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An analytical of an experimental method for evaluation and validation from measuring dissolution of Metformin and Empagliflozin for generic tablets: Confirmation the results by docking method

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Summary

Aim: Validation for assay and dissolution of metformin and Empagliflozin in tablet 500:5 mg for evaluating the efficacy and safety of linagliptin vs. placebo in patients with type 2 diabetes. **Materials and methods:** The HPLC system used was Shimadzu LC-2010 *HT* with UV detector and Empagliflozin standard stock 1: 28 mg of Empagliflozin was accurately prepared. Separately 10 µL of standard solution and sample solution was injected into the chromatographic system. Patients with inadequate glycaemia control despite stable-dose metformin received open-label Empagliflozin as add-on therapy for several weeks. All patients continued treatment with metformin and Empagliflozin or metformin and Empagliflozin. The primary endpoint was change from baseline. **Results:** The experiments were significantly

exhibited a reducing the problem in patients with type 2 diabetes. In addition, results of docking method confirmed also the effectiveness of Metformin/ Empagliflozin tablets to better controlling of diabete-type-2 disease. **Conclusions**: Empagliflozin in Metformin/Empagliflozin for 24 weeks improved glycaemia control and was well tolerated.

Keywords: Metformin; Empagliflozin; type 2 diabetes; HPLC; glycaemia control; docking simulation; molecular dynamic; drug design.

Resumen

Análisis de un método experimental para la evaluación y validación de la medición de la disolución de metformina y empagliflozina en comprimidos genéricos: confirmación de los resultados mediante el método de acoplamiento

Objetivo: Validación del ensayo y disolución de metformina y empagliflozina en comprimidos de 500:5 mg para evaluar la eficacia y seguridad de la linagliptina frente a placebo en pacientes con diabetes tipo 2. **Materiales y métodos:** El sistema HPLC utilizado fue Shimadzu LC-2010 HT con detector UV y para la solución madre estándar de empagliflozina 1: se prepararon con precisión 28 mg de empagliflozina. Se inyectaron por separado 10 µL de solución estándar y la solución de muestra en el sistema cromatográfico. Los pacientes con un control inadecuado de la glucemia a pesar de la administración de metformina en dosis estables recibieron empagliflozina de etiqueta abierta como terapia complementaria durante varias semanas. Todos los pacientes continuaron el tratamiento con metformina y empagliflozina o metformina y empagliflozina. El criterio de valoración principal fue el cambio con respecto al valor inicial. **Resultados:** Los experimentos mostraron una reducción significativa del problema en pacientes con diabetes tipo 2. Además, los resultados del método de acoplamiento confirmaron también la eficacia de los comprimidos de metformina/empagliflozina para controlar mejor la enfermedad diabética tipo 2. **Conclusiones:** La empagliflozina en combinación con metformina/empagliflozina durante 24 semanas mejoró el control de la glucemia y fue bien tolerada.

Palabras clave: metformina; empagliflozina; diabetes tipo 2; HPLC; control de la glucemia; simulación de acoplamiento; dinámica molecular; diseño de fármacos.

Resumo

Uma análise de um método experimental para avaliação e validação da medição da dissolução de Metformina e Empagliflozina para comprimidos genéricos: Confirmação dos resultados pelo método de encaixe

Objetivo: Validação para ensaio e dissolução de metformina e Empagliflozina em comprimido 500:5 mg para avaliar a eficácia e segurança da linagliptina vs. placebo em pacientes com diabetes tipo 2. **Materiais e métodos:** O sistema HPLC usado foi Shimadzu LC-2010 HT com detector UV e estoque padrão de Empagliflozina 1: 28 mg de Empagliflozina foram preparados com precisão. Separadamente, 10 µL de solução padrão e solução de amostra foram injetados no sistema cromatográfico. Pacientes com controle glicêmico inadequado, apesar da metformina em dose estável, receberam Empagliflozina de rótulo aberto como terapia complementar por várias semanas. Todos os pacientes continuaram o tratamento com metformina e Empagliflozina ou metformina e Empagliflozina. O desfecho primário foi a mudança da linha de base. **Resultados:** Os experimentos exibiram significativamente uma redução do problema em pacientes com diabetes tipo 2. Além disso, os resultados do método de encaixe confirmaram também a eficácia dos comprimidos de Metformina/Empagliflozina para melhor controle da doença diabética tipo 2. **Conclusões:** A empagliflozina em Metformina/Empagliflozina por 24 semanas melhorou o controle da glicemia e foi bem tolerada.

Palavras-chave: Metformina; Empagliflozina; diabetes tipo 2; HPLC; controle da glicemia; simulação de encaixe; dinâmica molecular; design de fármacos.

Introduction

Analytical Method

Analytical chemistry offers with the look at of the separation, quantification, and chemical identity pharmaceutical analysis could be very crucial within the dedication of prescription drugs formula and bulk drugs regarding quality manage and high-quality guarantee. The quick increase in pharmaceutical industries of herbal and non-natural substances composed with one or more compounds or element. It is categorized into two main types, a qualitative analysis that is concerned with the identification of chemical compounds present in the sample whereas quantitative the analysis determines the

number of certain elements or compounds in the sample and the producing of drugs in and around the sector cells searching for novel and advance analytical techniques in prescribed drugs. As a result, analytical method development has become a popular activity of analysis. Development in scientific and advanced analytical techniques is due to the advancement of analytical instruments [1-3]. The aim of the present work was to develop an accurate and sensitive HPLC analytical method validation for assay and dissolution of metformin and Empagliflozin in metformin/Empagliflozin tablet 500:5 mg that might be improved glycaemia controlling. Here we also described the optimization of the instrumental parameters as well as the extraction procedure for human type 2 diabetes disease through dissolution of Metformin and Empagliflozin in Metformin/Empagliflozin. The method had been validated by evaluating the precision, accuracy and other validation parameters [3-7].

