method was applied in the development of glucose response microparticles loaded dissolving microneedles

ABSTRACT

Metformin (MTF) is a first-line drug in the treatment of type 2 diabetes mellitus. Delivered through the oral route, MTF has several limitations, mainly due to the side effects in gastrointestinal, non-specific release and low intestinal permeability, resulting in the low. Here, we developed glucose-responsive microparticles (GR-MP) containing MTF delivered *via* dissolving microneedles (DMNs) to overcome these limitations. To support the development of the formula, in this study, a simple analytical method was developed using a UV-visible spectrophotometer which was validated in four different media, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose to mimic the normal and hyperglycemic condition. The method was further validated as per International Conference Harmonization (ICH). This analytical method was applied to quantify the amount of MTF in the GR-MP preparation, *in vitro* release, drug content in DMNs and, importantly, *ex vivo* permeation study in *in vitro* hyperglycemic conditions. The results exhibited that the calibration curves in all media showed a correlation coefficient (R2) of 0.998, indicating the linearity of the method. Moreover, LLOQ values in the four different media were 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL, and 2.88 µg/mL, respectively. Importantly, the method was precise and accurate with desired dilution integrity according to ICH, implying the validity of the methods. Finally, the method was successfully applied in the development of DMNs containing GR-MP of MTF, showing that the incorporation of MTF into this combination approach could selectively control the release of the drug according to the glucose concentration both in *in vitro* release and *ex vivo* permeation studies. Therefore, this approach could be a favorable system to solve the oral administration of MTF. Further *in vivo* analytical methods should now be developed to explore the effectiveness of this system in a suitable animal model.

Keywords: Metformin, UV-Vis spectrophotometric, Validation, Glucose-Response Microparticles, Dissolving Microneedles

1. Introduction

One of the biggest causes of death in the world is diabetes mellitus (DM). According to data from the International Diabetes Federation, there are 537 million people with DM in 2021, and it is estimated to increase to 783 million in 2045. DM causes various complications that are the direct cause of 1.5 million deaths in the world in 2019. Specifically, approximately 90% of DM cases are type 2 DM (T2DM) [1].

The first-line treatment for T2DM is metformin (MTF) tablets administered orally. MTF has been shown to be most effective in lowering blood glucose levels. MTF works in lowering blood sugar levels through various mechanisms. Consequently, the use of MTF in high doses and in the long term can potentially increase the risk of hypoglycemia. Research shows that 112 out of 4072 cases of MTF overdose can trigger hypoglycemia, which could potentially lead to intolerance to MTF [2]. Other side effects associated with the oral MTF therapy can cause undesired impacts on the gastrointestinal tract. MTF also has low permeability to cell membranes and, therefore, the absorption of MTF given orally does not occur optimally [3]. This causes an increase in the accumulation of drugs in the intestines, resulting in some dangerous side effects [4].

To overcome these problems, it is crucial to design a smart delivery system to deliver MTF. Recently, the development of a glucose-response delivery system has attracted the interest of numerous researchers to selectively control the release of antidiabetic drugs. To the best of our knowledge, there has been no glucose response system developed for MTF. In this study, we presented microparticles with glucose response ability which could release MTF in the presence of glucose. Accordingly, this could be beneficial in preventing hypoglycemia [5]. Some compounds have been explored to possess this characteristic, including glucose oxidase (GOD), concanavalin A (Con A), and phenylboronic acid (PBA) [6]. Among these three compounds, the use of PBA is more frequent because it is lower cost, biodegradable, and easy to fabricate compared to GOD and Con A. Importantly, since PBA is not a protein like GOD and Con A, disadvantages such as poor volatile inactivation, and the high cost can be avoided [7].

In designing a controlled release form of drug delivery, the choice of polymer is one of the crucial things to consider. A polymer with numerous potentials is chitosan (CS). CS is an attractive polymer used in the formulation of pharmaceutical preparations because apart from being non-toxic, biodegradable, and biocompatible. This polymer also possesses unique physical and chemical characteristics such as intermolecular hydrogen bonds and its polycationic charge in

acidic conditions [8]. This leads us to the binding of the hydroxyl and amino groups present in the CS chain which has a strong affinity for PBA. This binding resulted in a decrease in the pKa value of PBA and led to the manufacture of pH-responsive compounds which has broad application prospects, causing PBA-CS compounds to achieve glucose sensitivity below the physiological pH of the human body [9].

As previously explained, the oral administration of MTF resulted in several side effects. As a result, modified transdermal delivery of MTF was chosen to overcome the side effects of MTF related to the low permeability of MTF to cell membranes, resulting in reduced bioavailability [10]. The choice of subcutaneous administration of antidiabetic drugs is commonly used, such as insulin and GLP-1 agonist drugs. Subcutaneous administration of drugs can certainly help overcome previous MTF problems, but new problems arise, such as discomfort to the patient, bleeding, infection at the injection site, and many more [11].

As an innovative delivery system, dissolving microneedles have been widely explored as an alternative delivery system to the injection route [12–14]. This system is applied intradermally which would dissolve when applied and release the active substances [15]. Therefore, the fabrication of DMN as a drug delivery system is an interesting solution for the oral therapy of MTF. The use of DMN as a drug delivery system not only solves the problems of administration using injection, but can also reduce sharp object waste after use, because the needle used can dissolve due to its fabrication using water-soluble polymers [16]. However, the use of the DMN system can have an impact on the difficulty of drug encapsulation, and the dose control. Therefore, the DMN system could be collaborated with GR-MP in order to obtain an efficient drug delivery system and controlled drug release [17]. In this study, MTF loaded-GR-MP was further incorporated into DMN for a selective and efficient delivery system in the *in vitro* mimicking diabetes environment.

In the development of new drug delivery system, various tests and characterizations are required. One of the critical points is analyzing the active compounds. In this study, with regard to the analysis process, MTF was analyzed in the development of GR-MP, *in vitro* testing *and ex viv*o permeation test. With respect to the media used, in this study phosphate buffered saline (PBS) and PBS containing glucose media represented normal and diabetes conditions. Previously, there were studies that carried out the determination of pure MTF and from tablets quantitatively using the reversed phase-high performance liquid chromatography (RP-HPLC) method [18] and Ultra

Violet (UV) spectrophotometer. However, the chromatographic method has drawbacks such as requiring large costs, a lot of solvents, reliable power, and expensive instruments [19]. This could limit the application of the analytical method in the several laboratories which do not have access to use the HPLC. These studies also analyzed the MTF of tablet dosage forms and did not use specific media.

Considering several aspects mentioned previously, in this study, an analytical method of MTF was developed from GR-MP-DMN preparations in PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose mediums using a UV-Vis spectrophotometer. This analytical method has been widely used in the determination and has proven to be an analytical method that is simple, easy, and provides precise results in determining the number of samples [20]. Importantly, the application of a UV-Vis spectrophotometer has been widely used in almost all scientific laboratories, making it as a versatile tool in the drug development. To ensure that the developed analytical method provides appropriate results, this study was also conducted involving the validation of the analytical method based on the International Conference Harmonization (ICH) guidelines. Method validation parameters such as linearity, accuracy, precision, limit of detection (LOD), and the determined limit of quantification (LOQ), and were extensively applied in the determination of entrapment efficiency and drug loading in MP, drug content in MN preparations, and *in vitro* and *ex vivo* permeation profiles.

2. Material and Methods

2.1 Materials

Metformin HCl was obtained from Tokyo Chemical Industry Co., LTD, Tokyo, Japan. Chitosan (medium molecular weight), glutaraldehyde polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), potassium dihydrogen phosphate (KH_2PO_4), glucose, potassium chloride (KCl), disodium phosphate (Na_2HPO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Singapore). All other reagents used in this study were analytical grade,

2.2 Preparation of PBS and PBS Containing Glucose

PBS was prepared by dissolving 0.2 g of KCl, 8 g of NaCl, 2.4 g of KH_2PO_4, and 1.44 g of Na_2HPO_4 with ± 800 mL CO_2-free water. Following the solubilization, the solution pH was set to 7.4. Finally, CO_2-free water was added to make up the final volume to 1 l with. To prepare PBS containing glucose media, glucose with concentrations of 1% w/v, 2% w/v and 4% w/v were dissolved using PBS.

2.3 Preparation of MTF Stock Solution

The stock solution was prepared by dissolving 10 mg of MTF into 10 mL of different media separately to achieve 1000 µg/mL of MTF solution.

2.4 Determination of Maximum UV Absorption, Preparation of Calibration Solution, and Quality Control Solution

Initially, the stock solution of MTF in the respective media was diluted to achieve a concentration of 20 µg/mL. The determination of the maximum UV absorption in MTF in all media was carried out using UV-vis spectrophotometer (Dynamica, HALO XB-10). Thereafter, the calibration solution in all media was prepared by diluting MTF stock solution using the respective media to achieve the serial concentrations of 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL and 0.5 µg/mL

Quality control solutions were prepared in three different concentrations, such as 12 µg/mL for high-quality control (HQC), 7.5 µg/mL for medium-quality control (MQC), and 4 µg/mL for low-quality control (LQC).

2.5 Validation Method

The UV-Vis spectrophotometer validation method was carried out by measuring the validation parameters, such as linearity, specificity, LOD and LOQ, dilution integrity as well as accuracy and precision.

2.5.1 Linearity

Determination of linearity in the method validation was carried out by plotting the absorbance of three replications of the MTF calibration solution in three different media. From the curve results obtained, the correlation coefficient (R^2) was calculated. Linear parameters are considered valid if the value of R is close to 1 [21].

2.5.2 Specificity

The specifications need to be known to ensure that there is no interference from other materials in the sample [21]. Specificity was determined by comparing the UV spectra of GR-MP blank, DMN blank, and MTF in both GR-MP and DMN system. The UV spectra was scanned between 200-400 nm.

2.5.3 Limit of Detection (LOD)

The detection limit (LOD) was investigated to determine the smallest amount of analyte that could show absorption or absorbance in the instrument without having accuracy and precision

criteria. LOD was calculated by using equation 1. In the equation, 3.3 represents the factor for LOD, SD is the standard deviation of the blank, and b is the slope of the blank regression line [22].

$$LOD = \frac{3.3\ x\ SD}{b} \quad \text{Equation 1}$$

2.5.4 Lower Limit of Quantification (LLOQ)

LOQ is the smallest amount of analyte that can still be measured for its absorbance using an instrument and has accuracy and precision criteria. LLOQ can be calculated by using equation 2. In the equation, 10 represents the factor for LLOQ, SD represents the standard deviation of the blank, and b represents the slope of the regression line [22].

$$LLOQ = \frac{10\ x\ SD}{b} \quad \text{Equation 2}$$

2.5.5 Accuracy

Accuracy is a parameter that shows the degree of closeness of the analysis results to the actual analyte content. Accuracy is expressed as the percent recovery of the added analyte. The accuracy test was carried out by comparing the MTF concentration in LLOQ, LQC, MQC and HQC solutions from the absorbance measurement results with the theoretical concentration, then the relative standard deviation (% RSD) was calculated. the %RSD value should not be more than 15% of the theoretical concentration [21]. Measurements were done intra-day and inter-day.

2.5.6 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision test was the same as in the previous accuracy test, where the concentrations of the absorbance measurement results of LLOQ, LQC, MQC, and HQC solutions were compared with the theoretical concentrations. The relative error value (%RE) was calculated and the results obtained should not be more than 15% of the coefficient of variation (CV) [23]Measurements were carried out intra-day and inter-day.

2.5.7 Dilution Integrity

Dilution integrity was carried out by preparing 75 µg/mL MTF in all media. Then each solution was diluted 5 and 10 times, the experiment was carried out in triplicate, and the absorbance of the analyte was observed [24].

3. Application

3.1 Microparticle Formulation

Microparticles were prepared using CS. In this study, 5 formulations were prepared containing 100 mg of MTF with different amounts of CS, namely 100 mg, 150 mg, 200 mg, 250 mg and 300 mg for F1, F2, F3, F4 and F5, respectively. GR-MTF-MP were prepared by mixing MTF and CS with 5 mg of EDTA, added to 3 mL of acetic acid solution in water (1% v/v) under stirring condition at 500 rpm at room temperature. After that, 6 mL of ethanol was added to make a cloudy solution which indicated the formation of MPs. After that, 50 µL of glutaraldehyde (25%) solution were added as a crosslinker by forming a reaction between the aldehyde group and the amino group of the MP. Furthermore, the MP formed were centrifuged at 3000 rpm for 20 minutes and the sediment obtained was washed using distilled water to obtain pure MP CS [25].

To prepare GR-MP, PBA solution (11.2 mg) was dissolved in 1 mL of DMSO, reacted with EDC.HCl (15.5 mg) and NHS (9.3 mg) for 30 minutes (mixture 1). After that, the mixture 1 solution was added to 5 mL of MP CS solution, while stirring at 37˚C for 24 hours. Then, the PBA-decorated MP CS (MP PBA-CS) was dialyzed in distilled water for 48 h to remove unreacted PBA [25]. MP CS containing MTF was referred to as MP CS-MTF and PBA-CS MPs containing MTF were referred to as MP PBA-CS-MTF. Particle size and polydispersity index (PDI) were all calculated.

3.2 Determination of Entrapment Efficiency and Drug Loading

The entrapment efficiency (EE) of MTF in MP was determined using indirect method. In the washing steps, the supernatant was taken and the concentration of MTF was calculated using validated analytical method. Furthermore, the drug loading (DL) determination was carried out by mixing 50 mg of the formulation with 10 methanol. The mixture was sonicated for 30 minutes and diluted with PBS. ED and DL were calculated using the following calculations [26]:

$$\%EE = \frac{(Weight\ of\ initial\ drug - Weight\ of\ free\ drug)}{Weight\ of\ initial\ drug}\ x\ 100 \qquad \text{Equation 3}$$

$$\%DL = \frac{Amount\ of\ entrapped\ drug\ in\ microparticle}{Total\ weight\ of\ microparticle}\ x\ 100 \qquad \text{Equation 4}$$

3.3 *In vitro* Release Test

The *in vitro* release profile of MTF from MP was investigated using dialysis membrane method [13,27,28]. Briefly, MP formulations equal to 10 mg of MTF was placed inside dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO dialysis membrane). The membrane was further immersed into 100 mL of release media. Three different media were used, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose. The study was carried out in an orbital shaker at 100 rpm at 37°C. The media (1 mL) was sampled at certain time intervals and then the concentration of MTF was determined using a UV-Vis spectrophotometer. Fresh media was added after the sampling to ensure the sink condition during the study. The drug release mechanism was then analyzed using a variation of the mathematical kinetic model [29]

3.4 Mathematical Modelling for *In Vitro* Release Test

The data obtained from the *in vitro* assays were then fitted into five different mathematical models to determine the release kinetics of MTF from MP. The models applied were zero-order kinetics (Z0), first-order kinetics (F0), Krosmeyer-Peppas (KP), Higuchi, and Hixson-Crowell (HC). The equations of each model are described below:

$$Zero\ order\ kinetics: C_t = C_0 + K_0 t$$

$$First\ order\ kinetics: lnC_t = lnC_0 + k_1 t$$

$$Krosmeyer - Peppas\ model: C_t = k_{KP} t^n$$

$$Higuchi\ model: C_t = k_H \sqrt{t}$$

$$Hixson - Crowell\ model: C_t^{\frac{1}{3}} = C_0^{\frac{1}{3}} k_{HC} t$$

C_t represents the concentration of MTF at time t, C_0 represents the initial concentration of MTF in the medium (t = 0), k_0 represents the zero-order constant, k1 represents the first-order constant, k_{KP} represents the Korsmeyer–Peppas constant, k_H represents the Higuchi constant, and k_{HC} represents the Hixson constant. - Crowell. All calculations were performed using the DD-solver software. The release kinetics is determined from the correlation coefficient value (r2) obtained [24].

3.5 DMN Fabrication and Determination of Drug Content

In this study, two-layered DMNs containing MP CS-MTF and MP PBA-CS-MTF were fabricated using centrifugation method [30]. The formulation contained the aqueous gel of 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa) in distilled water mixed with 30% w/w

of MP. Initially, 100 mg formulation was poured on the top of DMN MN silicon mould (needle density 10 x 10, pyramidal needle with 700 μm of high and 200 μm wide on the base and 200 μm of spacing). Thereafter, the mold was centrifuged at 3000 rpm for 15 min at room temperature. The excess of the formulation was removed and dried for 6 h. Following this, an aqueous gel containing 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa) was poured as a second layer. The formulation was dried at room temperature for 24 h and removed from the mould. It was important to note that the DMNs used in this study possessed adequate mechanical and insertion properties.

In an attempt to measure the MTF content in DMNs, the formulation was initially dispersed in 5 mL of distilled water. Afterwards, the dispersion was mixed with 10 mL of methanol and sonicated for 30 min. The mixture was then centrifuged for 10 minutes at a speed of 5000 rpm. The supernatant was collected, and the absorbance was measured using the UV-Vis spectrum.

3.6 *Ex Vivo* Permeation Studies

Ex vivo permeation studies were performed using Franz diffusion cells, using rat skin [31–33]. PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose were used as the release medium. Prior to the experiment, skin was washed and soaked in the release medium for 30 minutes. Afterwards, the surface of the skin was dried and the skin was placed between the donor and recipient compartments. The experiment was conducted at 100 rpm at 37°C. During the study, the sampling was carried out at several time intervals, starting from 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours by taking 1 mL in the receiving compartment, then replaced with same volume of new media. The samples were then analyzed using UV-Vis spectrophotometry.

3.7 Statistical analysis

All data obtained were expressed in mean ± standard deviation (SD), all values were obtained using Microsoft excel® 2019 software (Microsoft Corporation, Redmond, USA). Graphpad Prism® version 6 (GraphPad Software, San Diego, California, USA) was used to analyze the data statistically, where statistical significance was indicated by p value < 0.05.

4. Results and Discussion

4.1 Validation of analytical method

4.1.1 Determination of Maximum UV Absorption and Specificity

The purpose of this study was to validate the UV-vis spectrophotometer as an instrument that allow the quantification of MTF in the development of a new dosage form, namely in the form of GR incorporated into DMN. Measurement of MTF levels was carried out in PBS media as a model medium for normal condition and PBS containing different concentration of glucose media as a model medium for hyperglycemia condition [34]. The results showed that MTF exhibited maximum absorption at 234 nm in all (Figure 1). Accordingly, the wavelength was used in the further steps in this study.

Specificity parameter was intended to ensure that the MTF analysis using a UV-Vis spectrophotometer from the MP and DMN formulations did not experience the interference from other compounds. As shown in Figure 2, the measurement results of the blank MP and MN showed a peak in the range of 210-220 nm, and did not indicate a possible interference at the MTF peak at 234 nm. In addition, the MTF peaks in both the MP and DMN formulas showed the same peaks as the pure MTF solution, which indicated that there was no peak shift due to additives or solvents used in the formulation. Therefore, the method developed in this study has been specific to the appropriate wavelength.