Metformin mono-therapy

Metformin mono-therapy, the recommended first-line pharmacotherapy for patients with type 2 diabetes (T2D), while initially effective, often fails to maintain glycaemia control as T2D progresses.1, 2 Therefore, additional effective and well tolerated therapies are required when metformin mono-therapy fails to maintain glycaemia control. There are no uniform recommendations regarding the best agent to combine with metformin [8]. Although metformin mono-therapy, for patients with type 2 diabetes (T2D) [8] initially was effective, but sometimes does not act to control the diabetes progresses properly [8, 9]. Therefore, additional drugs are needed when metformin mono-therapy fails to maintain glycaemia control [8, 10]. Empagliflozin, a potent and selective inhibitor of the sodium glucose co-transporter [9] (SGLT2), is one of the most recommended items for T2D [10] due to that SGLT2 inhibitors reduce renal glucose reabsorption, thereby increasing urinary glucose excretion and reducing hyperglycaemia [11]. In a unique testing in patients with T2D, Empagliflozin 10 mg and 25 mg given as add-on to metformin for 6 months provided clinically relevant reductions in HbA1c, fasting plasma glucose (FPG), weight and systolic blood pressure (SBP) vs. placebo effects, exactly [12]. If using dual mixtures of anti-diabetes agents did not work properly, triple combinations are required [8, 10]. Di-peptidyl peptidase-4 (DPP-4) inhibitors, which recommended as second- or third-line treatment option for patients with T2D is a potent and selective DPP-4 inhibitor [13, 14]. Since DPP-4 destroyed incretin hormones, in contrast linagliptin increases the concentrations of active incretin hormones, stimulating the release of insulin in a glucose-dependent manner and decreasing the levels of glucagon in the circulation, resulting in better regulation of glucose homeostasis [15]. For patients without controlling diabetes disease with metformin and a sulphonyl-urea, or with metformin and pioglitazone, linagliptin improved glycaemia control, with a low risk of hypo-glycaemia and a neutral effect on weight

[16-23]. The combination of SGLT2 inhibitors and DPP inhibitors with metformin is a recommended third-line treatment option for patients with T2D.

Docking Simulation

Although metformin is a compound that belongs to the class of drugs known as biguanidine and is the first therapeutic choice for Type 2 Diabetes Mellitus, unfortunately it has been used for more than four decades with several side effects. Therefore a novel drug with no side effects should be designed and replace. Since metformin inhibits hepatic gluconeogenesis and prevents hyper-glycaemia without impairing insulin secretion, hypoglycemia, or weight gain, it is an essential step to simulate the molecules, including 1-Carbamimidoyl-1,2-dimethyl-guanidine hydrochloride, Metformin hydrochloride, *N*1,*N*1-Dimethyl-*N*5-methylbiguanide hydrochloride, and *N*1,*N*1,*N*5,*N*5-Tetrakis (methyl-bi-guanine hydrochloride], via molecular docking simulation for analyzing the properties of these four analogues of metformin, as well as through a combination with Empagliflozin.

Materials and methods

HPLC system

The HPLC system (228 nm, 1.0 mL·min^{-1}) was used by Shimadzu LC-2010 *HT* with UV detector, including 2.8 g monobasic potassium phosphate at pH=4.5 with 10 μL acetonitrile as buffer. Using 'LC Solution software Version 1.21' data processing and chromatographic integration was carried out. A reciprocating shaker used for liquid–liquid extraction was procured from Trishul Equipment. The nitrogen evaporator used to evaporate the samples was procured from Takahe Analytical Instruments. Hitachi centrifuge machine was used for the sample preparation. Deep freezers used for storage of plasma samples were procured from Sanyo (Japan). Dissolution Condition: Medium: 900 mL, 6.08 gr monobasic potassium phosphate in 1000 mL water. PH=6.8 Apparatus: (2) paddle, Time: 30 min, RPM: 50

Preparation of standard solution

Empagliflozin standard stock 1: 28 mg of Empagliflozin WS was accurately weighed and taken in 50 mL clean and dry volumetric flask then adding containing 20 mL of Acetonitrile by Sonicated for 2 min and then the solution was made up to the mark by medium solution. Empagliflozin standard stock 2: 2 mL of stock solution were transferred as 1 to 20 mL volumetric flask and made up to mark by medium solution. Standard solution: 28 mg of Metformin HCl WS was accurately weighed and taken in a 50 mL clean and dry volumetric flask then adding to containing 20 mL of medium solu-

tion by sonication for 2 min and then adding 5 mL of Empagliflozin standard stock 2 to the flask. Thus, the solution was made up to the mark by medium solution (In assay preparation use Diluent instead of medium solution) (Metformin/ HCl: Empagliflozin standard; 0.56:0.0056 mg/mL).

Preparation of Test Solution (TS)

Assay: Not less than 10 tablets were taken, powdered and test stock solution of Metformin HCl: Empagliflozin was prepared by transferring weight equivalent to 500 mg of Metformin HCl and 5 mg of Empagliflozin to 70 mL of Diluent which is sonication and shake intermittently for 10 min and later made up to 100 mL with diluent. Transfer 1 mL of this solution to 10 volumetric flasks and fill to volume with diluent. This solution was filtered using a 0.22-micron syringe filter. (Metformin HCl, Empagliflozin standard, 0.50:0.005 mg/mL). Dissolution: Metformin HCl/ Empagliflozin tablet was dissolved in 900 mL medium for dissolution test (Metformin HCl: Empagliflozin standard; 0.56:0.0056 mg/mL).

Preparing stock solution

Not less than 10 tablets were taken, powdered and test stock solution was prepared by transferring weight equivalent to 500 mg of Metformin HCl and 5 mg of Empagliflozin to 80 mL of Diluent which is sonicated and shacked intermittently for 10 min and later made up to 100 mL with diluent.

Hydrolytic degradation under acidic condition

1 mL stock solution of Metformin HCl: Empagliflozin was prepared and taken in a 10 mL volumetric flask; 0.75 mL of 0.1 N HCl was added. Then the volumetric flask was kept at normal condition for 90 minutes and then neutralized with 0.1 N NaOH and the volume was made up to the mark with the Diluent. The resultant solution was filtered with 0.45-micron syringe filters and placed in the vials.

Hydrolytic degradation under alkaline condition

1 mL Metformin HCl: Empagliflozin stock solution was prepared and taken in a 10 mL volumetric flask; 0.75 mL of 0.1N NaOH was added. Then the volumetric flask was kept at normal condition for 90 minutes and then neutralized with 0.1 N HCl and the volume was made up to the mark with the Buffer. The resultant solution was filtered with 0.45-micron syringe filters and placed in the vials.

Thermal induced degradation

1 mL Metformin HCl: Empagliflozin stock solution was prepared and taken in a 10 mL volumetric flask; 1.5 mL of the Diluent was added. Then the volumetric flask was kept at reflex condition for 60 minutes and further the volume was made up to the mark with the Buffer. The resultant solution was filtered with 0.45 microns' syringe filters and placed in the vials.

Oxidative degradation

1 mL Metformin HCl: Empagliflozin stock solution was prepared and taken in a 10 mL volumetric flask; 0.5 mL of 3 % w/v of hydrogen peroxide solution was added and the volume was made up to the mark with Diluent. Further the volumetric flask was kept at room temperature for 15 min. The resultant solution was filtered with 0.45-micron syringe filters and placed in the vials.

Procedure

Separately inject 10 µL of standard solution and sample solution into the chromatographic system. Record the chromatograms and measure the peak responses. Calculate the assay and dissolution results of samples by the following formula: Result% = $(A_T/A_S)\times(C_S/C_T)\times100$, where: A_T = sample area, A_S = standard area, W_s = Concentration of standard, W_T = Concentration of sample.

Method precision

Method precision was determined by performing assay and dissolution of the sample under the tests of (i) repeatability (Intraday precision) and (ii) Intermediate precision (Inter day precision or ruggedness) performed during 2 consecutive days by two different analysts, at working concentration.

Method validation

Validation of the analytical method is the process that is established by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, intra-day precision, inter-day precision/ intermediate precision/ruggedness.

Results

Selectivity of the analytical method is defined as the degree to which a method can quantify the analyte in the presence of interferes. Specificity study of the chromatographic method is performed by the separation of the analyte from the other potential components such as impurities, degradants or excipients etc. In addition, forced degradation studies are carried out to challenge the method. The forced degradation studies are of particular importance when the impurities are not available. During forced degradation studies, the sample is subjected to the stressed conditions of light, heat, humidity, acid/base hydrolysis and oxidation.

Linearity

The linearity of the analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample. Standard solutions of Metformin HCl: Empagliflozin at different concentrations level (50-150%) were prepared. Calibration curve (Fig. 1) was constructed by plotting the concentration level of drug versus peak area. The results show an excellent correlation between peak area and concentration level of Metformin HCl: Empagliflozin within the concentration range (tables 1 and 2). The correlation coefficient was greater than 0.999, which meets the method's validation acceptance criteria and hence the method is said to be linear in the range of 0.28-0.84 mg/mL for Metformin HCl and 0.0028-0.0084 mg/mL for Empagliflozin (figures 1 and 2).

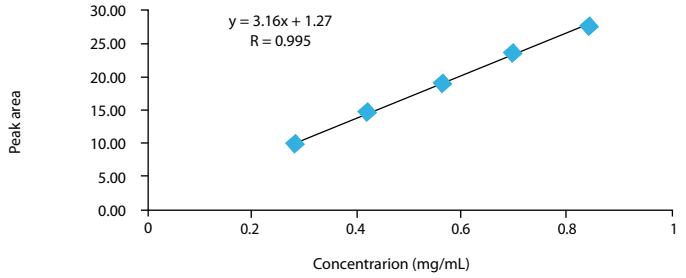

Figure 1. The Linearity plot of Metformin HCl

Table 1. Linearity results (part a)

Standards	1 (50%)	2 (75%)	3 (100%)	4 (125%)	5 (150%)
Metformin HCl Concentration (mg/mL)	0.28	0.42	0.56	0.7	0.84
Peak Area × 10^6	9.9	14.8	19	23.6	27.7

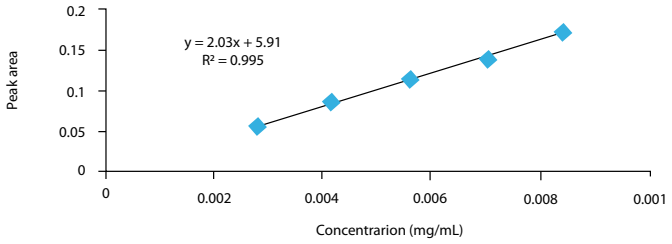

Figure 2. The Linearity plot of Metformin HCl

Table 2. Linearity results (part b).