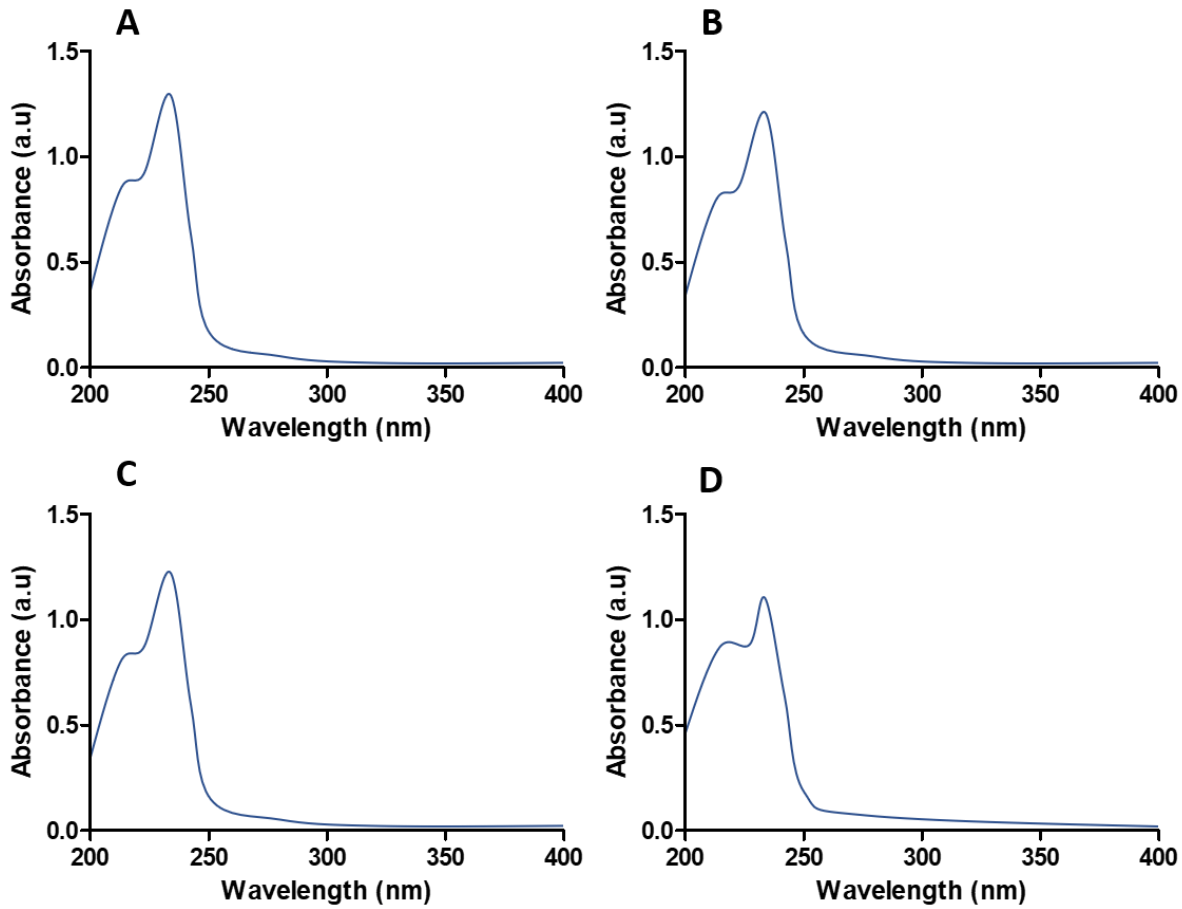

Figure 1. Maximum absorbance of MTF in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

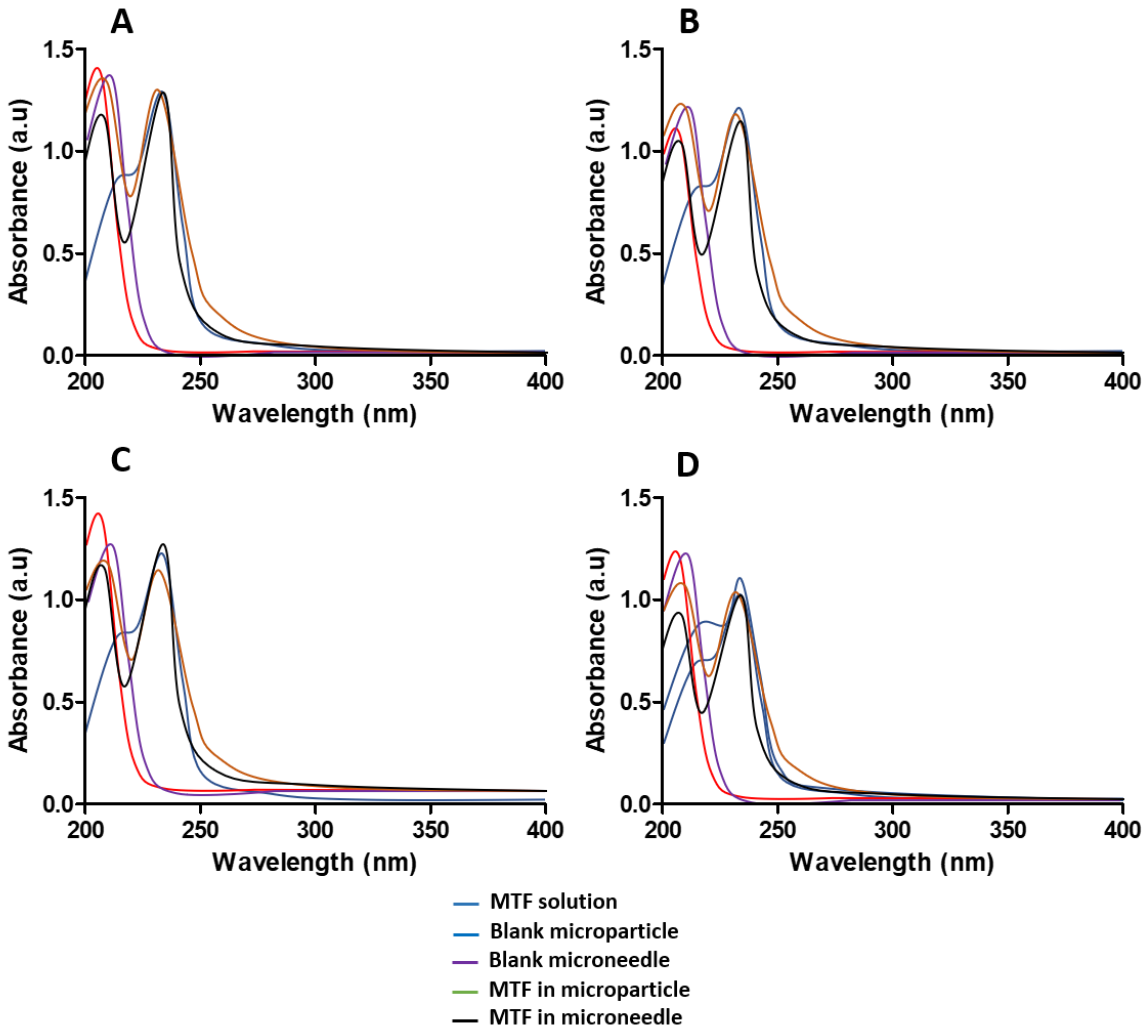

Fig 2. Representative UV-Spectra of pure MTF, blank MP, blank DMN, MTF in MP, and MTF in DMN in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

4.1.2 Linearity, LOD and LOQ

Linearity parameter is one of the characteristics required in the validation process of an analytical method. Based on the ICH guidelines, linearity needs to be evaluated by plotting a function of analyte concentration with the absorbance results obtained, then evaluated by mathematical modeling. Determination of linearity parameters from the validation of this method was carried out by measuring the absorbance of MTF in the concentration range of 0.5-16 µg/mL in all media. The spectrum of MTF in all media tested in various concentrations are depicted in Figure 3. The linearity acceptance criteria for active pharmaceutical ingredients is $R > 0.998$ [35]. The results of the linearity measurement of MTF showed the correlation coefficient valued of

0.9983 for PBS media, 0.9991 for PBS containing 1% w/v of glucose media, 0.9991 for PBS containing 2% w/v of glucose media, and 0.9981 for PBS containing 1% w/v of glucose media which of these four values met the criteria for linearity parameters.

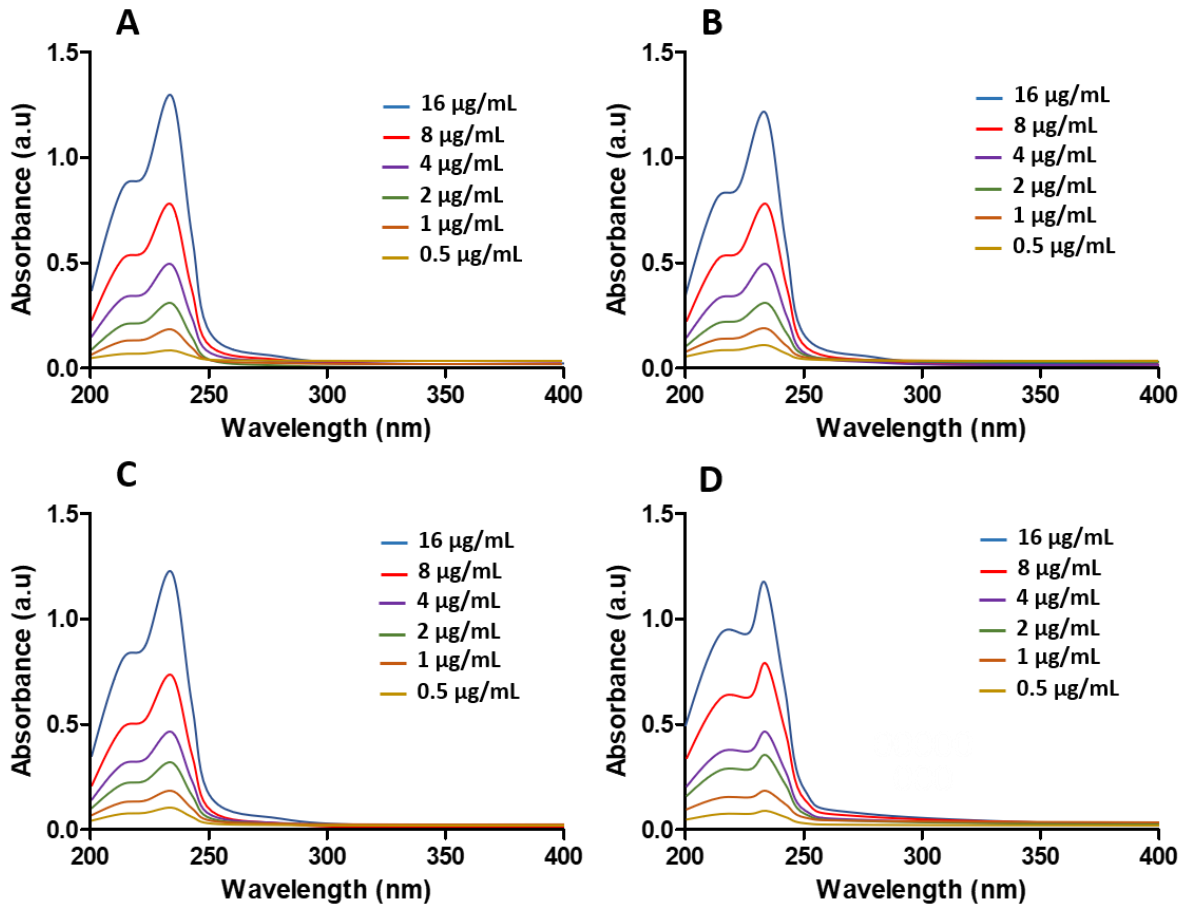

Figure 3. Spectrum of MTF standard solution in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

The LOD and LLOQ values in all media were calculated from the calibration data, as shown in Table 1. The LOD MTF values in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media were found to be 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL and 2.88 µg/mL respectively. LLOQ MTF values PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose were calculated as 0.74 µg/mL, 0.64 µg/mL, 0.64 µg/mL, and 0.95 µg/mL respectively.

Table 1. The calibration curve properties of MTF in different media with LOD and LOQ values of MTF

Media	Concentration range (µg/mL)	R^2	LOD (µg/mL)	LLOQ (µg/mL)
PBS	0.5-16	0.9989	0.74	2.23
PBS with 1% glucose	0.5-16	0.9991	0.64	1.95
PBS with 2% glucose	0.5-16	0.9991	0.64	1.94
PBS with 4% glucose	0.5-16	0.9981	0.95	2.88

4.1.3 Precision and Accuracy

Precision and accuracy were assessed for intra-day and inter-day. Intra-day determination was carried out to evaluate the repeatability of this analytical method whereas inter-day determination was conducted to investigate the variation of the day to the measurement. Precision and accuracy were analyzed in four different concentrations consisting of QC sample (HQC, MQC, and LQC) and LOQ sample which were measured in three replications respectively in one day for inter-day determination and three replications in three days for intra-day determination.

Precision was aimed to evaluate the closeness between a series of measurements from the homogenous sample under the same condition. The results were presented as %RSD values and are shown in Table S1, Table S2, Table S3, and Table S4 for PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media, respectively. It was found that all of the %RSD values were less than 15% and, thus, fulfilled the requirement from ICH [36–38]. As the analytical method was considered to be precise when %RSD is less 15%, this UV-Vis spectrophotometry method was therefore considered to have precision values to quantify MTF in all media used in this study.

Accuracy was carried out to evaluate the closeness between true value and value found from measurements. The accuracy values were reported as %RE. Table 3, Table 4, Table 5, and Table 6 show the accuracy of UV-Vis spectrophotometry method for MTF in for PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media, respectively. The analytical method was considered to be

accurate when the %RE values are ± 15% [21]. All the values were found to between the values and, therefore, the analytical method was considered to be accurate.

4.1.4 Dilution Integrity

Dilution integrity is the ability rate of the dilution process performed during the validation process as accurate, precise, and reliable [21]. Based on the dilution integrity data obtained in the Table 2, it was found that the results showed the satisfactory results, where the dilution integrity bias on all media were less than 15%. As per the precision parameter observed from the %RSD with values ranging from 1.62% - 4.68%, this indicated that the dilution in this validation method was accurate, precise. and reliable. In addition, it also implied that the analysis using this method can still be carried out on MTF with concentrations higher than the upper range of the calibration standard by using the appropriate dilutions.

Table 2. Dilution integrity data of UV-Vis spectrophotometry method for MTF in all media (mean ± SD, *n*=3)

Media	Dilution tested	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	%RSD	%RE
PBS	10	7.5	8.26 ± 0.15	1.87	-0.39
	5	15	15.36 ± 0.51	3.30	0.31
PBS with 1% of glucose	10	7.5	7.28 ± 0.30	4.11	-1.62
	5	15	14.25 ± 0.52	3.64	3.45
PBS with 2% of glucose	10	7.5	7.75 ± 0.36	4.68	-0.11
	5	15	14.76 ± 0.71	4.79	3.47
PBS with 4% of glucose	10	7.5	7.49 ± 0.12	1.62	1.66
	5	15	15.09 ± 0.64	4.23	3.22

4.2 Application of the analytical method

4.2.1 Entrapment Efficiency and Drug Loading

Following the successful validation of the spectrophotometric method, it was applied to characterize the EE and the DL capacities of the MP. In this study, in an attempt to achieve optimum parameters, we investigated five different concentrations of CS. The results of the characterization are depicted in Figure 4.1. With regard to the particle size, it was found that the increase of CS concentration could increase the particle size of the formulation. It might be due to the increase of CS concentration resulted in higher viscosity of the medium, leading to the decrease

of the energy to break the droplet into smaller size. This phenomenon was also observed in numerous studies investigating the concentration of the polymer in size of micro/nanoparticles [39,40]. The results showed that the particle size of F1, F2, F3, F4 and F5 were 2.87 ± 0.21 µm, 3.09 ± 0.28 µm, 4.87 ± 0.31 µm, 7.09 ± 0.59 µm and 10.19 ± 0.92 µm, respectively. Regarding the PDI values, despite the difference in size, all formulations exhibited narrow distribution pattern. Furthermore, the EE evaluation results showed the improvement of EE values following the increment of the CS concentrations. Following the application of the validated method, it was calculated that EE values were 27.49 ± 3.12% for F1, 39.16 ± 4.01% for F2, 53.19 ± 5.84% for F3, 55.18 ± 4.14% for F4, 58.42 ± 5.19% for F5. Furthermore, the DL values were 12.19 ± 1.31%, 13.92 ± 1.44%, 15.01 ± 1.59%, 14.39 ± 1.48% and 12.01 ± 1.37% for F1, F2, F3, F4 and F5, respectively. Analyzed statistically, the were statistical differences ($p < 0.05$) between the EE and the DL values in F1, F2 and F3, showing that the increase in CS concentration could potentially increase the EE and the DL of MP. However, the improvement of CS concentration in F3 and F4 did not significantly increase both parameters. Accordingly, considering the less amount of CS used in F3 compared to F4 and F5, based on the parameters evaluated here, F3 was considered as the optimum MP formulations.

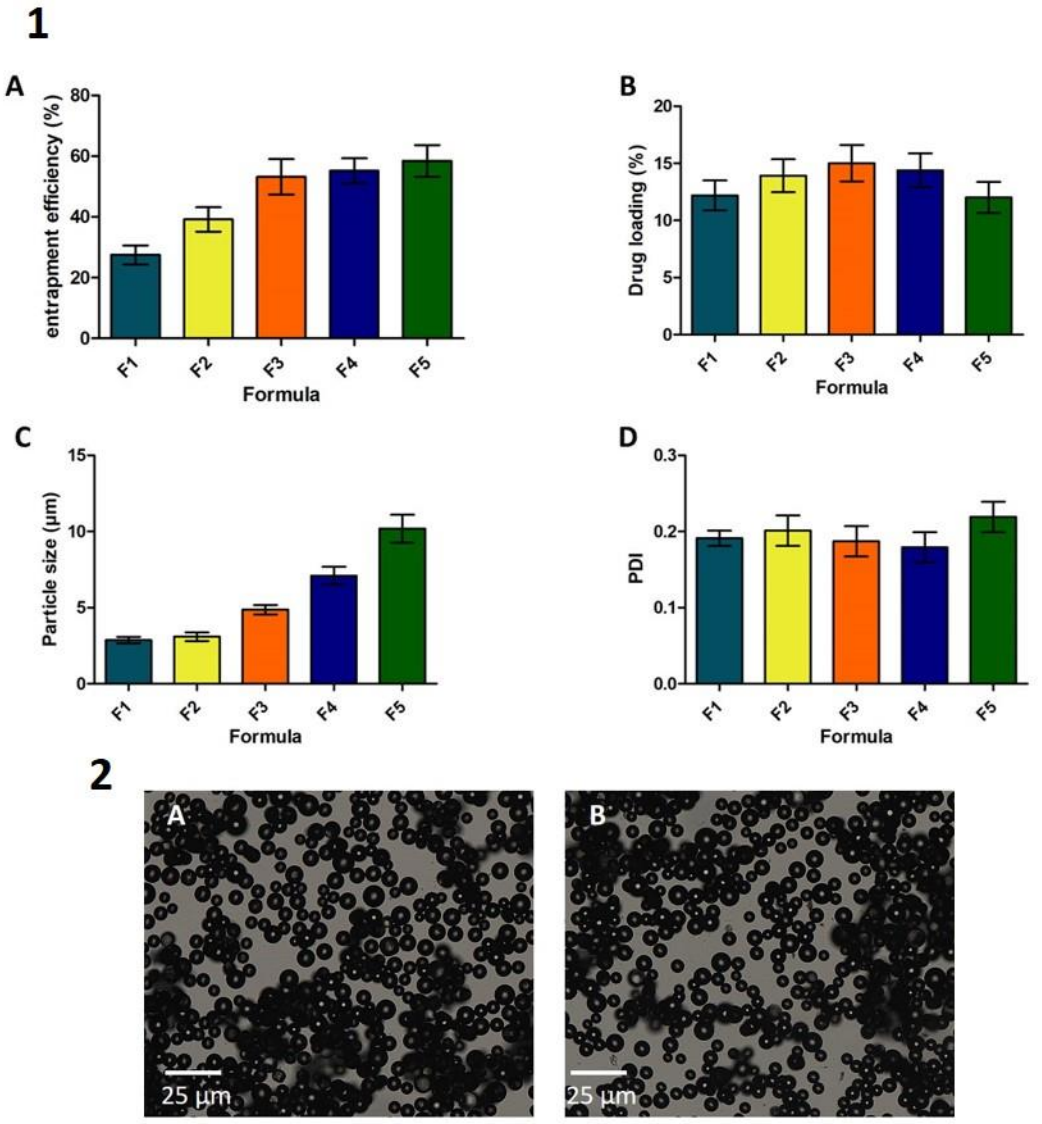

Figure 4. Entrapment efficiency (EE) (A); Drug loading (DL) (B); particle size (C); and polydispersity index (PDI) of MP formula (D) (mean ± SD, *n*=3) (**1**). The microscope images of microparticle F3 with PBA (A); and F3 without PBA (B) (**2**).

Furthermore, Figure 4.2 shows the microscopy images of F3 MP. Additionally, the formulation of F3 without PBA was also prepared. The images show the spherical shape of the MP. Importantly, the sizes of the microscopy images were in good agreement with the results from the particle size determination.

4.2.2 *In-vitro* Release Assay

A further validated UV-Vis spectrophotometer analysis method was applied to determine the amount of MTF released in an *in vitro* release assay. This test was carried out on GR-MP-MTF formulas F1, F2, F3, F4, F5 (the formulations with PBA), MP-MTF (formulation without PBA),

and free MTF solution in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media. The results are revealed in Figure 7, showing that after 24 hours, the release of MTF in PBS medium was 13.87 ± 1.25%, 5.94 ± 0.53%, 5.77 ± 0.52%, 4.93 ± 0.44%, 4.02 ± 0.36% for the GR-MP-MTF formula F1, F2, F3, F4, F5, respectively, and 99.19 ± 10.91% and 98.19 ± 9.03% for pure MTF solution and MP-MTF formula (F3 without PBA), respectively. Specifically, the release of MTF in PBS medium from free MTF solution reached almost 100% in just 2 hours. The MTF release from MP formula without glucose response polymer reached almost 100% after 24 hours. On the other hand, the MTF release from GR-MP in all formulations was only less than 15% MTF after 24 hours. Importantly, the increase glucose concentration in the release medium led to the enhancement of release of MTF from the MP-MTF formulation, indicating the successfulness of the development of GR-MP. As shown in Figure 5.1, after 24 hours, MTF released from all GR-MP-MTF formulations reached almost 100%, namely 99.91 ± 8.99%, 99.98 ± 9.00%, 99.18 ± 8.93%, 91.24 ± 8.21%, and 79.54 ± 7.16% for F1, F2, F3, F4, and F5, respectively. This amount was not significantly different ($p > 0.05$) from MTF released from MP-MTF without PBA and pure MTF solution. The difference in the amount of MTF released in the GR-MP-MTF and MP-MTF without PBA in the PBS and the PBS with glucose media was due to the presence of PBA in the formulation. PBA is a GR material that is sensitive to changes in glucose levels where the increment of glucose levels causes a break in the bond between the phenylboronic-diol in PBA which in turn causes the expansion of the polymer that binds to PBA and releases the MTF contained in the formulation [41]. Among all the MP formulations, with respect to the release pattern, F3 was considered as the optimum formulation. This was because due to the findings that F3 could control the release of MTF for up to 24 hours, reaching almost 100% of MTF released. On the other hand, F4 and F5 could only release around 80% of MTF after 24 h.

The results obtained from the *in vitro* release test were further fitted to five mathematical kinetic models to determine the MTF release model from the formulation. The values of coefficient correlacy for the *in vitro* released of MTF from F3 in PBS were 0.8257, 0.8193, 0.4580, 0.9988, and 0.8215 for Zero order (ZO), First order (FO), Higuchi (H), Korsmeyer-Peppas (KP), and Hixson-Crowell (HC), respectively. In PBS containing 1% w/v glucose, the value of coefficient correlacy were 0.9916, 0.9772, 0.7090, 0.9980, and 0.9829 for ZO, FO, H, KP, and HC, respectively. Regarding the release in PBS containing 2% w/v glucose, the values of coefficient

correlacy were found to be 0.9306, 0.9015, 0.7633, 0.9403, and 0.9333 for ZO, FO, H, KP, and HC, respectively. Finally, in PBS containing 4% w/v glucose, the values of coefficient correlacy were 0.8391, 0.9099, 0.7995, 0.9006, 0.9358 for ZO, FO, H, KP, and HC, respectively. The result obtained show that *in vitro* release of MTF in PBS, PBS containing 1% w/v of glucose media and PBS containing 2% w/v of glucose media followed Korsmeyer-Peppas model. This kinetic model was describe for drug release from polymeric matrix. Meanwhile, *in vitro* release of MTF inPBS containing 4% w/v of glucose media followed Hixson-Crowell, which kinetic model to describe drug release from systems that has change in surface area and diameter of particle, in the case of hydrophilic matrix swelling and erosion of the polymer occurs simultaneously [42]

4.2.3 DMN fabrication and drug content in MNs

Following the successful development of GR-MP, the formulations were further incorporated into DMNs to facilitate dermal delivery of MTF. Using the mixture of the aqueous gel of 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa), DMNs containing MPs possessed a complete and sharp needle shape (Figure 5.2) with adequate mechanical and insertion properties. As a control, MP without PBA and free MTF-loaded DMNs were also prepared. The combination of these polymers has shown the effectiveness of the DMNs formulation in numerous studies [43,44]. The validated spectrophotometer UV-Vis analysis method was further used to determine MTF content in formula MP-MTF-PBA and MP-MTF without PBA. As shown in Figure 5.2, the percentage of MTF recoveries were found to be 98.12 ± 1.13%, 98.09 ± 1.59%, and 99.23 ± 2.01% for MP-MTF-PBA, MP-MTF (without PBA) and pure MTF. This result indicated that the formulation of MTF in MP form and combine with PBA did not affect the concentration of MTF in the DMN formulations. The recovery percentage of all formulas also fulfil the required of acceptable recovery percentage from ICH which in range 95-105% [21]

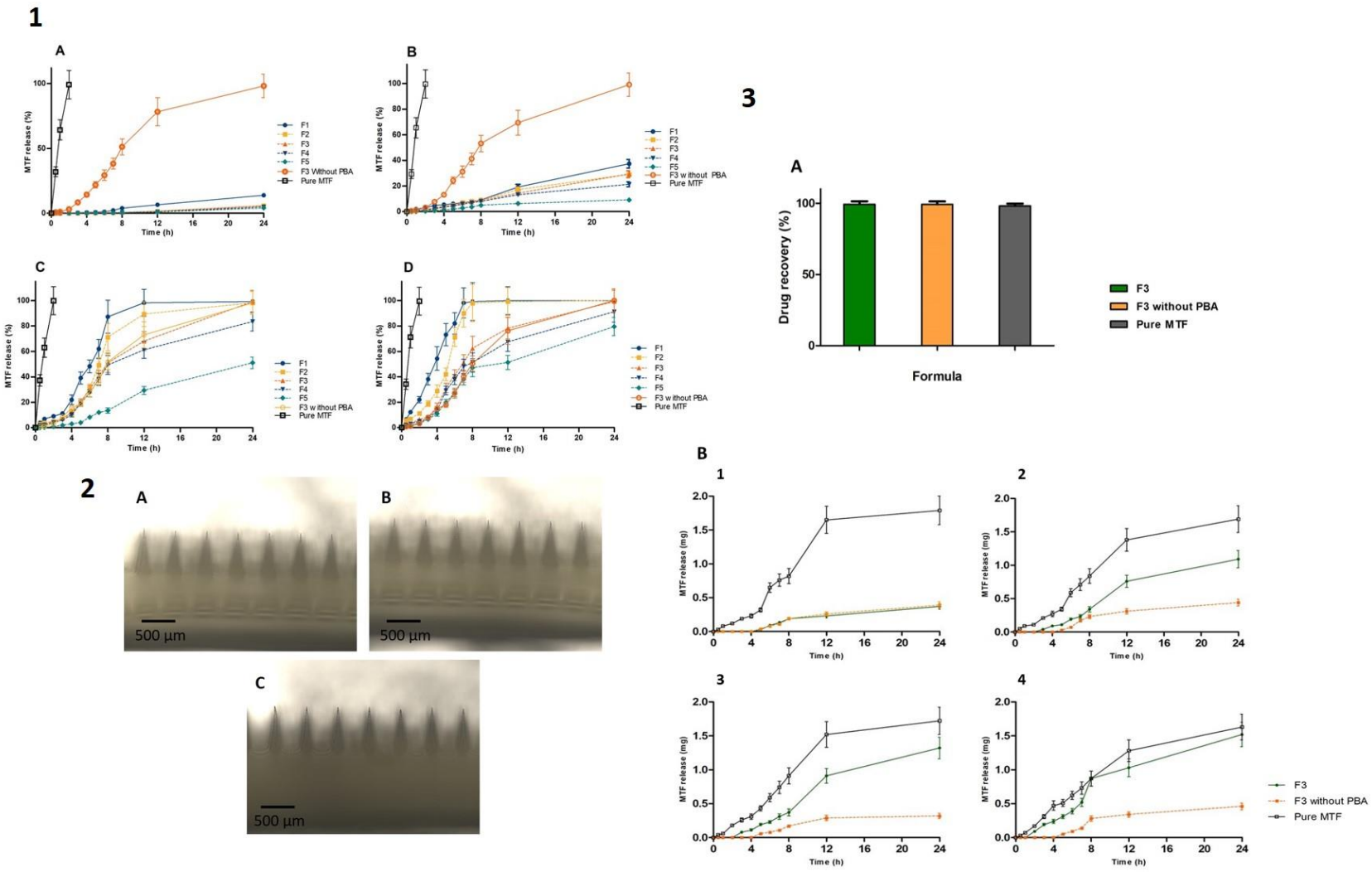

Figure 5. *In vitro* release profile of MTF from MP in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D) data (mean ± SD, *n*=3) (**1**). The microscope images of DMN containing F3 with PBA (A); F3 without PBA (B); and pure MTF (**2**). MTF recovery (%) from MN (A); In vitro permeation profile of MTF from DMN (B) in PBS media (1); PBS containing 1% w/v of glucose media (2); PBS containing 2% w/v of glucose media (3); PBS containing 4% w/v of glucose media (4) data (mean ± SD, *n*=3) (**3**).

4.2.4 *Ex vivo* Permeation Study

Ex vivo permeation test was carried out to investigate the penetration ability of MTF formulated into GR-MP-MTF, MP-MTF (without PBA), and pure MTF delivered using DMN. This evaluation was carried out to ensure the controlled release of MTF in the GR-MP-DMN formulation in *in vitro* hyperglycemic modeling media. Based on the results of the *ex vivo* permeation test in the four media presented in Figure 5.3, it was observed that the MTF permeation continued to increase with time. Specifically, the DMN containing the MP-MTF formulation (without PBA) showed a significant and similar increase in permeation in all media, indicating the non-specific release pattern of this formulation. Moreover, the DMNs containing pure MTF also showed non-specific release behavior with the highest amount of MTF released over 24 h. On the other hand, importantly, the formulation of GR-MP into DMNs could potentially control the permeation of MTF. After 24 h, the MTF permeation in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media were 0.37 ± 0.04, 1.09 ± 0.13, 1.32 ± 0.16, and 1.52 ± 0.18 mg. This increase in the amount of permeable MTF indicated the successfulness of the GR-MP-MTF formulation in controlling the release of MTF according to the amount of glucose contained in the media as a hyperglycemic model This shows that the modification of the formula in the form of CS-MP can help the controlled release system in MTF [8].

Here, for the first time, we successfully applied a simple validated analytical method using UV-vis spectrophotometer to quantify MTF in the development of GR-MP loaded DMNs. The method was found to be valid with desired accuracy, precision and dilution integrity results. Importantly, the application of the method indicated the successfulness of the selective delivery the approach in the *in vitro* hyperglycemic condition. Accordingly, this approach could be an alternative of the oral administration of MTF. Moving forward, the effectiveness of this system should be evaluated in appropriate *in vivo* models with suitable analytical models.

5. Conclusion

This research was conducted with the aim of developing and validating a UV-vis spectrophotometric method for the analysis of MTF in the development of GR-MP loaded DMNs. The method used was validated with the parameters of selectivity, accuracy, precision, linearity, LOD and LLOQ, and dilution integrity. The method was validated in *in vitro* normal physiological

and hyperglycemic conditions using PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose. Based on all the validation parameter tests, this method was found to meet the requirements of the ICH guidelines, indicating that this analytical method was valid for the application of the drug development of MTF. Specifically, the validated analytical method was successfully applied to evaluate EE, DL, *in vitro* release profile in MP system, drug recovery and *ex vivo* permeation in DMNs system. The results from the application of the method showed that the incorporation of MTF into the combination of GR-MP and DMNs could potentially improve the selective delivery and control the release as well as the permeation profile in *in vitro* hyperglycemic conditions, making it as an innovative approach to overcome the problems in oral administration of MTF. To further evaluate the efficacy, as the next step, in *vivo* analytical method must now be developed.

Author contributions:

Sumayya Binti Abd Azis: Conceptualization, Methodology, Funding acquisition, Writing – original draft. **Nur Syafika:** Methodology, Writing – original draft. **Hanin Azka Qonita:** Methodology, Writing – original draft. **Ahmad Abizart:** Methodology, Data curation. **Tiara Resky Anugrah Mahmud:** Data curation, Validation **Andi Dian Permana:** Conceptualization, Project administration, Funding acquisition, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of interests

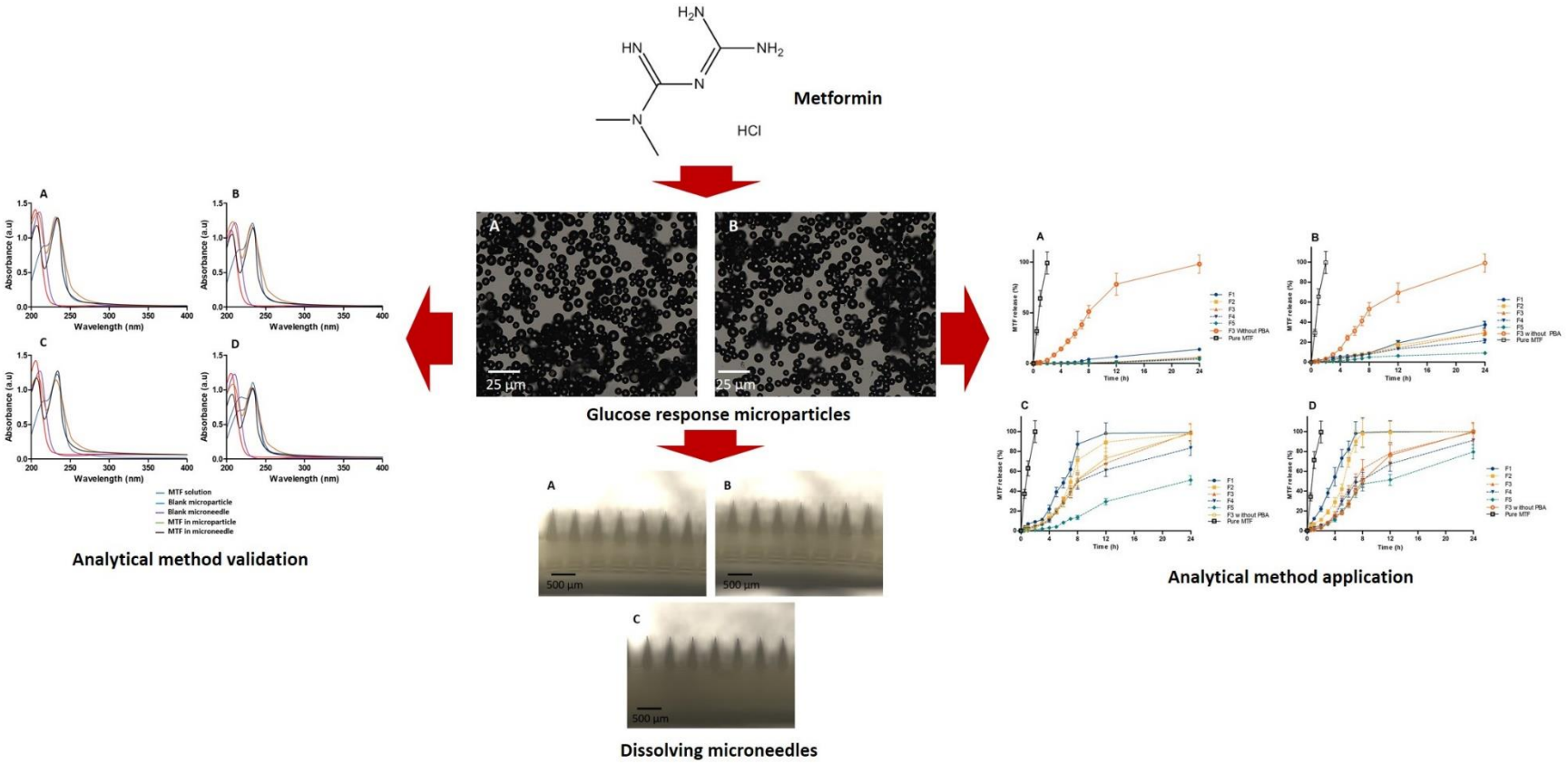
☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author contributions:

Sumayya Binti Abd Azis: Conceptualization, Methodology, Funding acquisition, Writing – original draft. **Nur Syafika:** Methodology, Writing – original draft. **Hanin Azka Qonita:** Methodology, Writing – original draft. **Ahmad Abizart:** Methodology, Data curation. **Tiara Resky Anugrah Mahmud:** Data curation, Validation **Andi Dian Permana:** Conceptualization, Project administration, Funding acquisition, Validation, Supervision.

Graphical Abstract



Application of validated spectrophotometric method to quantify metformin in the development of glucose-responsive microparticles loaded dissolving microneedles

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Highlights

- Spectrophotometric method was developed to determine metformin in *in vitro* hyperglycemic-mimicked conditions
- The spectrophotometric method was validated as per ICH guidelines
- The Spectrophotometric method was applied in the development of glucose-responsive microparticles loaded dissolving microneedles

ABSTRACT

Metformin (MTF) is a first-line drug in the treatment of type 2 diabetes mellitus. Delivered through the oral route, MTF has several limitations, mainly due to the side effects in gastrointestinal, non-specific release and low intestinal permeability, resulting in the low bioavailability of MTF in the body. Here, we developed glucose-responsive microparticles (GR-MP) containing MTF delivered *via* dissolving microneedles (DMNs) to overcome these limitations. To support the development of the formulation, in this study, a simple analytical method was developed using a UV-visible spectrophotometer. The method was validated in four different media, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose, to mimic the normal and hyperglycemic condition. The method was further validated as per International Conference Harmonization (ICH). This analytical method was applied to quantify the amount of MTF in the GR-MP preparation, *in vitro* release, drug content in DMNs and, importantly, *ex vivo* permeation study in *in vitro* hyperglycemic conditions. The results exhibited that the calibration curves in all media showed a correlation coefficient (R) of 0.998, indicating the linearity of the method. Moreover, LLOQ values in the four different media were 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL, and 2.88 µg/mL, respectively. Importantly, the method was precise and accurate with desired dilution integrity according to ICH, implying the validity of the methods. Finally, the method was successfully applied in the development of DMNs containing GR-MP of MTF, showing that the incorporation of MTF into this combination approach could selectively control the release of the drug according to the glucose concentration both in *in vitro* release and *ex vivo* permeation studies. Therefore, this approach could be a favorable system to solve the oral administration of MTF. Further *in vivo* analytical methods should now be developed to explore the effectiveness of this system in a suitable animal model.

Keywords: Metformin, UV-Vis spectrophotometric, Validation, Glucose-Responsive Microparticles, Dissolving Microneedles

1. Introduction

One of the biggest causes of death in the world is diabetes mellitus (DM). According to data from the International Diabetes Federation, 537 million people had DM in 2021, and it was estimated to increase to 783 million in 2045. DM causes various complications that were the direct cause of 1.5 million deaths worldwide in 2019. Specifically, approximately 90% of DM cases are type 2 DM (T2DM) [1].

The first-line treatment for T2DM is metformin (MTF) tablets administered orally. MTF has been shown to be most effective in lowering blood glucose levels. MTF works in lowering blood sugar levels through various mechanisms. Consequently, the use of MTF in high doses and in the long term can potentially increase the risk of hypoglycemia. Research shows that 112 out of 4072 cases of MTF overdose could trigger hypoglycemia, which could potentially lead to intolerance to MTF [2]. Other side effects associated with the oral MTF therapy can cause undesired impacts on the gastrointestinal tract. MTF also has low permeability to cell membranes and, therefore, the absorption of MTF given orally does not occur optimally [3]. This causes an increase in the accumulation of drugs in the intestines, resulting in some dangerous side effects [4]

To overcome these problems, it is crucial to design a smart delivery system to deliver MTF. Recently, the development of a glucose-responsive delivery system has attracted the interest of numerous researchers to selectively control the release of antidiabetic drugs. To the best of our knowledge, there has been no glucose-responsive system developed for MTF. In this study, we presented microparticles with glucose-responsive ability that could release MTF in the presence of glucose. Accordingly, this could be beneficial in preventing hypoglycemia [5]. Some compounds have been explored to possess this characteristic, including glucose oxidase (GOD), concanavalin A (Con A), and phenylboronic acid (PBA) [6]. Among these three compounds, the use of PBA is more frequent because it is lower cost, biodegradable, and easy to fabricate compared to GOD and Con A. Importantly, since PBA is not a protein like GOD and Con A, disadvantages such as poor volatile inactivation, and the high cost can be avoided [7].

In designing a controlled release form of drug delivery, the choice of polymer is one of the crucial things to consider [8]. There are many polymers that can be used in designing controlled release systems in the form of microparticles, one of which is a synthetic polymer in the form of poly(lactic) acid, which is a polymer with great potential, but controlling the particle size and drug adsorption efficiency of this polymer is quite difficult, and initial burst release may occur [9,10].