Standards	1 (50%)	2 (75%)	3 (100%)	4 (125%)	5 (150%)
Empagliflozin Concentration (mg/mL)	0.0028	0.0042	0.0056	0.007	0.0084
Peak Area × 10^4	5.74	8.62	11.36	14.34	17.10

System precision

The precision of an analytical procedure expresses the closeness of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions implied five replications of the standard solution at the target concentrations were injected. Results showed Relative Standard Deviation (RSD) was less than 2%, which indicates acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in tables 3-a,b)

Table 3. System precision

Y=3.16X+1.27, lim of RSD<2% (a)		
Area number	**Cal. conc.(mg/mL)**	**Assay %**
Area 1	0.5657	100.8
Area 2	0.5653	100.7
Area 3	0.5617	100.1
Area 4	0.5657	100.8
Area 5	0.5686	101.3
medium	1.9189	100.75
STDEV	7.86	0.41
RSD	0.44	0.41

(Continued)

Table 3. *Continuation*.

Y=2.0X+5.91, lim of RSD<2% (b)		
Area 1	0.00554	99.5
Area 2	0.00558	100.2
Area 3	0.00554	99.6
Area 4	0.00559	100.7
Area 5	0.00556	99.9
medium	0.00557	100
STDEV	530	0.47
RSD	0.47	0.47

Repeatability (Intraday precision)

Three replications of the sample solution at the three concentrations (50%, 100% and 150%) were injected. Results showed Relative Standard Deviation (RSD) was less than 2%, which indicates acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in (tables 4-a, b, c, d, e, f).

Table 4. Intraday precision of Empagliflozin/metformin·HCl

Y=3.16X+1.27, lim of RSD<2% a) Repeatability of 50%.			**Y=2.0X+5.91, lim of RSD<2% d) Repeatability of 50%.**		
Area number	Assay	Cal. conc. (mg/mL)	Assay %	Cal. conc. (mg/mL)	Assay %
Area 1	51.9	0.2721	100.8	0.00280	50.5
Area 2	52.1	0.2728	100.7	0.00280	50.6
Area 3	52.1	0.2729	100.1	0.00281	50.8
medium	52	0.273	100.75	0.00280	50.7
STDEV	0.08	16084	0.41	7.86	0.12
RSD	0.16	0.16	0.41	0.44	0.24
Y=3.16X+1.27, lim of RSD<2% b) Repeatability of 100%.			**Y=2.0X+5.91, lim of RSD<2% e) Repeatability of 100%.**		
Area 1	100.6	0.5644	99.5	0.00561	100.8
Area 2	100.5	0.5611	100.2	0.00559	100.3
Area 3	100	0.5640	99.6	0.00559	100.7
medium	100.4	0.5631	100	0.00560	100.6
STDEV	0.30	0.00	0.47	0.00	0.26
RSD	0.30	0.32	0.47	0.26	0.26

(Continued)

Table 4. *Continuation.*

Y=3.16X+1.27, lim of RSD<2% c) Repeatability of 150%.			Y=2.0X+5.91, lim of RSD<2% f) Repeatability of 150%.		
Area 1	147.3	0.8446	99.5	0.00843	151.3
Area 2	146.6	0.8407	100.2	0.00848	152.2
Area 3	148.1	0.8499	99.6	0.00851	152.6
medium	147.3	0.8452	100	0.00557	152
STDEV	0.77	0.00	0.47	0.00	0.67
RSD	0.52	0.55	0.47	0.44	0.44

Intermediate precision (Inter day precision/Ruggedness)

Precision between two consecutive days performed by different analysts of the sample showed % RSD less than 2, which indicate the method developed is inter day precise/ rugged (tables 5-a, b, c, d, e, f).

Table 5. Intermediate precision of Empagliflozin/metformin·HCl

Y=3.16X+1.27, lim of RSD<2% a) Repeatability of 50%			Y=2.0X+5.91, lim of RSD<2% d) Repeatability of 50%		
Area number	Assay	Cal. conc. (mg/mL)	Assay %	Cal. conc. (mg/mL)	Assay %
Area 1	51.9	0.2721	100.8	0.00280	50.5
Area 2	52.1	0.2728	100.7	0.00280	50.6
Area 3	52.1	0.2729	100.1	0.00281	50.8
medium	52	0.273	100.75	0.00280	50.7
STDEV	0.08	16084	0.41	7.86	0.12
RSD	0.16	0.16	0.41	0.44	0.24
Y=3.16X+1.27, lim of RSD<2% b) Repeatability of 100%			Y=2.0X+5.91, lim of RSD<2% e) Repeatability of 100%		
Area 1	100.6	0.5644	99.5	0.00561	100.8
Area 2	100.5	0.5611	100.2	0.00559	100.3
Area 3	100	0.5640	99.6	0.00559	100.7
medium	100.4	0.5631	100	0.00560	100.6
STDEV	0.30	0.00	0.47	0.00	0.26
RSD	0.30	0.32	0.47	0.26	0.26
Y=3.16X+1.27, lim of RSD<2% c) Repeatability of 150%			Y=2.0X+5.91, lim of RSD<2% f) Repeatability of 150%		
Area 1	147.3	0.8446	99.5	0.00843	151.3
Area 2	146.6	0.8407	100.2	0.00848	152.2
Area 3	148.1	0.8499	99.6	0.00851	152.6
medium	147.3	0.8452	100	0.00557	152
STDEV	0.77	0.00	0.47	0.00	0.67
RSD	0.52	0.55	0.47	0.44	0.44

Photolytic degradation

1 mL Metformin HCl: Empagliflozin stock solution was prepared and exposed to near ultraviolet lamp in photo stability chamber providing illumination for 1 hr. Then sample was dissolved in Diluent and the volume was made up to mark [10 mL] (Table 6).