Another polymer that is usually used in the manufacture of microparticles is ethyl cellulose. However, the structure of ethyl cellulose which does not have a carboxyl group, makes PBA compounds unable to be linked to gelatin polymers and form polymer complexes that are responsive to glucose [11]

In this study, a polymer in the form of chitosan (CS) was chosen due to several benefits, namely being non-toxic, biodegradable, and biocompatible. These polymers also have unique physical and chemical characteristics, such as intermolecular hydrogen bonding and their polycationic charge under acidic conditions [12]. This leads us to the binding of the hydroxyl and amino groups present in the CS chain, which has a strong affinity for PBA. This binding resulted in a decrease in the pKa value of PBA and led to the manufacture of pH-responsive compounds, which has broad application prospects, causing PBA-CS combinations to achieve glucose sensitivity below the physiological pH of the human body [13]. Previously, several studies have been carried out in formulating metformin in the form of microparticles. One of them is research conducted by Avram et al 2017 who formulated MTF in the form of microparticles using a syringe technique using chitosan polymer [14,15]. However, the particles were not developed for selective delivery for hyperglycemic conditions. Therefore, further development is required to selectively release MTF based on glucose concentration.

As previously explained, the oral administration of MTF resulted in several side effects. As a result, modified transdermal delivery of MTF was chosen to overcome the side effects of MTF related to the low permeability of MTF to cell membranes, resulting in reduced bioavailability [16]. The choice of subcutaneous administration of antidiabetic drugs is commonly used, such as insulin and GLP-1 agonist drugs. Subcutaneous administration of drugs can certainly help overcome previous MTF problems, but new problems arise, such as discomfort to the patient, bleeding, infection at the injection site, and many more [17].

As an innovative delivery system, dissolving microneedles have been widely explored as an alternative delivery system to the injection route [18–20]. This system is applied intradermally which would dissolve when applied and release the active substances [21]. Therefore, the fabrication of DMN as a drug delivery system is an interesting solution for the oral therapy of MTF. The use of DMN as a drug delivery system not only solves the problems of administration using injection, but can also reduce sharp object waste after use because the needle used can dissolve due to its fabrication using water-soluble polymers [22]. However, the use of the DMN

system can have an impact on the difficulty of drug encapsulation and dose control. Therefore, the DMN system could be collaborated with GR-MP in order to obtain an efficient drug delivery system and controlled drug release [23]. In this study, MTF-loaded-GR-MP was further incorporated into DMN for a selective and efficient delivery system in the *in vitro* mimicking diabetes environment.

In the development of a new drug delivery system, various tests and characterizations are required. One of the critical points is analyzing the active compounds. In this study, with regard to the analysis process, MTF was analyzed in the development of GR-MP, *in vitro* testing, and *ex vivo* permeation test. With respect to the media used, in this study phosphate buffered saline (PBS) and PBS containing glucose media represented normal and diabetes conditions. Previously, there were studies that carried out the determination of pure MTF and from tablets quantitatively using the reversed phase-high performance liquid chromatography (RP-HPLC) method [24] and Ultra Violet (UV) spectrophotometer. However, the chromatographic method has drawbacks, such as requiring large costs, a lot of solvents, reliable power, and expensive instruments [25]. This could limit the application of the analytical method in the several laboratories which do not have access to use the HPLC. These studies also analyzed the MTF of tablet dosage forms and did not use specific media. It has been reported previously that the results of research conducted by Georgia et al., 2021 in quantifying irinotecan from human plasma using UV-vis spectrophotometric techniques show that this technique was still relevant and valid in analyzing drugs from blood plasma [26].

Considering several aspects mentioned previously, in this study, an analytical method of MTF was developed from GR-MP-DMN preparations in PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose mediums using a UV-Vis spectrophotometer. This analytical method has been widely used in the determination and has proven to be an analytical method that is simple, easy, and provides precise results in determining the number of samples [27]. Importantly, the application of a UV-Vis spectrophotometer has been widely used in almost all scientific laboratories, making it a versatile tool in the drug development. To ensure that the developed analytical method provides appropriate results, this study was also conducted involving the validation of the analytical method based on the International Conference Harmonization (ICH) guidelines. Method validation parameters such as linearity, accuracy, precision, limit of detection (LOD), and the determined limit of quantification (LOQ), and were extensively applied

in the determination of entrapment efficiency and drug loading in MP, drug content in DMN preparations, and *in vitro* and *ex vivo* permeation profiles.

2. Material and Methods

2.1 Materials

Metformin HCl was obtained from Tokyo Chemical Industry Co., LTD, Tokyo, Japan. Chitosan (medium molecular weight), glutaraldehyde polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), potassium dihydrogen phosphate (KH_2PO_4), glucose, potassium chloride (KCl), disodium phosphate (Na_2HPO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Singapore). All other reagents used in this study were analytical grade,

2.2 Preparation of PBS and PBS Containing Glucose

PBS was prepared by dissolving 0.2 g of KCl, 8 g of NaCl, 2.4 g of KH_2PO_4, and 1.44 g of Na_2HPO_4 with ± 800 mL CO_2-free water. Following the solubilization, the solution pH was set to 7.4. Finally, CO_2-free water was added to make up the final volume to 1 l. To prepare PBS containing glucose media, glucose with concentrations of 1% w/v, 2% w/v and 4% w/v were dissolved using PBS.

2.3 Preparation of MTF Stock Solution

The stock solution was prepared by dissolving 10 mg of MTF into 10 mL of different media separately to achieve 1000 µg/mL of MTF solution.

2.4 Determination of Maximum UV Absorption, Preparation of Calibration Solution, and Quality Control Solution

Initially, the stock solution of MTF in the respective media was diluted to achieve a concentration of 20 µg/mL. The determination of the maximum UV absorption in MTF in all media was carried out using a UV-vis spectrophotometer (Dynamica, HALO XB-10). Thereafter, the calibration solution in all media was prepared by diluting MTF stock solution using the respective media to achieve the serial concentrations of 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL and 0.5 µg/mL

Quality control solutions were prepared in three different concentrations, such as 12 µg/mL for high-quality control (HQC), 7.5 µg/mL for medium-quality control (MQC), and 4 µg/mL for low-quality control (LQC).

2.5 Validation Method

The UV-Vis spectrophotometer validation method was carried out by measuring the validation parameters, such as linearity, specificity, LOD and LOQ, dilution integrity, as well as accuracy and precision.

2.5.1 Linearity

Determination of linearity in the method validation was carried out by plotting the absorbance of three replications of the MTF calibration solution in three different media. From the curve results obtained, the correlation coefficient (R) was calculated. Linear parameters are considered valid if the value of R is close to 1 [28]

2.5.2 Specificity

The specificity needs to be known to ensure that there is no interference from other materials in the sample [28]. Specificity was determined by comparing the UV spectra of GR-MP blank, DMN blank, and MTF in both GR-MP and DMN system. The UV spectra was scanned between 200-400 nm.

2.5.3 Limit of Detection (LOD)

The detection limit (LOD) was investigated to determine the smallest amount of analyte that could show absorption or absorbance in the instrument without having accuracy and precision criteria. LOD was calculated by using equation 1. In the equation, 3.3 represents the factor for LOD, SD is the standard deviation of the blank, and b is the slope of the blank regression line [29]

$$LOD = \frac{3.3\ x\ SD}{b} \qquad \text{Equation 1}$$

2.5.4 Lower Limit of Quantification (LLOQ)

LOQ is the smallest amount of analyte that can still be measured for its absorbance using an instrument and has accuracy and precision criteria. LLOQ can be calculated by using equation 2. In the equation, 10 represents the factor for LLOQ, SD represents the standard deviation of the blank, and b represents the slope of the regression line [29]

$$LLOQ = \frac{10\ x\ SD}{b} \qquad \text{Equation 2}$$

2.5.5 Accuracy

Accuracy is a parameter that shows the degree of closeness of the analysis results to the actual analyte content. Accuracy is expressed as the percent recovery of the added analyte. The

accuracy test was carried out by comparing the MTF concentration in LLOQ, LQC, MQC and HQC solutions from the absorbance measurement results with the theoretical concentration, then the relative standard deviation (% RSD) was calculated. the %RSD value should not be more than 15% of the theoretical concentration [28]. Measurements were done intra-day and inter-day.

2.5.6 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision test was the same as in the previous accuracy test, where the concentrations of the absorbance measurement results of LLOQ, LQC, MQC, and HQC solutions were compared with the theoretical concentrations. The relative error value (%RE) was calculated, and the results obtained should not be more than 15% of the coefficient of variation (CV) [30]. Measurements were carried out intra-day and inter-day.

2.5.7 Dilution Integrity

Dilution integrity was carried out by preparing 75 µg/mL MTF in all media. Then each solution was diluted 5 and 10 times, the experiment was carried out in triplicate, and the absorbance of the analyte was observed [31].

3. Application

3.1 Microparticle Formulation

Microparticles were prepared using CS. In this study, 5 formulations were prepared containing 100 mg of MTF with different amounts of CS, namely 100 mg, 150 mg, 200 mg, 250 mg and 300 mg for F1, F2, F3, F4 and F5, respectively. GR-MTF-MP were prepared by mixing MTF and CS with 5 mg of EDTA, and added to 3 mL of acetic acid solution in water (1% v/v) under the stirring condition at 500 rpm at room temperature. After that, 6 mL of ethanol was added to make a cloudy solution which indicated the formation of MPs. After that, 50 µL of glutaraldehyde (25%) solution were added as a crosslinker by forming a reaction between the aldehyde group and the amino group of the MP. Furthermore, the MP formed were centrifuged at 3000 rpm for 20 minutes, and the sediment obtained was washed using distilled water to obtain pure MP CS [32].

To prepare GR-MP, PBA solution (11.2 mg) was dissolved in 1 mL of DMSO, and reacted with EDC.HCl (15.5 mg) and NHS (9.3 mg) for 30 minutes (mixture 1). After that, the mixture 1 solution was added to 5 mL of MP CS solution, while stirring at 37˚C for 24 hours. Then, the PBA-

decorated MP CS (MP PBA-CS) was dialyzed in distilled water for 48 h to remove unreacted PBA [32]. MP CS containing MTF was referred to as MP CS-MTF, and PBA-CS MPs containing MTF were referred to as MP PBA-CS-MTF. Particle size and polydispersity index (PDI) were all calculated.

3.2 Determination of Entrapment Efficiency and Drug Loading

The entrapment efficiency (EE) of MTF in MP was determined using the indirect method. In the washing steps, the supernatant was taken, and the concentration of MTF was calculated using a validated analytical method. Furthermore, the drug loading (DL) determination was carried out by mixing 50 mg of the formulation with 10 mL methanol. The mixture was sonicated for 30 minutes and diluted with PBS. ED and DL were calculated using the following calculations [33]:

$$\%EE = \frac{(Weight\ of\ initial\ drug - Weight\ of\ free\ drug)}{Weight\ of\ initial\ drug}\ x\ 100 \quad \text{Equation 3}$$

$$\%DL = \frac{Amount\ of\ entrapped\ drug\ in\ microparticle}{Total\ weight\ of\ microparticle}\ x\ 100 \quad \text{Equation 4}$$

3.3 *In vitro* Release Test

The *in vitro* release profile of MTF from MP was investigated using dialysis membrane method [21,33,34]. Briefly, MP formulations equal to 10 mg of MTF were placed inside dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO dialysis membrane). The membrane was further immersed into 100 mL of release media. Three different media were used, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose. The study was carried out in an orbital shaker at 100 rpm at 37°C. The media (1 mL) was sampled at certain time intervals, and then the concentration of MTF was determined using a UV-Vis spectrophotometer. Fresh media was added after the sampling to ensure the sink condition during the study. The drug release mechanism was then analyzed using a variation of the mathematical kinetic model [33]

3.4 Mathematical Modelling for *In Vitro* Release Test

The data obtained from the *in vitro* assays were then fitted into five different mathematical models to determine the release kinetics of MTF from MP. The models applied were zero-order kinetics (Z0), first-order kinetics (F0), Krosmeyer-Peppas (KP), Higuchi, and Hixson-Crowell (HC). The equations of each model are described below:

$$Zero\ order\ kinetics: C_t = C_0 + K_0 t$$

$$First\ order\ kinetics: lnC_t = lnC_0 + k_1 t$$

$$Krosmeyer - Peppas\ model: C_t = k_{KP} t^n$$

$$Higuchi\ model: C_t = k_H \sqrt{t}$$

$$Hixson - Crowell\ model: C_t^{\frac{1}{3}} = C_0^{\frac{1}{3}} k_{HC} t$$

C_t represents the concentration of MTF at time t, C_0 represents the initial concentration of MTF in the medium (t = 0), k_0 represents the zero-order constant, k1 represents the first-order constant, k_{KP} represents the Korsmeyer–Peppas constant, k_H represents the Higuchi constant, and k_{HC} represents the Hixson constant. - Crowell. All calculations were performed using the DD-solver software. The release kinetics is determined from the correlation coefficient value (R) obtained [31]

3.5 DMN Fabrication and Determination of Drug Content

In this study, two-layered DMNs containing MP CS-MTF and MP PBA-CS-MTF were fabricated using the centrifugation method [35]. The formulation contained the aqueous gel of 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa) in distilled water mixed with 30% w/w of MP. Initially, 100 mg formulation was poured on the top of DMN MN silicon mould (needle density 10 x 10, the pyramidal needle with 700 μm of high and 200 μm wide on the base and 200 μm of spacing). Thereafter, the mould was centrifuged at 3000 rpm for 15 min at room temperature. The excess of the formulation was removed and dried for 6 h. Following this, an aqueous gel containing 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa) was poured as a second layer. The formulation was dried at room temperature for 24 h and removed from the mould. It was important to note that the DMNs used in this study possessed adequate mechanical and insertion properties.

In an attempt to measure the MTF content in DMNs, the formulation was initially dispersed in 5 mL of distilled water. Afterwards, the dispersion was mixed with 10 mL of methanol and sonicated for 30 min. The mixture was then centrifuged for 10 minutes at a speed of 5000 rpm. The supernatant was collected, and the absorbance was measured using the UV-Vis spectrum.

3.6 *Ex Vivo* Permeation Studies

Ex vivo permeation studies were performed using Franz diffusion cells, using rat skin [36–38]. PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose were used as the

release medium. Prior to the experiment, skin was washed and soaked in the release medium for 30 minutes. Afterwards, the surface of the skin was dried, and the skin was placed between the donor and recipient compartments. The experiment was conducted at 100 rpm at 37°C. During the study, the sampling was carried out at several time intervals, starting from 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours by taking 1 mL in the receiving compartment, then replaced with the same volume of new media. The samples were then analyzed using UV-Vis spectrophotometry.

3.7 Statistical analysis

All data obtained were expressed in mean ± standard deviation (SD), all values were obtained using Microsoft excel® 2019 software (Microsoft Corporation, Redmond, USA). Graphpad Prism® version 6 (GraphPad Software, San Diego, California, USA) was used to analyze the data statistically, where statistical significance was indicated by p value < 0.05.

4. Results and Discussion

4.1 Validation of analytical method

4.1.1 Determination of Maximum UV Absorption and Specificity

The purpose of this study was to validate the UV-vis spectrophotometer as an instrument that allow the quantification of MTF in the development of a new dosage form, namely in the form of GR incorporated into DMN. Measurement of MTF levels was carried out in PBS media as a model medium for normal condition and PBS containing different concentration of glucose media as a model medium for hyperglycemia condition [39]. The results showed that MTF exhibited maximum absorption at 234 nm in all media (Figure 1). Accordingly, the wavelength was used in the further steps in this study.

Specificity parameter was intended to ensure that the MTF analysis using a UV-Vis spectrophotometer from the MP and DMN formulations did not experience interference from other compounds. As shown in Figure 2, the measurement results of the blank MP and MN showed a peak in the range of 210-220 nm, and did not indicate a possible interference at the MTF peak at 234 nm. In addition, the MTF peaks in both the MP and DMN formulas showed the same peaks as the pure MTF solution, which indicated that there was no peak shift due to additives or solvents used in the formulation. Therefore, the method developed in this study has been specific to the appropriate wavelength.

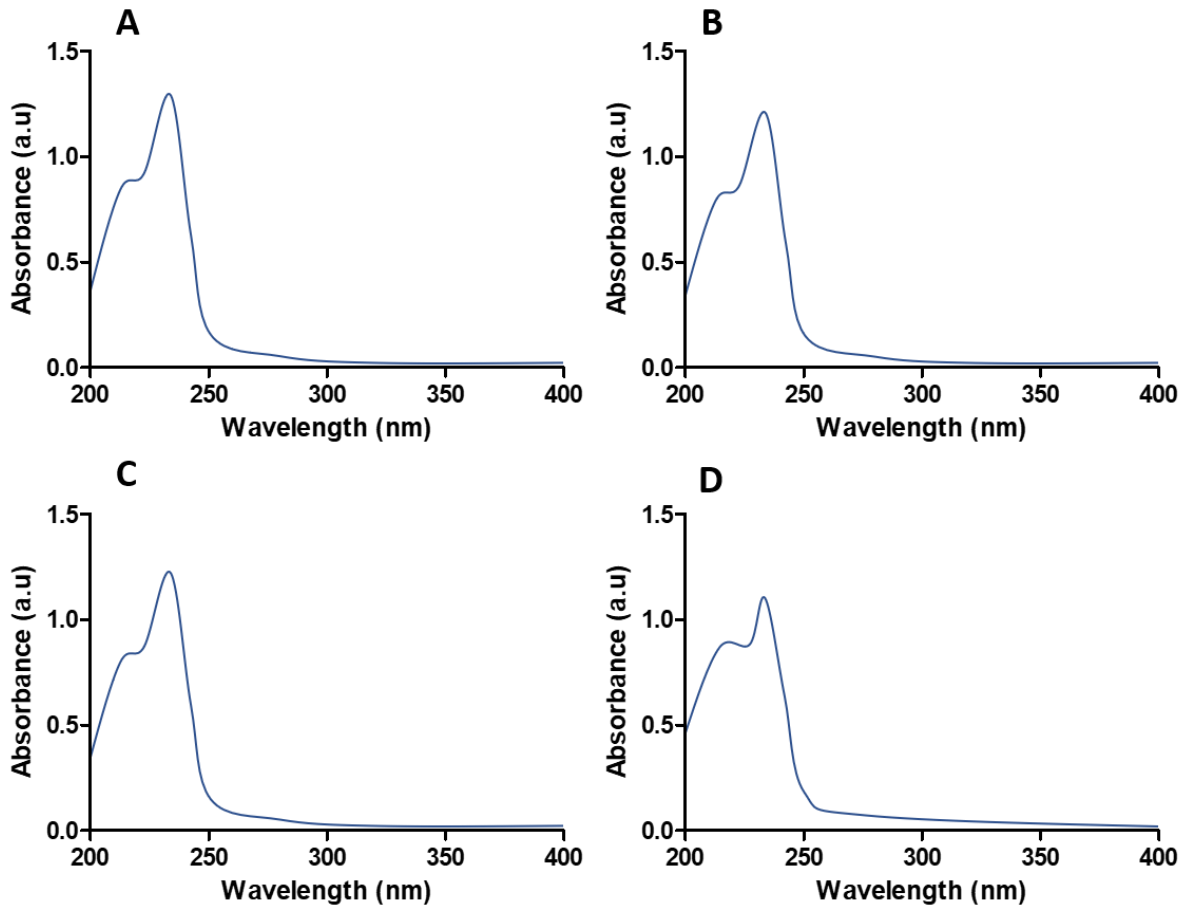


Figure 1. Maximum absorbance of MTF in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