Table 6. Specificity of Metformin HCl: Empagliflozin in tablet

No	Degradation studies	Metformin HCl Result%	Empagliflozin Result%
1	Hydrolytic degradation alkaline	101.9	99.5
2	Hydrolytic degradation acidic	101.3	99.5
3	Thermal degradation	101.5	100.0
4	Oxidative degradation	100.7	101.1
5	Photolytic degradation	100.0	99.8

Evaluating the accuracy of methods

The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value found. Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of sample by standard addition method at three different levels. At each level, three determinations were performed. Percent mean recovery was calculated as shown in table 7. The accepted limits of recovery are 98%-102% and all observed data are within the required range, which indicates good recovery values and hence the accuracy of the method developed.

Table 7. Evaluating the accuracy of methods in Metformin/Empagliflozin tablet

Control solution of 50%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=3.16X+1.27 (a)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^7	1.016	1.011	1.005	1.013	55624	0.550
Observed conc. (mg/mL)	0.280	0.270	0.280	0.270	0.0	0.0
Calculated conc. (mg/mL)	0.280	0.278	0.277	0.278	1.37	0.27
Relative error	-0.25	0.36	1.00	0.37		
Accuracy	100.26	99.64	99.00	99.63		

(Continued)

Table 7. *Continuation*.

Control solution of 100%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=3.16X+1.27 (b)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^7	1.89	1.90	1.91	1.90	56430	0.290
Observed conc. (mg/mL)	0.560	0.560	0.560	0.560	0.0	0.0
Calculated conc. (mg/mL)	0.559	0.561	0.562	0.561	1.38	0.27
Relative error	0.14	-0.28	-0.48	-0.21		
Accuracy	99.86	100.28	100.48	100.21		
Control solution of 150%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=3.16X+1.27 (c)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^7	2.83	2.83	2.83	2.83	25153	0.089
Observed conc. (mg/mL)	0.8400	0.8400	0.8400	0.8400	0.0	0.0
Calculated conc. (mg/mL)	0.85597	0.85478	0.85446	0.85507	0.77	0.27
Relative error	-1.90	-1.76	-1.72	-1.79		
Accuracy	101.90	101.76	101.72	101.79		
Control solution of 50%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=2.0X+5.91 (d)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^4	5.74	5.77	5.73	5.75	183	0.32
Observed conc. (mg/mL) × 10^{-3}	2.80	2.80	2.80	2.80	0.0	0.0
Calculated conc. (mg/mL)	0.003	0.003	0.003	0.003	0.273	0.27
Relative error	-0.05	-0.44	0.2	-0.1		
Accuracy	100.05	100.44	99.8	100.1		
Control solution of 100%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=2.0X+5.91 (e)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^5	1.13	1.13	1.14	1.13	594.6	0.52
Observed conc. (mg/mL) × 10^{-3}	5.6	5.6	5.6	5.6	0.0	0.0
Calculated conc. (mg/mL)	0.006	0.006	0.006	0.006	0.28	0.27
Relative error	0.61	1.04	0.0	0.55		
Accuracy	99.39	98.96	100	99.45		

(Continued)

Table 7. *Continuation*.

Control solution of 150%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=2.0X+5.91 (f)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^5	1.71	1.71	1.71	1.71	430	0.25
Observed conc. (mg/mL) × 10^{-3}	8.4	8.4	8.4	8.4	0.0	0.0
Calculated conc. (mg/mL)	0.008	0.008	0.008	0.008	0.28	0.27
Relative error	-0.28	0.19	-0.20	-0.10		
Accuracy	100.28	99.81	100.20	100.10		

Limit of detection (LOD) and Limit of quantitation (LOQ)

There are several terms that have been used to define LOD and LOQ. In general, the LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test. For a linear calibration curve, it is assumed that the instrument response y is linearly related to the standard concentration x for a limited range of concentration. It can be expressed in a model such as y=a+bx. This model is used to compute the sensitivity b and the LOD and LOQ. Therefore, the LOD and LOQ can be expressed as: LOD = 3 × Sa / b, LOQ = 10 × Sa / b, where Sa is the standard deviation of the response and b is the slope of the calibration curve. The standard deviation of the response can be estimated by the standard deviation of either y-residuals, or y-intercepts, of regression lines. This method can be applied in all cases, and it is most applicable when the analysis method does not involve background noise. It uses a range of low values close to zero for calibration curve, and with a more homogeneous distribution will result in a more relevant assessment (Figure 3).

Ranges of testing: Range of analytical method can be obtained from Linearity, precision and accuracy data. Report the range in % with respect to sample concentration. According to the result it can be concluded that the range of analytical method for the determination of assay and dissolution of Metformin HCl/Empagliflozin tablet 500/5 mg is 50-150% of target concentration. The null hypotheses of no treatment effect for the primary and key secondary endpoint were tested hierarchically to control diabetes type 2. For each endpoint, the superiority of linagliptin 5 mg was tested. The trial was powered separately for linagliptin 5 mg vs. placebo as add-on to Empagliflozin 10 mg and metformin and linagliptin 5 mg vs. placebo as add-on to Empagliflozin 25 mg and metformin (Figure 4).

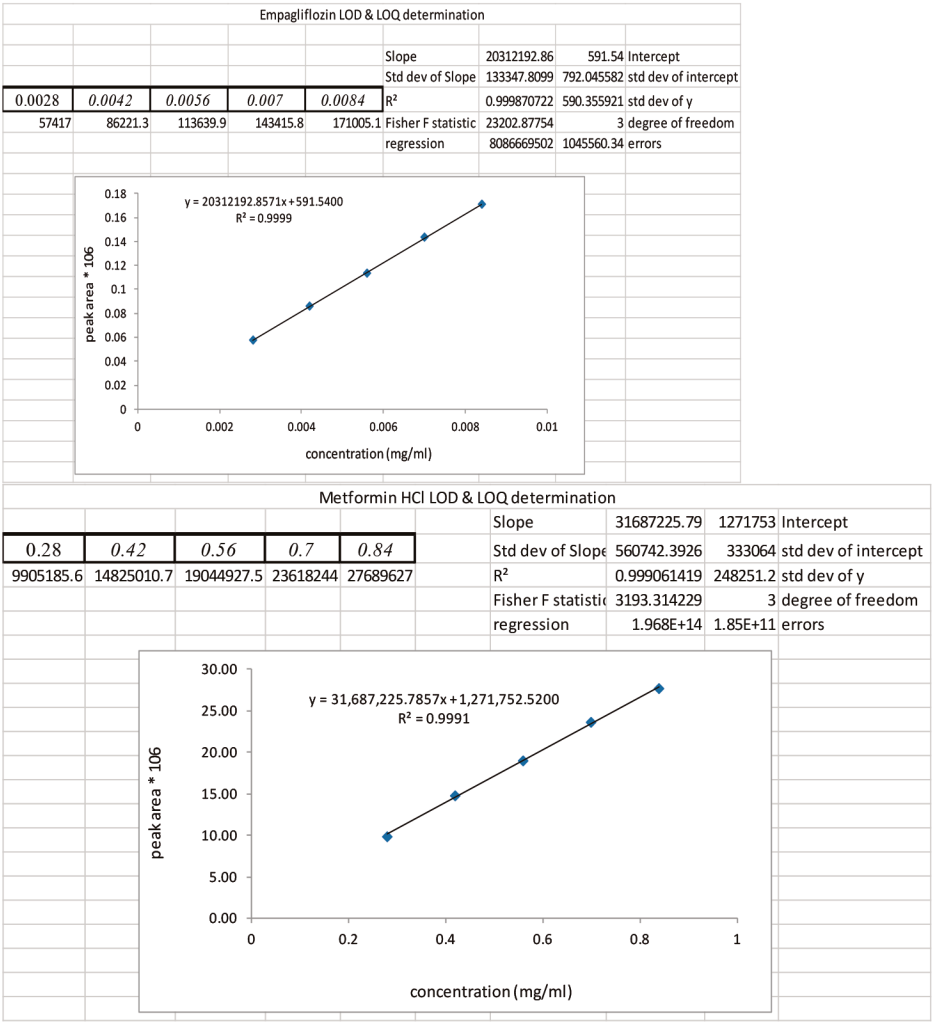

Figure 3. Peak versus concentration

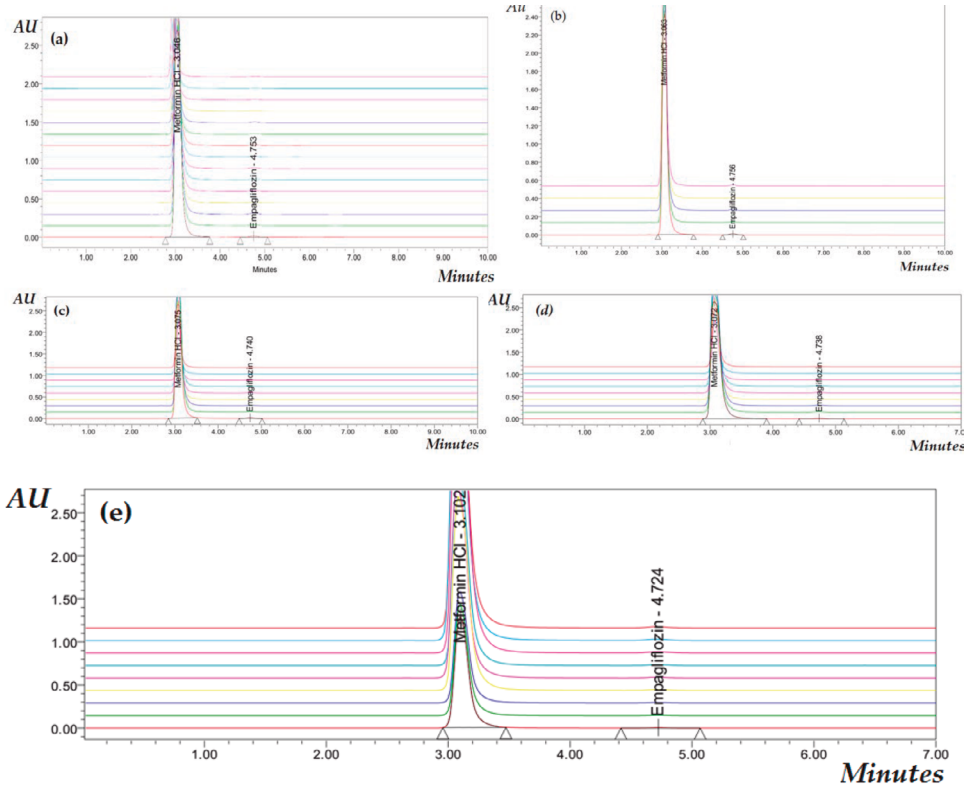

Figure 4. Several sample peaks

Results of Docking Simulation

The Metformin and its five analogues were downloaded from Pub-Chem database (Table 8), and then were converted into PDB format. In addition, XRD structure of 7VSI.pdb, that is structure of human SGLT2-MAP17 complex bound with Empagliflozin, and 8JTS.pdb a crystal structure in complex with metformin in outward open conformation, were gotten from Brookhaven Protein Data Bank (Figure 5). Finally all the analogues were converted into PDBQT file format using Auto Dock Tool (ADT) for further analysis and also docking was performed with iGemdock algorithms. At the first step the interactive maps of the docked molecules (five molecules in table 1) were done by two mentioned PDB files, and the docked minimum energies were then analyzed for finding the suitable places, which any types of peptides could attached with higher affinity to the both SGLT2-MAP17 and human organic cation transporter. NMR data of Empagliflozin and Metformin cation have plotted in figure 6.

Table 8. The Metformin and its five analogues were downloaded from Pub-Chem database

No.	Compounds analogs
1	Metformin cation