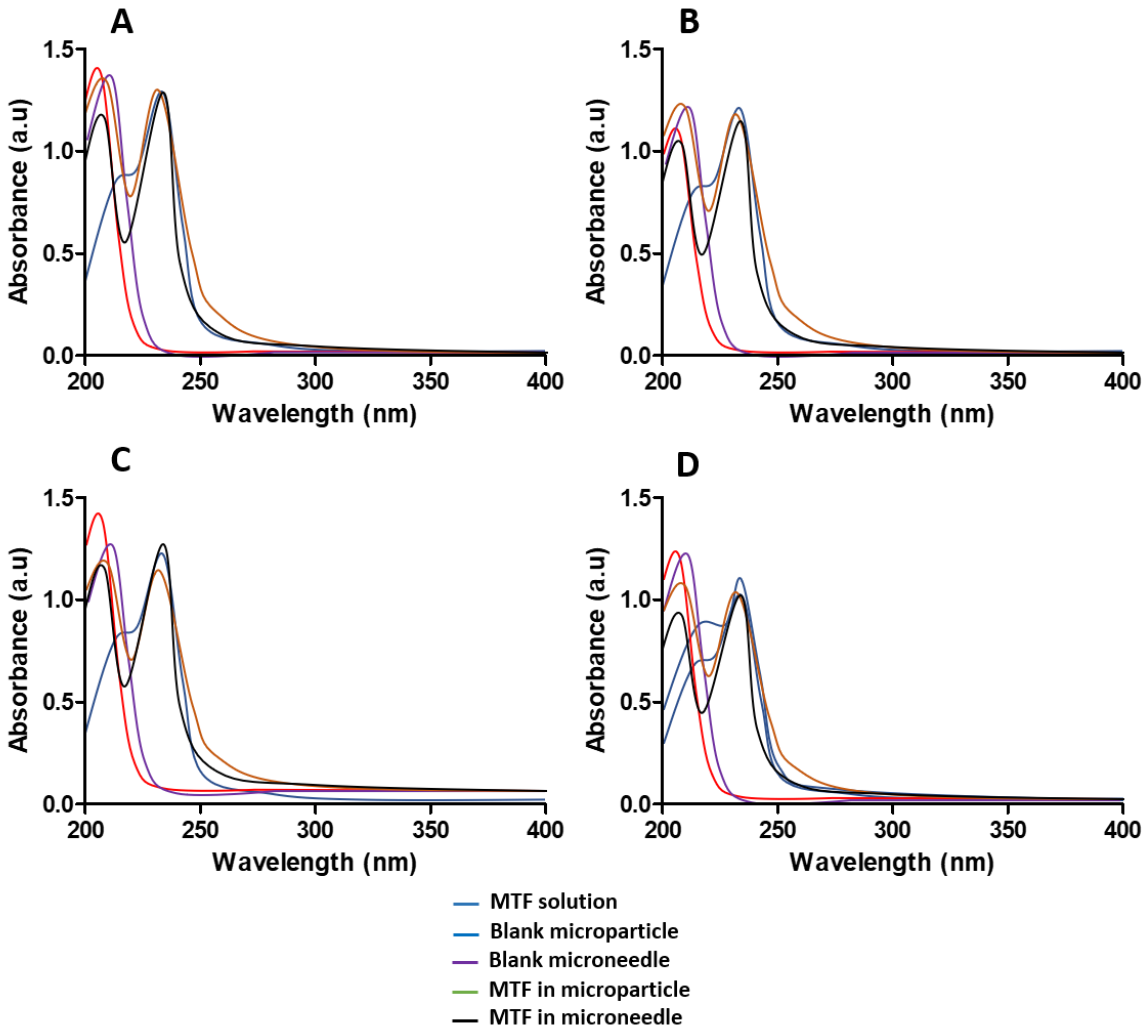


Figure 2. Representative UV-Spectra of pure MTF, blank MP, blank DMN, MTF in MP, and MTF in DMN in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

4.1.2 Linearity, LOD and LOQ

Linearity parameter is one of the characteristics required in the validation process of an analytical method. Based on the ICH guidelines, linearity needs to be evaluated by plotting a function of analyte concentration with the absorbance results obtained, then evaluated by mathematical modeling. Determination of linearity parameters from the validation of this method was carried out by measuring the absorbance of MTF in the concentration range of 0.5-16 µg/mL in all media. The spectrum of MTF in all media tested in various concentrations are depicted in Figure 3. The linearity acceptance criteria for active pharmaceutical ingredients is $R > 0.998$ [40]. The results of the linearity measurement of MTF showed the correlation coefficient value of

0.9983 for PBS media, 0.9991 for PBS containing 1% w/v of glucose media, 0.9991 for PBS containing 2% w/v of glucose media, and 0.9981 for PBS containing 1% w/v of glucose media which of these four values met the criteria for linearity parameters.

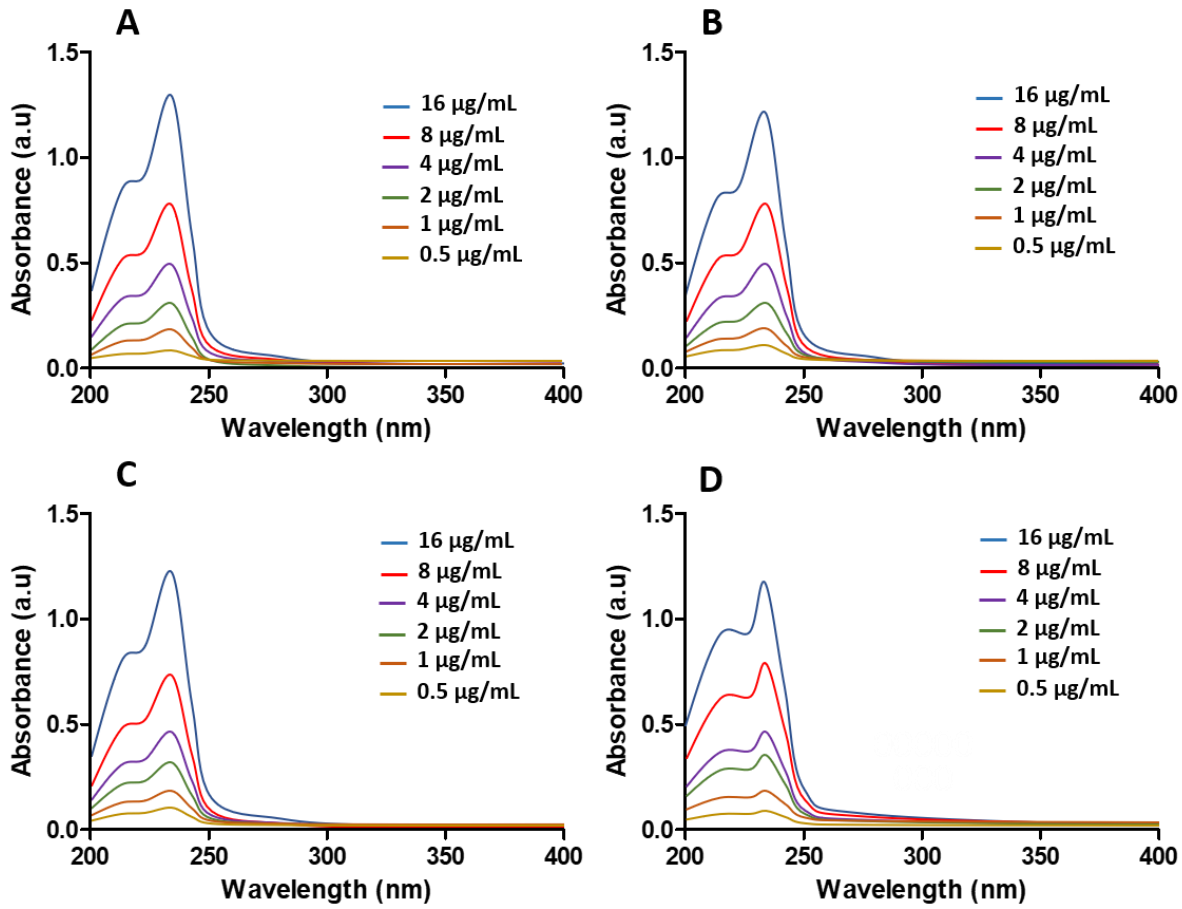


Figure 3. Spectrum of MTF standard solution in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

The LOD and LLOQ values in all media were calculated from the calibration data, as shown in Table 1. The LOD MTF values in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media were found to be 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL and 2.88 µg/mL respectively. LLOQ MTF values PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose were calculated as 0.74 µg/mL, 0.64 µg/mL, 0.64 µg/mL, and 0.95 µg/mL respectively.

Table 1. The calibration curve properties of MTF in different media with LOD and LOQ values of MTF

Media	Concentration range (µg/mL)	R	LOD (µg/mL)	LLOQ (µg/mL)
PBS	0.5-16	0.9989	0.74	2.23
PBS with 1% glucose	0.5-16	0.9991	0.64	1.95
PBS with 2% glucose	0.5-16	0.9991	0.64	1.94
PBS with 4% glucose	0.5-16	0.9981	0.95	2.88

4.1.3 Precision and Accuracy

Precision and accuracy were assessed for intra-day and inter-day. Intra-day determination was carried out to evaluate the repeatability of this analytical method, whereas inter-day determination was conducted to investigate the variation of the day to the measurement. Precision and accuracy were analyzed in four different concentrations consisting of QC sample (HQC, MQC, and LQC) and LOQ sample which were measured in three replications respectively in one day for inter-day determination and three replications in three days for intra-day determination.

Precision was aimed to evaluate the closeness between a series of measurements from the homogenous sample under the same condition. The results were presented as %RSD values and are shown in Table S1, Table S2, Table S3, and Table S4 for PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media, and PBS containing 4% w/v of glucose media, respectively. It was found that all of the %RSD values were less than 15% and, thus, fulfilled the requirement from ICH [41–43]. As the analytical method was considered to be precise when %RSD is less than 15%, this UV-Vis spectrophotometry method was therefore considered to have precision values to quantify MTF in all media used in this study.

Accuracy was carried out to evaluate the closeness between true value and value found from measurements. The accuracy values were reported as %RE. Table S1, Table S2, Table S3, and Table S4 show the accuracy of UV-Vis spectrophotometry method for MTF in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media, respectively. The analytical method was considered to be accurate when the %RE values are ± 15% [28]. All the values were found to be ± 15%; therefore, the analytical method was considered accurate.

4.1.4 Dilution Integrity

Dilution integrity is the ability rate of the dilution process performed during the validation process as accurate, precise, and reliable [28]. Based on the dilution integrity data obtained in Table 2, it was found that the results showed the satisfactory results, where the dilution integrity bias on all media was less than 15%. The precision parameter observed from the %RSD with values ranging from 1.62% - 4.68% indicated that the dilution in this validation method was accurate and precise. In addition, it also implied that the analysis using this method can still be carried out on MTF with concentrations higher than the upper range of the calibration standard by using the appropriate dilutions.

Table 2. Dilution integrity data of UV-Vis spectrophotometry method for MTF in all media (mean ± SD, *n*=3)

Media	Dilution tested	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	%RSD	%RE
PBS	10	7.5	8.26 ± 0.15	1.87	-0.39
	5	15	15.36 ± 0.51	3.30	0.31
PBS with 1% of glucose	10	7.5	7.28 ± 0.30	4.11	-1.62
	5	15	14.25 ± 0.52	3.64	3.45
PBS with 2% of glucose	10	7.5	7.75 ± 0.36	4.68	-0.11
	5	15	14.76 ± 0.71	4.79	3.47
PBS with 4% of glucose	10	7.5	7.49 ± 0.12	1.62	1.66
	5	15	15.09 ± 0.64	4.23	3.22

4.2 Application of the analytical method

4.2.1 Entrapment Efficiency and Drug Loading

Following the successful validation of the spectrophotometric method, it was applied to characterize the MP's EE and DL capacities. In this study, in an attempt to achieve optimum parameters, we investigated five different concentrations of CS. The results of the characterization are depicted in Figure S1.1. With regard to the particle size, it was found that the increase of CS concentration could increase the particle size of the formulation. It might be due to the increased CS concentration resulting in a higher viscosity of the medium, leading to the decrease of the energy to break the droplet into a smaller size. This phenomenon was also observed in numerous studies investigating the concentration of the polymer in the size of micro/nanoparticles [30,44]. The results showed that the particle size of F1, F2, F3, F4 and F5 were 2.87 ± 0.21 µm, 3.09 ±

0.28 µm, 4.87 ± 0.31 µm, 7.09 ± 0.59 µm and 10.19 ± 0.92 µm, respectively. Regarding the PDI values, despite the difference in size, all formulations exhibited a narrow distribution pattern. Furthermore, the EE evaluation results showed the improvement of EE values following the increment of the CS concentrations. Following the application of the validated method, it was calculated that EE values were 27.49 ± 3.12% for F1, 39.16 ± 4.01% for F2, 53.19 ± 5.84% for F3, 55.18 ± 4.14% for F4, 58.42 ± 5.19% for F5. Furthermore, the DL values were 12.19 ± 1.31%, 13.92 ± 1.44%, 15.01 ± 1.59%, 14.39 ± 1.48% and 12.01 ± 1.37% for F1, F2, F3, F4 and F5, respectively. Analyzed statistically, statistical differences ($p < 0.05$) between the EE and the DL values in F1, F2 and F3 showed that the increase in CS concentration could potentially increase the EE and the DL of MP. However, the improvement of CS concentration in F3 and F4 did not significantly increase both parameters. Accordingly, considering the less amount of CS used in F3 compared to F4 and F5, based on the parameters evaluated here, F3 was considered as the optimum MP formulations.

Furthermore, Figure S1.2 shows the microscopy images of F3 MP. Additionally, the formulation of F3 without PBA was also prepared. The images show the spherical shape of the MP. Importantly, the sizes of the microscopy images were in good agreement with the results from the particle size determination.

4.2.2 *In-vitro* Release Assay

A further validated UV-Vis spectrophotometer analysis method was applied to determine the amount of MTF released in an *in vitro* release assay. This test was carried out on GR-MP-MTF formulas F1, F2, F3, F4, F5 (the formulations with PBA), MP-MTF (formulation without PBA), and free MTF solution in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media. The results are revealed in Figure 5.1, showing that after 24 hours, the release of MTF in PBS medium was 13.87 ± 1.25%, 5.94 ± 0.53%, 5.77 ± 0.52%, 4.93 ± 0.44%, 4.02 ± 0.36% for the GR-MP-MTF formula F1, F2, F3, F4, F5, respectively, and 99.19 ± 10.91% and 98.19 ± 9.03% for pure MTF solution and MP-MTF formula (F3 without PBA), respectively. Specifically, the release of MTF in PBS medium from free MTF solution reached almost 100% in just 2 hours. The MTF release from MP formula without glucose responsive polymer reached almost 100% after 24 hours. On the other hand, the MTF release from GR-MP in all formulations was only less than 15% MTF after 24 hours.

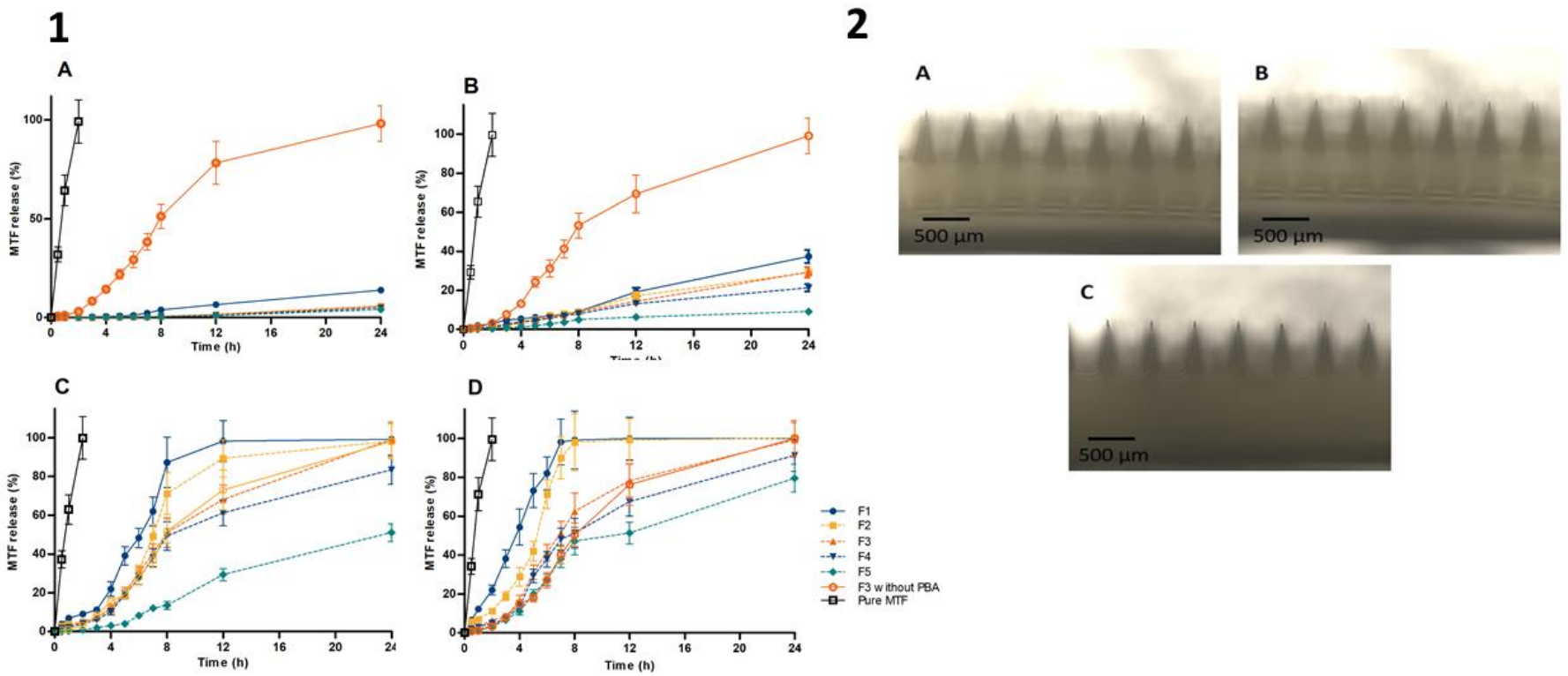
Importantly, the increased glucose concentration in the release medium led to the enhancement of release of MTF from the MP-MTF formulation, indicating the successful development of GR-MP. As shown in Figure 4.1, after 24 hours, MTF released from all GR-MP-MTF formulations reached almost 100%, namely 99.91 ± 8.99%, 99.98 ± 9.00%, 99.18 ± 8.93%, 91.24 ± 8.21%, and 79.54 ± 7.16% for F1, F2, F3, F4, and F5, respectively. This amount was not significantly different ($p > 0.05$) from MTF released from MP-MTF without PBA and pure MTF solution. The difference in the amount of MTF released in the GR-MP-MTF and MP-MTF without PBA in the PBS and the PBS with glucose media was due to the presence of PBA in the formulation. PBA is a GR material that is sensitive to changes in glucose levels where the increment of glucose levels causes a break in the bond between the phenylboronic-diol in PBA, which in turn causes the expansion of the polymer that binds to PBA and releases the MTF contained in the formulation [45]. Among all the MP formulations, with respect to the release pattern, F3 was considered as the optimum formulation. This was because due to the findings that F3 could control the release of MTF for up to 24 hours, reaching almost 100% of MTF released. On the other hand, F4 and F5 could only release around 80% of MTF after 24 h.

The results obtained from the *in vitro* release test were further fitted to five mathematical kinetic models to determine the MTF release model from the formulation. The values of the correlacy coefficient for the *in vitro* released of MTF from F3 in PBS were 0.8257, 0.8193, 0.4580, 0.9988, and 0.8215 for Zero order (ZO), First order (FO), Higuchi (H), Korsmeyer-Peppas (KP), and Hixson-Crowell (HC), respectively. In PBS containing 1% w/v glucose, the value of the correlacy coefficient were 0.9916, 0.9772, 0.7090, 0.9980, and 0.9829 for ZO, FO, H, KP, and HC, respectively. Regarding the release in PBS containing 2% w/v glucose, the values of the correlacy coefficient were found to be 0.9306, 0.9015, 0.7633, 0.9403, and 0.9333 for ZO, FO, H, KP, and HC, respectively. Finally, in PBS containing 4% w/v glucose, the values of the correlacy coefficient were 0.8391, 0.9099, 0.7995, 0.9006, 0.9358 for ZO, FO, H, KP, and HC, respectively. The result obtained show that *in vitro* release of MTF in PBS, PBS containing 1% w/v of glucose media and PBS containing 2% w/v of glucose media followed Korsmeyer-Peppas model. This kinetic model described the mechanism of drug release from the polymeric matrix. Meanwhile, *in vitro* release of MTF in PBS containing 4% w/v of glucose media followed Hixson-Crowell, which kinetic model to describe drug release from systems that has a change in surface area and diameter

of the particle, in the case of hydrophilic matrix swelling and erosion of the polymer occurs simultaneously [46]

4.2.3 DMN fabrication and drug content in MNs

Following the successful development of GR-MP, the formulations were further incorporated into DMNs to facilitate dermal delivery of MTF. Using the mixture of the aqueous gel of 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa), DMNs containing MPs possessed a complete and sharp needle shape (Figure 4.2) with adequate mechanical and insertion properties. As a control, MP without PBA and free MTF-loaded DMNs were also prepared. The combination of these polymers has shown the effectiveness of the DMNs formulation in numerous studies [47,48]. The validated spectrophotometer UV-Vis analysis method was further used to determine MTF content in the formula MP-MTF-PBA and MP-MTF without PBA. As shown in Figure 6.1, the percentage of MTF recoveries was found to be 98.12 ± 1.13%, 98.09 ± 1.59%, and 99.23 ± 2.01% for MP-MTF-PBA, MP-MTF (without PBA) and pure MTF. This result indicated that the formulation of MTF in MP form and combined with PBA did not affect the concentration of MTF in the DMN formulations. The recovery percentage of all formulas also fulfilled the acceptable recovery percentage from ICH, which is 95-105% [28].