2	Metformin-d6 hydrochloride
3	Metformin Hydrochloride
4	Empagliflozin
5	*N*1,*N*1-Dimethyl-*N*5-methylbiguanide hydrochloride

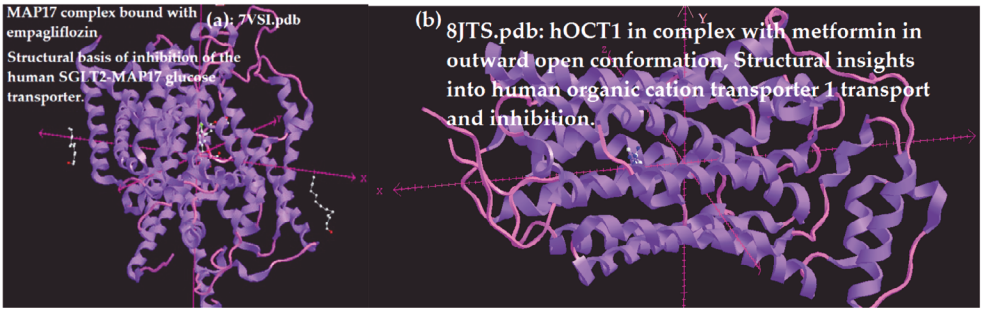

Figure 5. Brookhaven Protein Data Bank of (a): human SGLT2-MAP17 complex bound with Empagliflozin, and (b): Structural insights into human organic cation transporter 1 (HOCT).

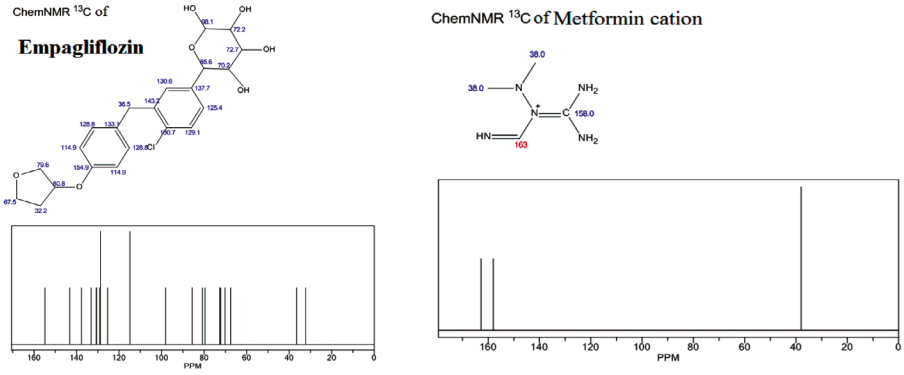

Figure 6. NMR data of Empagliflozin and Metformin cation

The potential binding sites were calculated and plotted using Acsite software. Docking was performed with iGemdock algorithms. At the first step the interactive maps of the docked molecules were done by LIGPLOT, and the docked minimum energies were then analyzed for finding the suitable places, which any types of each molecules from

Table 8 could attached with higher affinity to the SGLT2-MAP17 complex .Three of these molecules including, Metformin cation, Metformin-Hydrochloride, and Empagliflozin docked, exhibited suitable binding energies, and docking were repeated by increased stringency using a reduced cavity size as a receptor. This resulted in one matching peptide again modeled with SGLT2-MAP17 Data Bank, and HOCT a template using MODELLER software. The scoring function, X-score, was used to compare the binding energies Table 9 and Figure 7.

Table 9. Docking energies for three complexes

Molecules No	Name	Complex	Ka ($(10_4$ M	VDW (kcal/mol)	H-bond (kcal/mol)	Docking Fitness (kcal/mol)
(1)	Metformin cation	Metformin cation- SGLT2	3.15	-46.74	-16.52	-63.24
(3)	Metformin Hydrochloride	Metformin Hydrochloride-SGLT2	1.45	-29.12	-14.18	-43.28
(4)	Empagliflozin	Empagliflozin--SGLT2	2.45	-57.61	-13.59	-71.194

Compound	Energy	H-M CYS 536	H-M LEU 539	H-S THR 543	V-M LEU 539	V-S LEU 539	V-M LEU 540	V-S LEU 540
cav7vsi_PLM-Metformin -1.pdb	-43.3	-6.9	-3.5	-3.5	-4.7	-4.5	-5.4	-6.7

Compound	Energy	H-M VAL 532	H-M PHE 535	H-M LEU 539	V-M LEU 533	V-S PHE 535	V-M SER 537	V-M GLY 538
cav7vsi_PLM-Empagliflozin-0.pdb	-71.2	-3.5	-6.6	-3.5	-7.5	-8.2	-6.8	-14.1

Compound	Energy	H-M GLY 491	H-M GLY 492	H-M CYS 536	V-M GLY 491	V-M CYS 536	V-S LEU 540
cav7vsi_PLM-Empagliflozin-0.pdb	-63.3	-2.5	-3.5	-5	-6.9	-4.6	-4.8

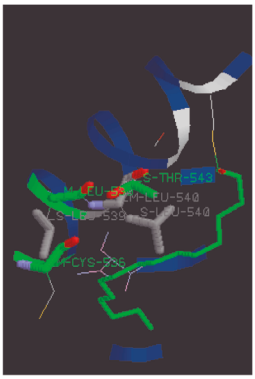

Figure 7. Docking position of amino acids for binding of three complexes

Discussion

In these two-Phase III studies, designed to evaluate the efficacy and safety of linagliptin 5 mg vs placebo as add-on to Empagliflozin 10 mg or 25 mg and metformin for 24 weeks

in patients with T2D, use of linagliptin resulted in statistically significant reductions in HbA1c and FPG vs placebo. Importantly, the proportion of patients with baseline HbA1c ≥7% who reached HbA1c <7% after 24 weeks with linagliptin was more than twice that with placebo as add-on to Empagliflozin 10 or 25 mg and metformin. These improvements in glycaemic control were observed despite a smaller proportion of patients receiving rescue therapy in the linagliptin vs the placebo as add-on to Empagliflozin and metformin groups. At week 24, the reduction from baseline in HbA1c observed with linagliptin as add-on to Empagliflozin 10 mg or 25 mg and metformin was >0.5%. The impact on weight is an important consideration when choosing a treatment for T2D. Metformin, the recommended first-line treatment for patients with T2D, is considered weight neutral. However, some second- and third-line therapies such as sulphonylureas, glinides, thiazolidinediones and insulin, are associated with weight gain. Weight loss or avoidance of weight gain is desirable for patients with T2D, weight gain being associated with decreased treatment satisfaction and health-related quality of life [24]. In line with the mechanism of action of DPP-4 inhibitors [25-28] and data from linagliptin Phase III trials [29], linagliptin did not markedly change weight during the double-blind treatment periods, while Empagliflozin has been shown to reduce weight [30].

Docking technique needs selecting the three-dimensional coordinate space of the binding site in the target and measuring the binding interactions of the molecule's resultant orientation within the binding site, forming the complex. The importance and sensitivity of binding affinity values are calculated by the maximum magnitude negative number (highest binding affinity or lowest binding energy), which represents the most advantageous conformation of the complex produced when the ligand involved binds efficiently to the target's active pockets. Additionally, docking simulations were used to confirm the anti-diabetic potency of Metformin cation- SGLT2, Metformin Hydrochloride- SGLT2, and Empagliflozin-SGLT2, by examining the binding affinity and orientation of ligands inside the SGLT2 receptor pocket. The docking poses were rated as per their score values, and Table 9 summarized the binding affinities of the best pose for each of the four analogues with the SGLT2 target (Figure 7). Additionally, the molecules in this analysis have a higher binding affinity, comparable to that of a regular drug (metformin).The four compounds could dock into the active site of SGLT2 successfully. The tight binding can be explained in terms of hydrogen bonding with target protein. All the three complexes were involved in the hydrogen bonding with a residue. Metformin hydrochloride interacted with SGLT2 forming H-bonds at active site region involving residues CYS, LEU, and THR (7). Analysis of these interaction results confirmed that the selected four analogues showed the efficient binding with

diabetic target protein SGLT2 like the standard drug metformin. So it was confirmed that, these analogues might be a potential lead compounds for experimentally validation for diabetes management. Our docking results confirmed the experimental data significantly.

Conclusion

The present study was carried out to develop a sensitive, precise and accurate HPLC method for the analysis of Metformin HCl and Empagliflozin in Bulk as well as in Metformin HCl /Empagliflozin tablets. In order to method development under gradient conditions, was tested as mobile phase on a (L10 (250×4.6 mm); column. The retention times obtained for Metformin HCl/Empagliflozin was around 3.09 and 4.7 min respectively. In conclusion, linagliptin improved glycaemia control compared with placebo as add-on to Empagliflozin 10 or 25 mg and metformin and was well tolerated. Therefore, linagliptin could provide a valuable treatment option for patients with T2D who are inadequately controlled with Empagliflozin and metformin, improving glycaemia control with a low risk of hypo glycaemia and no increase in weight.

Author contributions

Motahareh Dehghandar, Parisa Latifi, and Iman Eskandary designed, measured, and drawn the experimental data, images, graph, and experimental activities and methods. Sara Shahriari and Fatemeh Mollaamin checked the molecular structures and chemical abbreviation in viewpoint of chemistry and biochemistry . Majid Monajjemi accomplished the molecular docking simulation. Majid Monajjemi and Fatemeh Mollaamin revised the English, grammar and dictation of the manuscript.

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Conflict of interest

All authors report that they do not have any conflicts of interest.

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