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486 **Figure 4.** *In vitro* release profile of MTF from MP in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media
487 (C); PBS containing 4% w/v of glucose media (D) data (mean ± SD, *n*=3) (**1**). The microscope images of DMN containing F3 with PBA (A); F3 without PBA
488 (B); and pure MTF (**2**).

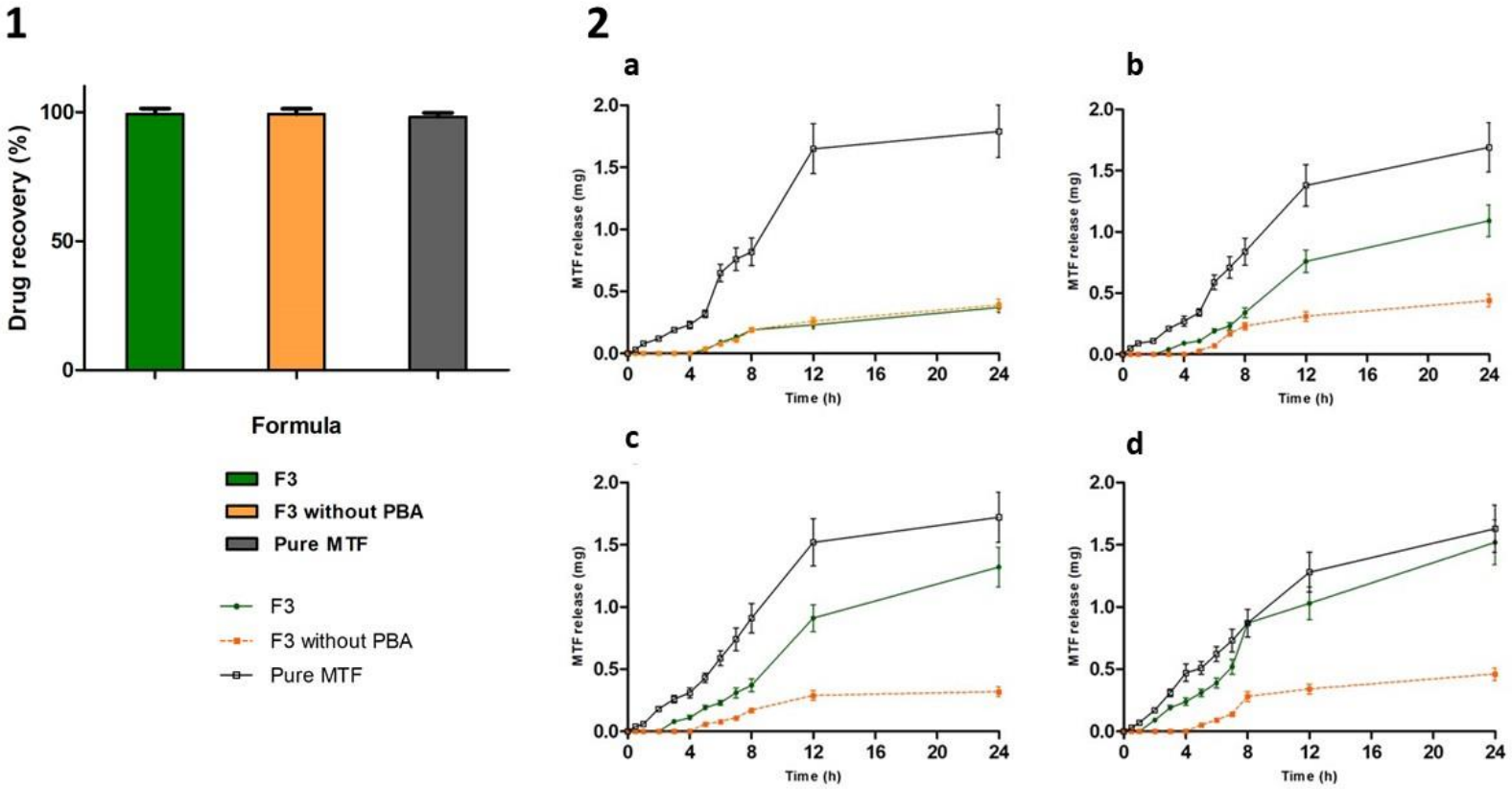

Figure 5. MTF recovery (%) from MN (**1**); Ex vivo permeation profile of MTF from DMN (B) in PBS media (a); PBS containing 1% w/v of glucose media (b); PBS containing 2% w/v of glucose media (c); PBS containing 4% w/v of glucose media (d) data (mean ± SD, *n*=3) (**2**).

4.2.4 *Ex vivo* Permeation Study

Ex vivo permeation test was carried out to investigate the penetration ability of MTF formulated into GR-MP-MTF, MP-MTF (without PBA), and pure MTF delivered using DMN. This evaluation was carried out to ensure the controlled release of MTF in the GR-MP-DMN formulation in *in vitro* hyperglycemic modeling media. Based on the results of the *ex vivo* permeation test in the four media presented in Figure 5.2, it was observed that the MTF permeation continued to increase with time. Specifically, the DMN containing the MP-MTF formulation (without PBA) showed a significant and similar increase in permeation in all media, indicating the non-specific release pattern of this formulation. Moreover, the DMNs containing pure MTF also showed non-specific release behavior, with the highest amount of MTF released over 24 h. On the other hand, importantly, the formulation of GR-MP into DMNs could potentially control the permeation of MTF. After 24 h, the MTF permeation in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media were 0.37 ± 0.04, 1.09 ± 0.13, 1.32 ± 0.16, and 1.52 ± 0.18 mg. This increase in the amount of permeable MTF indicated the successfulness of the GR-MP-MTF formulation in controlling the release of MTF according to the amount of glucose contained in the media as a hyperglycemic model This shows that the modification of the formula in the form of CS-MP can help the controlled release system in MTF [12].

Here, for the first time, we successfully applied a simple validated analytical method using UV-vis spectrophotometer to quantify MTF in the development of GR-MP loaded DMNs. The method was found to be valid with desired accuracy, precision and dilution integrity results. Importantly, the application of the method indicated the successfulness of the selective delivery the approach in the *in vitro* hyperglycemic condition. Accordingly, this approach could be an alternative of the oral administration of MTF. Moving forward, the effectiveness of this system should be evaluated in appropriate *in vivo* models with suitable analytical models.

5. Conclusion

This research was conducted with the aim of developing and validating a UV-vis spectrophotometric method for the analysis of MTF in the development of GR-MP loaded DMNs. The method used was validated with the parameters of selectivity, accuracy, precision, linearity, LOD and LLOQ, and dilution integrity. The method was validated in *in vitro* normal physiological

and hyperglycemic conditions using PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose. Based on all the validation parameter tests, this method was found to meet the requirements of the ICH guidelines, indicating that this analytical method was valid for the application of the drug development of MTF. Specifically, the validated analytical method was successfully applied to evaluate EE, DL, *in vitro* release profile in MP system, drug recovery and *ex vivo* permeation in DMNs system. The results from the application of the method showed that the incorporation of MTF into the combination of GR-MP and DMNs could potentially improve the selective delivery and control the release as well as the permeation profile in *in vitro* hyperglycemic conditions, making it as an innovative approach to overcome the problems in oral administration of MTF. To further evaluate the efficacy, as the next step, in *vivo* analytical method must now be developed.

Author contributions:

Sumayya Binti Abd Azis: Conceptualization, Methodology, Funding acquisition, Writing – original draft. **Nur Syafika:** Methodology, Writing – original draft. **Hanin Azka Qonita:** Methodology, Writing – original draft. **Ahmad Abizart:** Methodology, Data curation. **Tiara Resky Anugrah Mahmud:** Data curation, Validation **Andi Dian Permana:** Conceptualization, Project administration, Funding acquisition, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Application of validated spectrophotometric method to quantify metformin in the development of glucose-responsive microparticles loaded dissolving microneedles

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Highlights

- Spectrophotometric method was developed to determine metformin in *in vitro* hyperglycemic-mimicked conditions
- The spectrophotometric method was validated as per ICH guidelines
- The Spectrophotometric method was applied in the development of glucose-responsive microparticles loaded dissolving microneedles

ABSTRACT

Metformin (MTF) is a first-line drug in the treatment of type 2 diabetes mellitus. Delivered through the oral route, MTF has several limitations, mainly due to the side effects in gastrointestinal, non-specific release and low intestinal permeability, resulting in the low bioavailability of MTF in the body. Here, we developed glucose-responsive microparticles (GR-MP) containing MTF delivered *via* dissolving microneedles (DMNs) to overcome these limitations. To support the development of the formulation, in this study, a simple analytical method was developed using a UV-visible spectrophotometer. The method was validated in four different media, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose, to mimic the normal and hyperglycemic condition. The method was further validated as per International Conference Harmonization (ICH). This analytical method was applied to quantify the amount of MTF in the GR-MP preparation, *in vitro* release, drug content in DMNs and, importantly, *ex vivo* permeation study in *in vitro* hyperglycemic conditions. The results exhibited that the calibration curves in all media showed a correlation coefficient (R) of 0.998, indicating the linearity of the method. Moreover, LLOQ values in the four different media were 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL, and 2.88 µg/mL, respectively. Importantly, the method was precise and accurate with desired dilution integrity according to ICH, implying the validity of the methods. Finally, the method was successfully applied in the development of DMNs containing GR-MP of MTF, showing that the incorporation of MTF into this combination approach could selectively control the release of the drug according to the glucose concentration both in *in vitro* release and *ex vivo* permeation studies. Therefore, this approach could be a favorable system to solve the oral administration of MTF. Further *in vivo* analytical methods should now be developed to explore the effectiveness of this system in a suitable animal model.

Keywords: Metformin, UV-Vis spectrophotometric, Validation, Glucose-Responsive Microparticles, Dissolving Microneedles

1. Introduction

One of the biggest causes of death in the world is diabetes mellitus (DM). According to data from the International Diabetes Federation, 537 million people had DM in 2021, and it was estimated to increase to 783 million in 2045. DM causes various complications that were the direct cause of 1.5 million deaths worldwide in 2019. Specifically, approximately 90% of DM cases are type 2 DM (T2DM) [1].

The first-line treatment for T2DM is metformin (MTF) tablets administered orally. MTF has been shown to be most effective in lowering blood glucose levels. MTF works in lowering blood sugar levels through various mechanisms. Consequently, the use of MTF in high doses and in the long term can potentially increase the risk of hypoglycemia. Research shows that 112 out of 4072 cases of MTF overdose could trigger hypoglycemia, which could potentially lead to intolerance to MTF [2]. Other side effects associated with the oral MTF therapy can cause undesired impacts on the gastrointestinal tract. MTF also has low permeability to cell membranes and, therefore, the absorption of MTF given orally does not occur optimally [3]. This causes an increase in the accumulation of drugs in the intestines, resulting in some dangerous side effects [4]

To overcome these problems, it is crucial to design a smart delivery system to deliver MTF. Recently, the development of a glucose-responsive delivery system has attracted the interest of numerous researchers to selectively control the release of antidiabetic drugs. To the best of our knowledge, there has been no glucose-responsive system developed for MTF. In this study, we presented microparticles with glucose-responsive ability that could release MTF in the presence of glucose. Accordingly, this could be beneficial in preventing hypoglycemia [5]. Some compounds have been explored to possess this characteristic, including glucose oxidase (GOD), concanavalin A (Con A), and phenylboronic acid (PBA) [6]. Among these three compounds, the use of PBA is more frequent because it is lower cost, biodegradable, and easy to fabricate compared to GOD and Con A. Importantly, since PBA is not a protein like GOD and Con A, disadvantages such as poor volatile inactivation, and the high cost can be avoided [7].

In designing a controlled release form of drug delivery, the choice of polymer is one of the crucial things to consider [8]. There are many polymers that can be used in designing controlled release systems in the form of microparticles, one of which is a synthetic polymer in the form of poly(lactic) acid, which is a polymer with great potential, but controlling the particle size and drug adsorption efficiency of this polymer is quite difficult, and initial burst release may occur [9,10].

Another polymer that is usually used in the manufacture of microparticles is ethyl cellulose. However, the structure of ethyl cellulose which does not have a carboxyl group, makes PBA compounds unable to be linked to gelatin polymers and form polymer complexes that are responsive to glucose [11]

In this study, a polymer in the form of chitosan (CS) was chosen due to several benefits, namely being non-toxic, biodegradable, and biocompatible. These polymers also have unique physical and chemical characteristics, such as intermolecular hydrogen bonding and their polycationic charge under acidic conditions [12]. This leads us to the binding of the hydroxyl and amino groups present in the CS chain, which has a strong affinity for PBA. This binding resulted in a decrease in the pKa value of PBA and led to the manufacture of pH-responsive compounds, which has broad application prospects, causing PBA-CS combinations to achieve glucose sensitivity below the physiological pH of the human body [13]. Previously, several studies have been carried out in formulating metformin in the form of microparticles. One of them is research conducted by Avram et al 2017 who formulated MTF in the form of microparticles using a syringe technique using chitosan polymer [14,15]. However, the particles were not developed for selective delivery for hyperglycemic conditions. Therefore, further development is required to selectively release MTF based on glucose concentration.

As previously explained, the oral administration of MTF resulted in several side effects. As a result, modified transdermal delivery of MTF was chosen to overcome the side effects of MTF related to the low permeability of MTF to cell membranes, resulting in reduced bioavailability [16]. The choice of subcutaneous administration of antidiabetic drugs is commonly used, such as insulin and GLP-1 agonist drugs. Subcutaneous administration of drugs can certainly help overcome previous MTF problems, but new problems arise, such as discomfort to the patient, bleeding, infection at the injection site, and many more [17].

As an innovative delivery system, dissolving microneedles have been widely explored as an alternative delivery system to the injection route [18–20]. This system is applied intradermally which would dissolve when applied and release the active substances [21]. Therefore, the fabrication of DMN as a drug delivery system is an interesting solution for the oral therapy of MTF. The use of DMN as a drug delivery system not only solves the problems of administration using injection, but can also reduce sharp object waste after use because the needle used can dissolve due to its fabrication using water-soluble polymers [22]. However, the use of the DMN

system can have an impact on the difficulty of drug encapsulation and dose control. Therefore, the DMN system could be collaborated with GR-MP in order to obtain an efficient drug delivery system and controlled drug release [23]. In this study, MTF-loaded-GR-MP was further incorporated into DMN for a selective and efficient delivery system in the *in vitro* mimicking diabetes environment.

In the development of a new drug delivery system, various tests and characterizations are required. One of the critical points is analyzing the active compounds. In this study, with regard to the analysis process, MTF was analyzed in the development of GR-MP, *in vitro* testing, and *ex vivo* permeation test. With respect to the media used, in this study phosphate buffered saline (PBS) and PBS containing glucose media represented normal and diabetes conditions. Previously, there were studies that carried out the determination of pure MTF and from tablets quantitatively using the reversed phase-high performance liquid chromatography (RP-HPLC) method [24] and Ultra Violet (UV) spectrophotometer. However, the chromatographic method has drawbacks, such as requiring large costs, a lot of solvents, reliable power, and expensive instruments [25]. This could limit the application of the analytical method in the several laboratories which do not have access to use the HPLC. These studies also analyzed the MTF of tablet dosage forms and did not use specific media. It has been reported previously that the results of research conducted by Georgia et al., 2021 in quantifying irinotecan from human plasma using UV-vis spectrophotometric techniques show that this technique was still relevant and valid in analyzing drugs from blood plasma [26].

Considering several aspects mentioned previously, in this study, an analytical method of MTF was developed from GR-MP-DMN preparations in PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose mediums using a UV-Vis spectrophotometer. This analytical method has been widely used in the determination and has proven to be an analytical method that is simple, easy, and provides precise results in determining the number of samples [27]. Importantly, the application of a UV-Vis spectrophotometer has been widely used in almost all scientific laboratories, making it a versatile tool in the drug development. To ensure that the developed analytical method provides appropriate results, this study was also conducted involving the validation of the analytical method based on the International Conference Harmonization (ICH) guidelines. Method validation parameters such as linearity, accuracy, precision, limit of detection (LOD), and the determined limit of quantification (LOQ), and were extensively applied

in the determination of entrapment efficiency and drug loading in MP, drug content in DMN preparations, and *in vitro* and *ex vivo* permeation profiles.

2. Material and Methods

2.1 Materials

Metformin HCl was obtained from Tokyo Chemical Industry Co., LTD, Tokyo, Japan. Chitosan (medium molecular weight), glutaraldehyde polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), potassium dihydrogen phosphate (KH_2PO_4), glucose, potassium chloride (KCl), disodium phosphate (Na_2HPO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Singapore). All other reagents used in this study were analytical grade,

2.2 Preparation of PBS and PBS Containing Glucose

PBS was prepared by dissolving 0.2 g of KCl, 8 g of NaCl, 2.4 g of KH_2PO_4, and 1.44 g of Na_2HPO_4 with ± 800 mL CO_2-free water. Following the solubilization, the solution pH was set to 7.4. Finally, CO_2-free water was added to make up the final volume to 1 l. To prepare PBS containing glucose media, glucose with concentrations of 1% w/v, 2% w/v and 4% w/v were dissolved using PBS.

2.3 Preparation of MTF Stock Solution

The stock solution was prepared by dissolving 10 mg of MTF into 10 mL of different media separately to achieve 1000 µg/mL of MTF solution.

2.4 Determination of Maximum UV Absorption, Preparation of Calibration Solution, and Quality Control Solution

Initially, the stock solution of MTF in the respective media was diluted to achieve a concentration of 20 µg/mL. The determination of the maximum UV absorption in MTF in all media was carried out using a UV-vis spectrophotometer (Dynamica, HALO XB-10). Thereafter, the calibration solution in all media was prepared by diluting MTF stock solution using the respective media to achieve the serial concentrations of 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL and 0.5 µg/mL

Quality control solutions were prepared in three different concentrations, such as 12 µg/mL for high-quality control (HQC), 7.5 µg/mL for medium-quality control (MQC), and 4 µg/mL for low-quality control (LQC).

2.5 Validation Method

The UV-Vis spectrophotometer validation method was carried out by measuring the validation parameters, such as linearity, specificity, LOD and LOQ, dilution integrity, as well as accuracy and precision.

2.5.1 Linearity

Determination of linearity in the method validation was carried out by plotting the absorbance of three replications of the MTF calibration solution in three different media. From the curve results obtained, the correlation coefficient (R) was calculated. Linear parameters are considered valid if the value of R is close to 1 [28]

2.5.2 Specificity

The specificity needs to be known to ensure that there is no interference from other materials in the sample [28]. Specificity was determined by comparing the UV spectra of GR-MP blank, DMN blank, and MTF in both GR-MP and DMN system. The UV spectra was scanned between 200-400 nm.

2.5.3 Limit of Detection (LOD)

The detection limit (LOD) was investigated to determine the smallest amount of analyte that could show absorption or absorbance in the instrument without having accuracy and precision criteria. LOD was calculated by using equation 1. In the equation, 3.3 represents the factor for LOD, SD is the standard deviation of the blank, and b is the slope of the blank regression line [29]

$$LOD = \frac{3.3\ x\ SD}{b} \qquad \text{Equation 1}$$

2.5.4 Lower Limit of Quantification (LLOQ)

LOQ is the smallest amount of analyte that can still be measured for its absorbance using an instrument and has accuracy and precision criteria. LLOQ can be calculated by using equation 2. In the equation, 10 represents the factor for LLOQ, SD represents the standard deviation of the blank, and b represents the slope of the regression line [29]

$$LLOQ = \frac{10\ x\ SD}{b} \qquad \text{Equation 2}$$

2.5.5 Accuracy

Accuracy is a parameter that shows the degree of closeness of the analysis results to the actual analyte content. Accuracy is expressed as the percent recovery of the added analyte. The

accuracy test was carried out by comparing the MTF concentration in LLOQ, LQC, MQC and HQC solutions from the absorbance measurement results with the theoretical concentration, then the relative standard deviation (% RSD) was calculated. the %RSD value should not be more than 15% of the theoretical concentration [28]. Measurements were done intra-day and inter-day.

2.5.6 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision test was the same as in the previous accuracy test, where the concentrations of the absorbance measurement results of LLOQ, LQC, MQC, and HQC solutions were compared with the theoretical concentrations. The relative error value (%RE) was calculated, and the results obtained should not be more than 15% of the coefficient of variation (CV) [30]. Measurements were carried out intra-day and inter-day.

2.5.7 Dilution Integrity

Dilution integrity was carried out by preparing 75 µg/mL MTF in all media. Then each solution was diluted 5 and 10 times, the experiment was carried out in triplicate, and the absorbance of the analyte was observed [31].

3. Application

3.1 Microparticle Formulation

Microparticles were prepared using CS. In this study, 5 formulations were prepared containing 100 mg of MTF with different amounts of CS, namely 100 mg, 150 mg, 200 mg, 250 mg and 300 mg for F1, F2, F3, F4 and F5, respectively. GR-MTF-MP were prepared by mixing MTF and CS with 5 mg of EDTA, and added to 3 mL of acetic acid solution in water (1% v/v) under the stirring condition at 500 rpm at room temperature. After that, 6 mL of ethanol was added to make a cloudy solution which indicated the formation of MPs. After that, 50 µL of glutaraldehyde (25%) solution were added as a crosslinker by forming a reaction between the aldehyde group and the amino group of the MP. Furthermore, the MP formed were centrifuged at 3000 rpm for 20 minutes, and the sediment obtained was washed using distilled water to obtain pure MP CS [32].

To prepare GR-MP, PBA solution (11.2 mg) was dissolved in 1 mL of DMSO, and reacted with EDC.HCl (15.5 mg) and NHS (9.3 mg) for 30 minutes (mixture 1). After that, the mixture 1 solution was added to 5 mL of MP CS solution, while stirring at 37˚C for 24 hours. Then, the PBA-

decorated MP CS (MP PBA-CS) was dialyzed in distilled water for 48 h to remove unreacted PBA [32]. MP CS containing MTF was referred to as MP CS-MTF, and PBA-CS MPs containing MTF were referred to as MP PBA-CS-MTF. Particle size and polydispersity index (PDI) were all calculated.

3.2 Determination of Entrapment Efficiency and Drug Loading

The entrapment efficiency (EE) of MTF in MP was determined using the indirect method. In the washing steps, the supernatant was taken, and the concentration of MTF was calculated using a validated analytical method. Furthermore, the drug loading (DL) determination was carried out by mixing 50 mg of the formulation with 10 mL methanol. The mixture was sonicated for 30 minutes and diluted with PBS. ED and DL were calculated using the following calculations [33]:

$$\%EE = \frac{(Weight\ of\ initial\ drug - Weight\ of\ free\ drug)}{Weight\ of\ initial\ drug}\ x\ 100 \quad \text{Equation 3}$$

$$\%DL = \frac{Amount\ of\ entrapped\ drug\ in\ microparticle}{Total\ weight\ of\ microparticle}\ x\ 100 \quad \text{Equation 4}$$

3.3 *In vitro* Release Test

The *in vitro* release profile of MTF from MP was investigated using dialysis membrane method [21,33,34]. Briefly, MP formulations equal to 10 mg of MTF were placed inside dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO dialysis membrane). The membrane was further immersed into 100 mL of release media. Three different media were used, namely PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose. The study was carried out in an orbital shaker at 100 rpm at 37°C. The media (1 mL) was sampled at certain time intervals, and then the concentration of MTF was determined using a UV-Vis spectrophotometer. Fresh media was added after the sampling to ensure the sink condition during the study. The drug release mechanism was then analyzed using a variation of the mathematical kinetic model [33]

3.4 Mathematical Modelling for *In Vitro* Release Test

The data obtained from the *in vitro* assays were then fitted into five different mathematical models to determine the release kinetics of MTF from MP. The models applied were zero-order kinetics (Z0), first-order kinetics (F0), Krosmeyer-Peppas (KP), Higuchi, and Hixson-Crowell (HC). The equations of each model are described below:

$$Zero\ order\ kinetics: C_t = C_0 + K_0 t$$

$$First\ order\ kinetics: lnC_t = lnC_0 + k_1 t$$

$$Krosmeyer - Peppas\ model: C_t = k_{KP} t^n$$

$$Higuchi\ model: C_t = k_H \sqrt{t}$$

$$Hixson - Crowell\ model: C_t^{\frac{1}{3}} = C_0^{\frac{1}{3}} k_{HC} t$$

C_t represents the concentration of MTF at time t, C_0 represents the initial concentration of MTF in the medium (t = 0), k_0 represents the zero-order constant, k1 represents the first-order constant, k_{KP} represents the Korsmeyer–Peppas constant, k_H represents the Higuchi constant, and k_{HC} represents the Hixson constant. - Crowell. All calculations were performed using the DD-solver software. The release kinetics is determined from the correlation coefficient value (R) obtained [31]

3.5 DMN Fabrication and Determination of Drug Content

In this study, two-layered DMNs containing MP CS-MTF and MP PBA-CS-MTF were fabricated using the centrifugation method [35]. The formulation contained the aqueous gel of 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa) in distilled water mixed with 30% w/w of MP. Initially, 100 mg formulation was poured on the top of DMN MN silicon mould (needle density 10 x 10, the pyramidal needle with 700 µm of high and 200 µm wide on the base and 200 µm of spacing). Thereafter, the mould was centrifuged at 3000 rpm for 15 min at room temperature. The excess of the formulation was removed and dried for 6 h. Following this, an aqueous gel containing 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa) was poured as a second layer. The formulation was dried at room temperature for 24 h and removed from the mould. It was important to note that the DMNs used in this study possessed adequate mechanical and insertion properties.

In an attempt to measure the MTF content in DMNs, the formulation was initially dispersed in 5 mL of distilled water. Afterwards, the dispersion was mixed with 10 mL of methanol and sonicated for 30 min. The mixture was then centrifuged for 10 minutes at a speed of 5000 rpm. The supernatant was collected, and the absorbance was measured using the UV-Vis spectrum.

3.6 *Ex Vivo* Permeation Studies

Ex vivo permeation studies were performed using Franz diffusion cells, using rat skin [36–38]. PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose were used as the

release medium. Prior to the experiment, skin was washed and soaked in the release medium for 30 minutes. Afterwards, the surface of the skin was dried, and the skin was placed between the donor and recipient compartments. The experiment was conducted at 100 rpm at 37°C. During the study, the sampling was carried out at several time intervals, starting from 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours by taking 1 mL in the receiving compartment, then replaced with the same volume of new media. The samples were then analyzed using UV-Vis spectrophotometry.

3.7 Statistical analysis

All data obtained were expressed in mean ± standard deviation (SD), all values were obtained using Microsoft excel® 2019 software (Microsoft Corporation, Redmond, USA). Graphpad Prism® version 6 (GraphPad Software, San Diego, California, USA) was used to analyze the data statistically, where statistical significance was indicated by p value < 0.05.

4. Results and Discussion

4.1 Validation of analytical method

4.1.1 Determination of Maximum UV Absorption and Specificity

The purpose of this study was to validate the UV-vis spectrophotometer as an instrument that allow the quantification of MTF in the development of a new dosage form, namely in the form of GR incorporated into DMN. Measurement of MTF levels was carried out in PBS media as a model medium for normal condition and PBS containing different concentration of glucose media as a model medium for hyperglycemia condition [39]. The results showed that MTF exhibited maximum absorption at 234 nm in all media (Figure 1). Accordingly, the wavelength was used in the further steps in this study.

Specificity parameter was intended to ensure that the MTF analysis using a UV-Vis spectrophotometer from the MP and DMN formulations did not experience interference from other compounds. As shown in Figure 2, the measurement results of the blank MP and MN showed a peak in the range of 210-220 nm, and did not indicate a possible interference at the MTF peak at 234 nm. In addition, the MTF peaks in both the MP and DMN formulas showed the same peaks as the pure MTF solution, which indicated that there was no peak shift due to additives or solvents used in the formulation. Therefore, the method developed in this study has been specific to the appropriate wavelength.

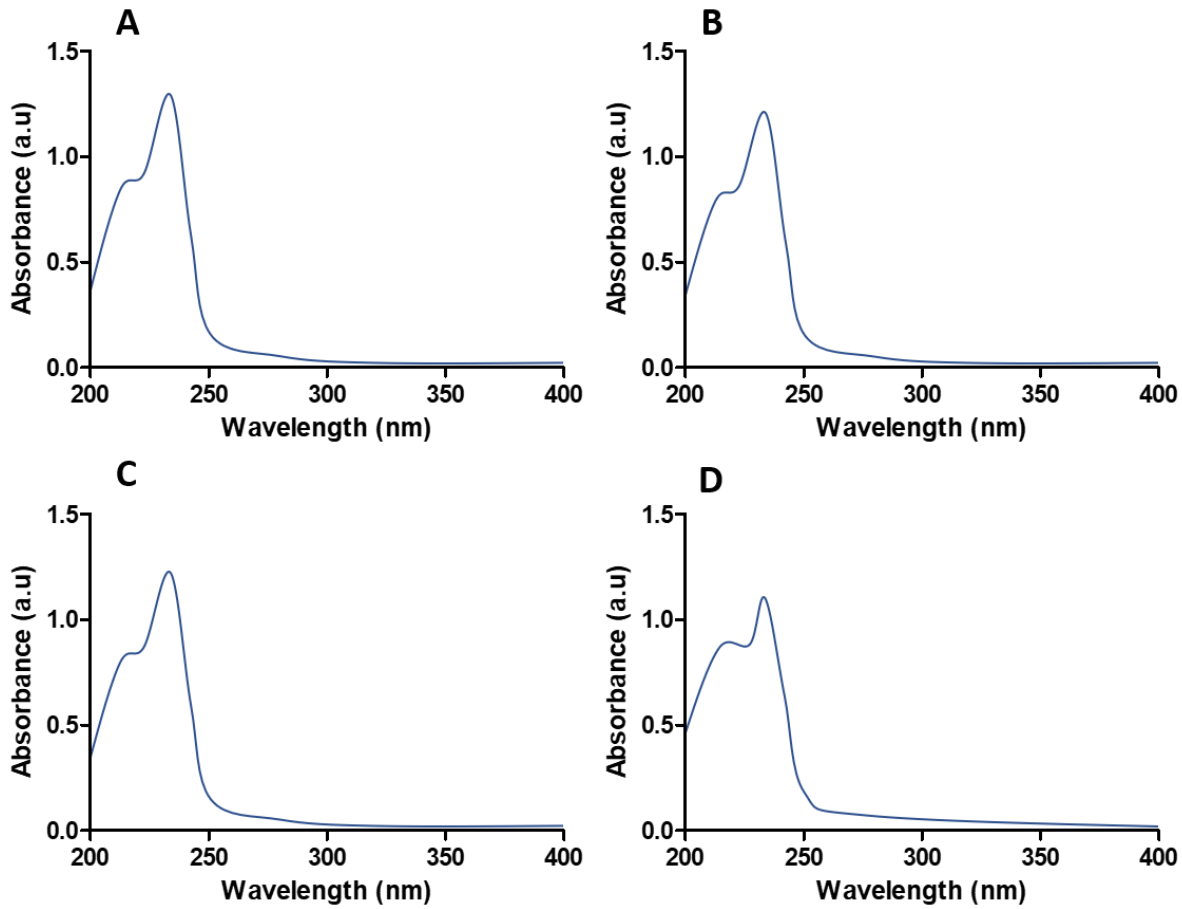


Figure 1. Maximum absorbance of MTF in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

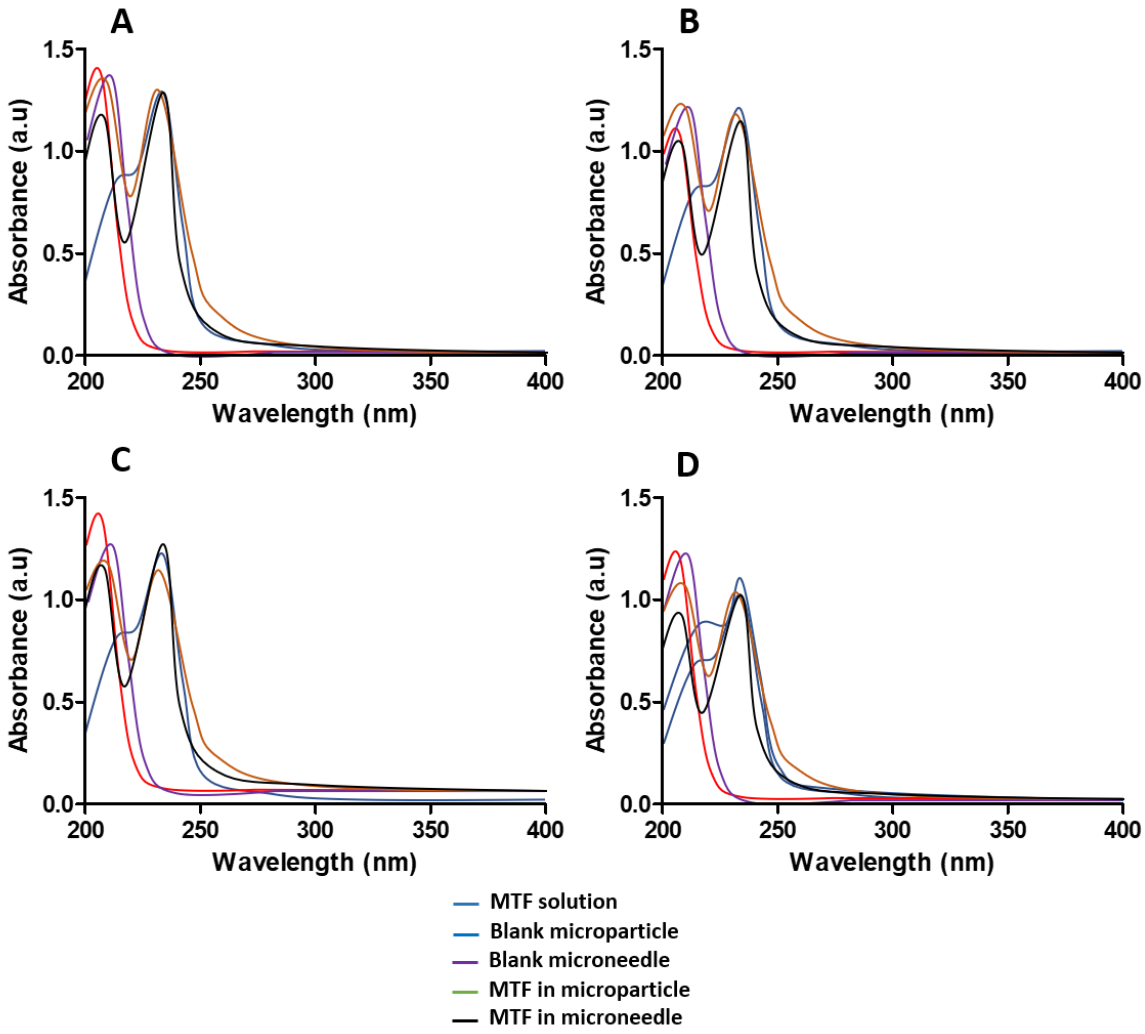


Figure 2. Representative UV-Spectra of pure MTF, blank MP, blank DMN, MTF in MP, and MTF in DMN in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

4.1.2 Linearity, LOD and LOQ

Linearity parameter is one of the characteristics required in the validation process of an analytical method. Based on the ICH guidelines, linearity needs to be evaluated by plotting a function of analyte concentration with the absorbance results obtained, then evaluated by mathematical modeling. Determination of linearity parameters from the validation of this method was carried out by measuring the absorbance of MTF in the concentration range of 0.5-16 µg/mL in all media. The spectrum of MTF in all media tested in various concentrations are depicted in Figure 3. The linearity acceptance criteria for active pharmaceutical ingredients is $R > 0.998$ [40]. The results of the linearity measurement of MTF showed the correlation coefficient value of

0.9983 for PBS media, 0.9991 for PBS containing 1% w/v of glucose media, 0.9991 for PBS containing 2% w/v of glucose media, and 0.9981 for PBS containing 1% w/v of glucose media which of these four values met the criteria for linearity parameters.

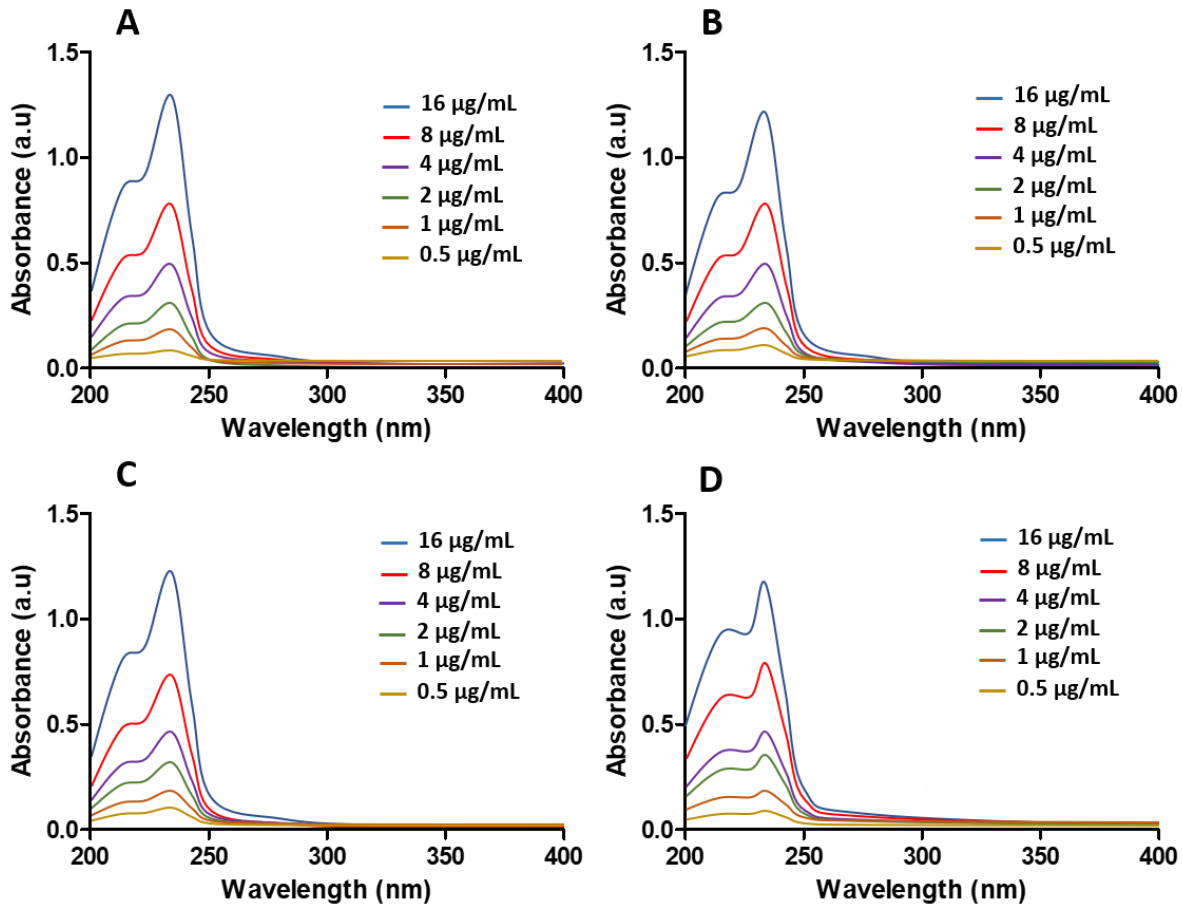


Figure 3. Spectrum of MTF standard solution in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D).

The LOD and LLOQ values in all media were calculated from the calibration data, as shown in Table 1. The LOD MTF values in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media were found to be 2.23 µg/mL, 1.95 µg/mL, 1.94 µg/mL and 2.88 µg/mL respectively. LLOQ MTF values PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose were calculated as 0.74 µg/mL, 0.64 µg/mL, 0.64 µg/mL, and 0.95 µg/mL respectively.

Table 1. The calibration curve properties of MTF in different media with LOD and LOQ values of MTF

Media	Concentration range (µg/mL)	R	LOD (µg/mL)	LLOQ (µg/mL)
PBS	0.5-16	0.9989	0.74	2.23
PBS with 1% glucose	0.5-16	0.9991	0.64	1.95
PBS with 2% glucose	0.5-16	0.9991	0.64	1.94
PBS with 4% glucose	0.5-16	0.9981	0.95	2.88

4.1.3 Precision and Accuracy

Precision and accuracy were assessed for intra-day and inter-day. Intra-day determination was carried out to evaluate the repeatability of this analytical method, whereas inter-day determination was conducted to investigate the variation of the day to the measurement. Precision and accuracy were analyzed in four different concentrations consisting of QC sample (HQC, MQC, and LQC) and LOQ sample which were measured in three replications respectively in one day for inter-day determination and three replications in three days for intra-day determination.

Precision was aimed to evaluate the closeness between a series of measurements from the homogenous sample under the same condition. The results were presented as %RSD values and are shown in Table S1, Table S2, Table S3, and Table S4 for PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media, and PBS containing 4% w/v of glucose media, respectively. It was found that all of the %RSD values were less than 15% and, thus, fulfilled the requirement from ICH [41–43]. As the analytical method was considered to be precise when %RSD is less than 15%, this UV-Vis spectrophotometry method was therefore considered to have precision values to quantify MTF in all media used in this study.

Accuracy was carried out to evaluate the closeness between true value and value found from measurements. The accuracy values were reported as %RE. Table S1, Table S2, Table S3, and Table S4 show the accuracy of UV-Vis spectrophotometry method for MTF in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media, respectively. The analytical method was considered to be accurate when the %RE values are ± 15% [28]. All the values were found to be ± 15%; therefore, the analytical method was considered accurate.

4.1.4 Dilution Integrity

Dilution integrity is the ability rate of the dilution process performed during the validation process as accurate, precise, and reliable [28]. Based on the dilution integrity data obtained in Table 2, it was found that the results showed the satisfactory results, where the dilution integrity bias on all media was less than 15%. The precision parameter observed from the %RSD with values ranging from 1.62% - 4.68% indicated that the dilution in this validation method was accurate and precise. In addition, it also implied that the analysis using this method can still be carried out on MTF with concentrations higher than the upper range of the calibration standard by using the appropriate dilutions.

Table 2. Dilution integrity data of UV-Vis spectrophotometry method for MTF in all media (mean ± SD, *n*=3)

Media	Dilution tested	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	%RSD	%RE
PBS	10	7.5	8.26 ± 0.15	1.87	-0.39
	5	15	15.36 ± 0.51	3.30	0.31
PBS with 1% of glucose	10	7.5	7.28 ± 0.30	4.11	-1.62
	5	15	14.25 ± 0.52	3.64	3.45
PBS with 2% of glucose	10	7.5	7.75 ± 0.36	4.68	-0.11
	5	15	14.76 ± 0.71	4.79	3.47
PBS with 4% of glucose	10	7.5	7.49 ± 0.12	1.62	1.66
	5	15	15.09 ± 0.64	4.23	3.22

4.2 Application of the analytical method

4.2.1 Entrapment Efficiency and Drug Loading

Following the successful validation of the spectrophotometric method, it was applied to characterize the MP's EE and DL capacities. In this study, in an attempt to achieve optimum parameters, we investigated five different concentrations of CS. The results of the characterization are depicted in Figure S1.1. With regard to the particle size, it was found that the increase of CS concentration could increase the particle size of the formulation. It might be due to the increased CS concentration resulting in a higher viscosity of the medium, leading to the decrease of the energy to break the droplet into a smaller size. This phenomenon was also observed in numerous studies investigating the concentration of the polymer in the size of micro/nanoparticles [30,44]. The results showed that the particle size of F1, F2, F3, F4 and F5 were 2.87 ± 0.21 µm, 3.09 ±

0.28 µm, 4.87 ± 0.31 µm, 7.09 ± 0.59 µm and 10.19 ± 0.92 µm, respectively. Regarding the PDI values, despite the difference in size, all formulations exhibited a narrow distribution pattern. Furthermore, the EE evaluation results showed the improvement of EE values following the increment of the CS concentrations. Following the application of the validated method, it was calculated that EE values were 27.49 ± 3.12% for F1, 39.16 ± 4.01% for F2, 53.19 ± 5.84% for F3, 55.18 ± 4.14% for F4, 58.42 ± 5.19% for F5. Furthermore, the DL values were 12.19 ± 1.31%, 13.92 ± 1.44%, 15.01 ± 1.59%, 14.39 ± 1.48% and 12.01 ± 1.37% for F1, F2, F3, F4 and F5, respectively. Analyzed statistically, statistical differences ($p < 0.05$) between the EE and the DL values in F1, F2 and F3 showed that the increase in CS concentration could potentially increase the EE and the DL of MP. However, the improvement of CS concentration in F3 and F4 did not significantly increase both parameters. Accordingly, considering the less amount of CS used in F3 compared to F4 and F5, based on the parameters evaluated here, F3 was considered as the optimum MP formulations.

Furthermore, Figure S1.2 shows the microscopy images of F3 MP. Additionally, the formulation of F3 without PBA was also prepared. The images show the spherical shape of the MP. Importantly, the sizes of the microscopy images were in good agreement with the results from the particle size determination.

4.2.2 *In-vitro* Release Assay

A further validated UV-Vis spectrophotometer analysis method was applied to determine the amount of MTF released in an *in vitro* release assay. This test was carried out on GR-MP-MTF formulas F1, F2, F3, F4, F5 (the formulations with PBA), MP-MTF (formulation without PBA), and free MTF solution in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media. The results are revealed in Figure 5.1, showing that after 24 hours, the release of MTF in PBS medium was 13.87 ± 1.25%, 5.94 ± 0.53%, 5.77 ± 0.52%, 4.93 ± 0.44%, 4.02 ± 0.36% for the GR-MP-MTF formula F1, F2, F3, F4, F5, respectively, and 99.19 ± 10.91% and 98.19 ± 9.03% for pure MTF solution and MP-MTF formula (F3 without PBA), respectively. Specifically, the release of MTF in PBS medium from free MTF solution reached almost 100% in just 2 hours. The MTF release from MP formula without glucose responsive polymer reached almost 100% after 24 hours. On the other hand, the MTF release from GR-MP in all formulations was only less than 15% MTF after 24 hours.

Importantly, the increased glucose concentration in the release medium led to the enhancement of release of MTF from the MP-MTF formulation, indicating the successful development of GR-MP. As shown in Figure 4.1, after 24 hours, MTF released from all GR-MP-MTF formulations reached almost 100%, namely 99.91 ± 8.99%, 99.98 ± 9.00%, 99.18 ± 8.93%, 91.24 ± 8.21%, and 79.54 ± 7.16% for F1, F2, F3, F4, and F5, respectively. This amount was not significantly different (p > 0.05) from MTF released from MP-MTF without PBA and pure MTF solution. The difference in the amount of MTF released in the GR-MP-MTF and MP-MTF without PBA in the PBS and the PBS with glucose media was due to the presence of PBA in the formulation. PBA is a GR material that is sensitive to changes in glucose levels where the increment of glucose levels causes a break in the bond between the phenylboronic-diol in PBA, which in turn causes the expansion of the polymer that binds to PBA and releases the MTF contained in the formulation [45]. Among all the MP formulations, with respect to the release pattern, F3 was considered as the optimum formulation. This was because due to the findings that F3 could control the release of MTF for up to 24 hours, reaching almost 100% of MTF released. On the other hand, F4 and F5 could only release around 80% of MTF after 24 h.

The results obtained from the *in vitro* release test were further fitted to five mathematical kinetic models to determine the MTF release model from the formulation. The values of the correlacy coefficient for the *in vitro* released of MTF from F3 in PBS were 0.8257, 0.8193, 0.4580, 0.9988, and 0.8215 for Zero order (ZO), First order (FO), Higuchi (H), Korsmeyer-Peppas (KP), and Hixson-Crowell (HC), respectively. In PBS containing 1% w/v glucose, the value of the correlacy coefficient were 0.9916, 0.9772, 0.7090, 0.9980, and 0.9829 for ZO, FO, H, KP, and HC, respectively. Regarding the release in PBS containing 2% w/v glucose, the values of the correlacy coefficient were found to be 0.9306, 0.9015, 0.7633, 0.9403, and 0.9333 for ZO, FO, H, KP, and HC, respectively. Finally, in PBS containing 4% w/v glucose, the values of the correlacy coefficient were 0.8391, 0.9099, 0.7995, 0.9006, 0.9358 for ZO, FO, H, KP, and HC, respectively. The result obtained show that *in vitro* release of MTF in PBS, PBS containing 1% w/v of glucose media and PBS containing 2% w/v of glucose media followed Korsmeyer-Peppas model. This kinetic model described the mechanism of drug release from the polymeric matrix. Meanwhile, *in vitro* release of MTF in PBS containing 4% w/v of glucose media followed Hixson-Crowell, which kinetic model to describe drug release from systems that has a change in surface area and diameter

of the particle, in the case of hydrophilic matrix swelling and erosion of the polymer occurs simultaneously [46]

4.2.3 DMN fabrication and drug content in MNs

Following the successful development of GR-MP, the formulations were further incorporated into DMNs to facilitate dermal delivery of MTF. Using the mixture of the aqueous gel of 15% w/w of PVA (31–50 kDa) and 25% w/w of PVP (58 kDa), DMNs containing MPs possessed a complete and sharp needle shape (Figure 4.2) with adequate mechanical and insertion properties. As a control, MP without PBA and free MTF-loaded DMNs were also prepared. The combination of these polymers has shown the effectiveness of the DMNs formulation in numerous studies [47,48]. The validated spectrophotometer UV-Vis analysis method was further used to determine MTF content in the formula MP-MTF-PBA and MP-MTF without PBA. As shown in Figure 6.1, the percentage of MTF recoveries was found to be 98.12 ± 1.13%, 98.09 ± 1.59%, and 99.23 ± 2.01% for MP-MTF-PBA, MP-MTF (without PBA) and pure MTF. This result indicated that the formulation of MTF in MP form and combined with PBA did not affect the concentration of MTF in the DMN formulations. The recovery percentage of all formulas also fulfilled the acceptable recovery percentage from ICH, which is 95-105% [28].

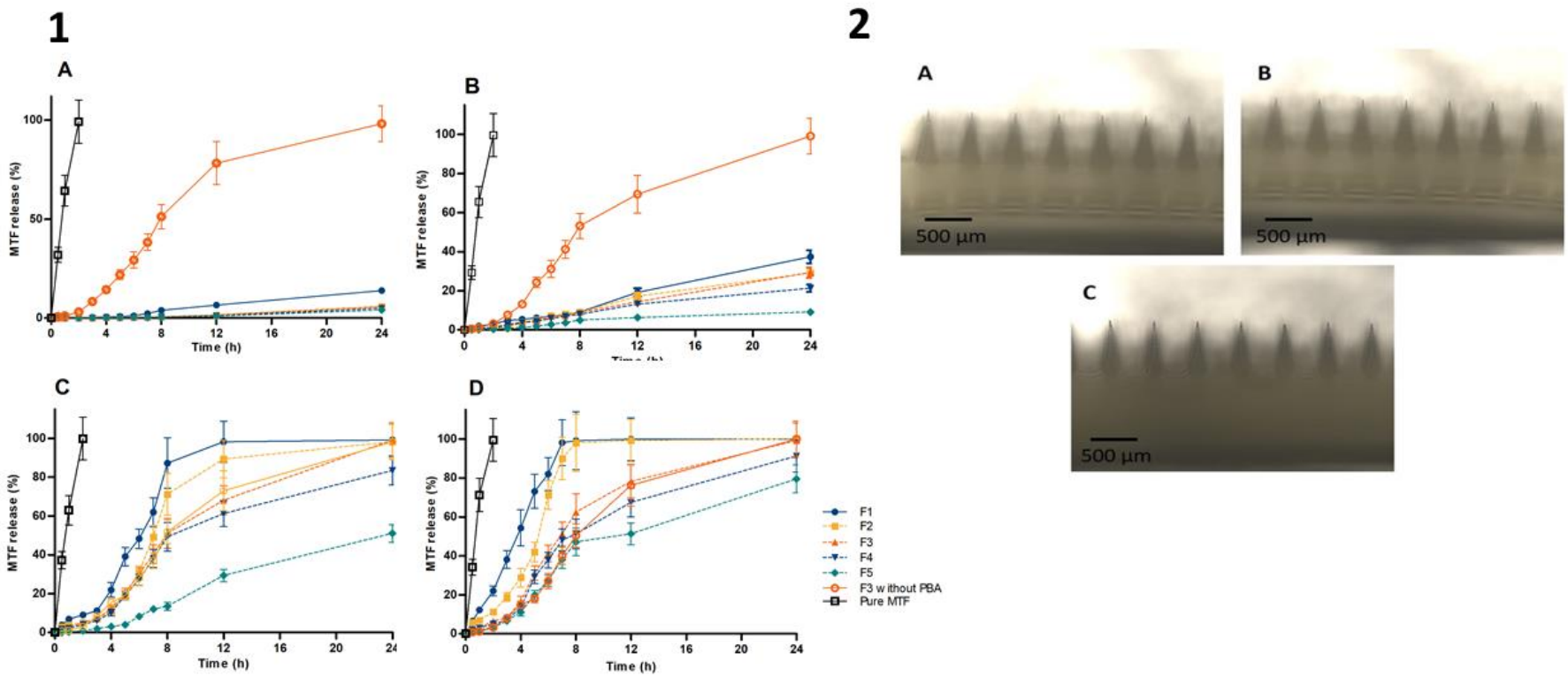
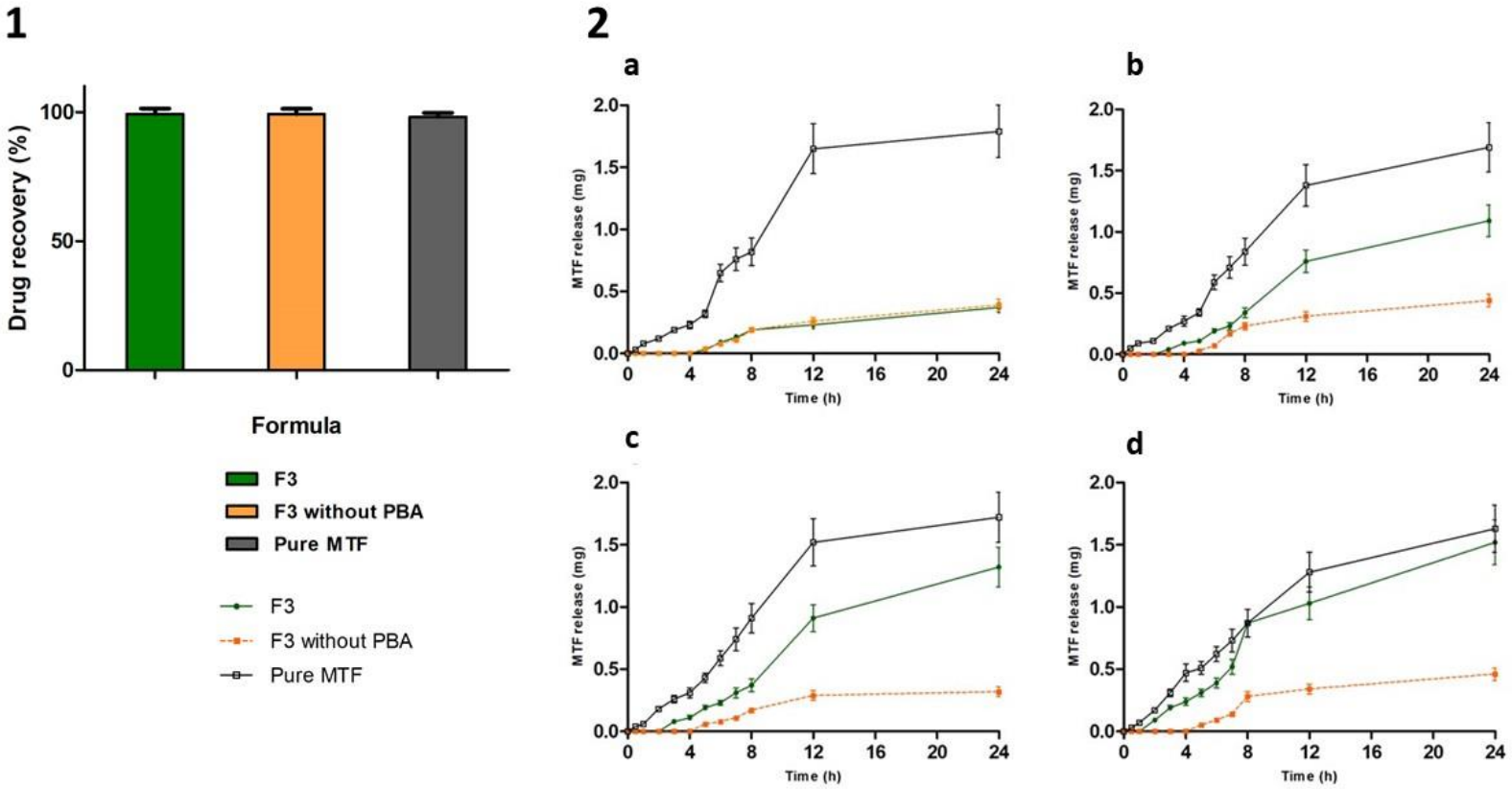


Figure 4. *In vitro* release profile of MTF from MP in PBS media (A); PBS containing 1% w/v of glucose media (B); PBS containing 2% w/v of glucose media (C); PBS containing 4% w/v of glucose media (D) data (mean ± SD, *n*=3) (**1**). The microscope images of DMN containing F3 with PBA (A); F3 without PBA (B); and pure MTF (**2**).



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490 **Figure 5.** MTF recovery (%) from MN (**1**); Ex vivo permeation profile of MTF from DMN (B) in PBS media (a); PBS containing 1% w/v of glucose media (b);
491 PBS containing 2% w/v of glucose media (c); PBS containing 4% w/v of glucose media (d) data (mean ± SD, *n*=3) (**2**).

4.2.4 *Ex vivo* Permeation Study

Ex vivo permeation test was carried out to investigate the penetration ability of MTF formulated into GR-MP-MTF, MP-MTF (without PBA), and pure MTF delivered using DMN. This evaluation was carried out to ensure the controlled release of MTF in the GR-MP-DMN formulation in *in vitro* hyperglycemic modeling media. Based on the results of the *ex vivo* permeation test in the four media presented in Figure 5.2, it was observed that the MTF permeation continued to increase with time. Specifically, the DMN containing the MP-MTF formulation (without PBA) showed a significant and similar increase in permeation in all media, indicating the non-specific release pattern of this formulation. Moreover, the DMNs containing pure MTF also showed non-specific release behavior, with the highest amount of MTF released over 24 h. On the other hand, importantly, the formulation of GR-MP into DMNs could potentially control the permeation of MTF. After 24 h, the MTF permeation in PBS media, PBS containing 1% w/v of glucose media, PBS containing 2% w/v of glucose media and PBS containing 4% w/v of glucose media were 0.37 ± 0.04, 1.09 ± 0.13, 1.32 ± 0.16, and 1.52 ± 0.18 mg. This increase in the amount of permeable MTF indicated the successfulness of the GR-MP-MTF formulation in controlling the release of MTF according to the amount of glucose contained in the media as a hyperglycemic model This shows that the modification of the formula in the form of CS-MP can help the controlled release system in MTF [12].

Here, for the first time, we successfully applied a simple validated analytical method using UV-vis spectrophotometer to quantify MTF in the development of GR-MP loaded DMNs. The method was found to be valid with desired accuracy, precision and dilution integrity results. Importantly, the application of the method indicated the successfulness of the selective delivery the approach in the *in vitro* hyperglycemic condition. Accordingly, this approach could be an alternative of the oral administration of MTF. Moving forward, the effectiveness of this system should be evaluated in appropriate *in vivo* models with suitable analytical models.

5. Conclusion

This research was conducted with the aim of developing and validating a UV-vis spectrophotometric method for the analysis of MTF in the development of GR-MP loaded DMNs. The method used was validated with the parameters of selectivity, accuracy, precision, linearity, LOD and LLOQ, and dilution integrity. The method was validated in *in vitro* normal physiological

and hyperglycemic conditions using PBS, PBS containing 1% w/v glucose, 2% w/v glucose and 4% w/v glucose. Based on all the validation parameter tests, this method was found to meet the requirements of the ICH guidelines, indicating that this analytical method was valid for the application of the drug development of MTF. Specifically, the validated analytical method was successfully applied to evaluate EE, DL, *in vitro* release profile in MP system, drug recovery and *ex vivo* permeation in DMNs system. The results from the application of the method showed that the incorporation of MTF into the combination of GR-MP and DMNs could potentially improve the selective delivery and control the release as well as the permeation profile in *in vitro* hyperglycemic conditions, making it as an innovative approach to overcome the problems in oral administration of MTF. To further evaluate the efficacy, as the next step, in *vivo* analytical method must now be developed.

Author contributions:

Sumayya Binti Abd Azis: Conceptualization, Methodology, Funding acquisition, Writing – original draft. **Nur Syafika:** Methodology, Writing – original draft. **Hanin Azka Qonita:** Methodology, Writing – original draft. **Ahmad Abizart:** Methodology, Data curation. **Tiara Resky Anugrah Mahmud:** Data curation, Validation **Andi Dian Permana:** Conceptualization, Project administration, Funding acquisition, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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