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Adınızı soyadınızı giriniz

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(MASTER THESIS)

"SIMULTANEOUS DETERMINATION OF NEW GENERATION ANTIDIABETICS IN BIO-FLUIDS BY HPLC"

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THESIS APPROVAL

(Bu sayfa yerine, başarılı geçen Tez Sınavı sonrası sınav tutanağı ekinde yer alan Tez Onay sayfası gelecektir.)

DECLARATION

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DECLARATION

I declare that this thesis is my own study, I did not engage in any unethical acts from the planning stage to the actual writing stage of this thesis, I acquired all information in this thesis in compliance with academic and ethical rules, I have provided references for all other information and comments other than the ones I have obtained as a result of my study and I have listed them in the references section and I did not infringe any patent rights or copyrights

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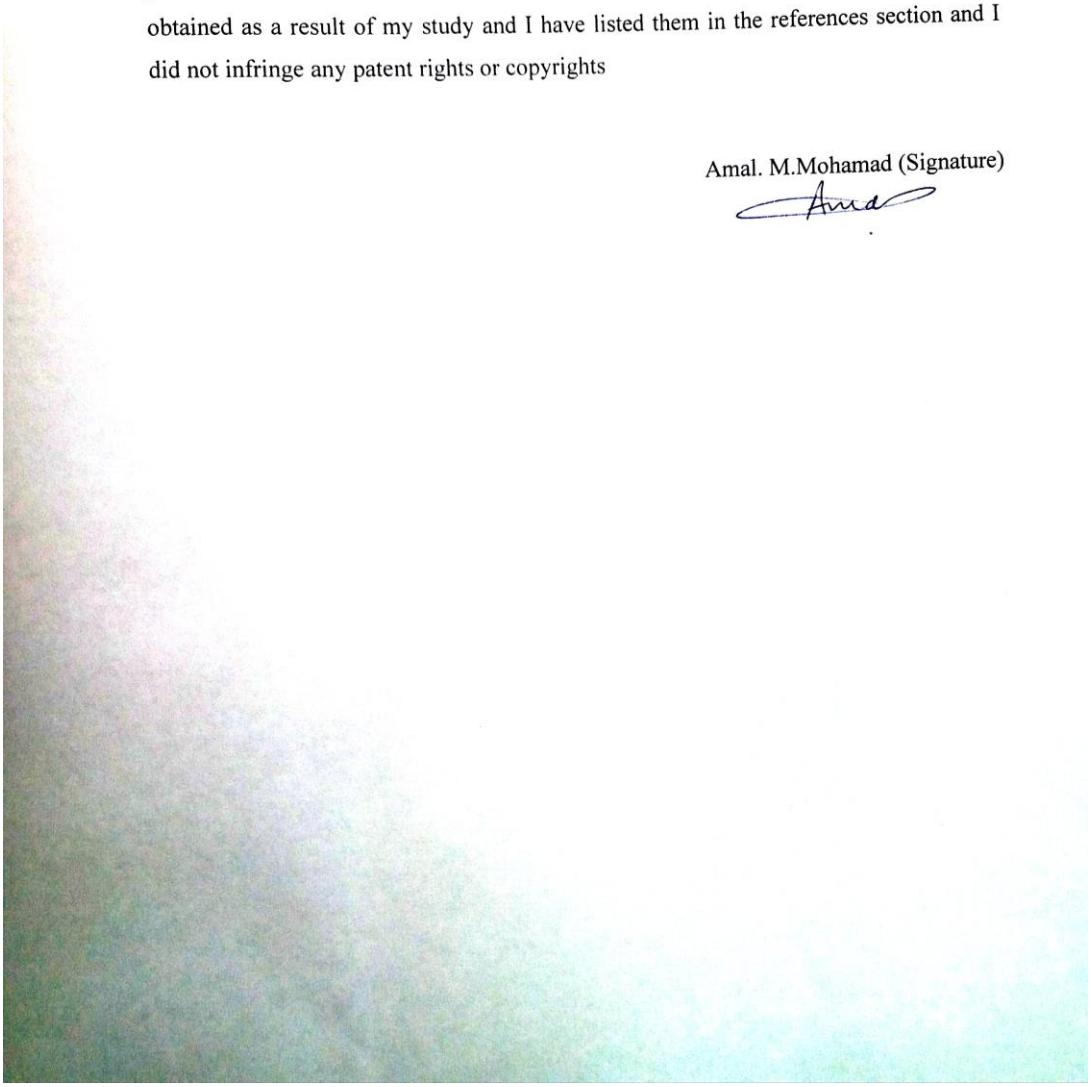

DEDICATION

I dedicate my dissertation work to my family and many friends and who support me every morning by words or even smile.

A special feeling of gratitude to my loving parents, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

My sister and brothers have never left my side and are very specialI also dedicate this dissertation to my many friends and church family who have supported me throughout the process.

I dedicate this work and give special thanks to my best Teacher **Assoc.Prof. Dr. Sena CAGLAR ANDAC**

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On a more personal note, I would like to dedicate this study to the individual who were kind enough to support me from the first day of my study, who didn't leave me alone in this life I mean **my husband.** I am truly thankful for having you in my life.

I also want to dedicate this project to my colleagues who offered unwavering encouragement and support, especially my best friends **Asma** and **Yathrep**

Also I want to dedicate my project to my daughter **Dana** and my son **Layth** who they have made me stronger, better and more fulfilled than I could have ever imagined. I love you to the moon and back.

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LIST OF SYMBOLS AND ABBREVIATIONS

DPP-4: Dipeptidyl peptidase-4

DDI: Drug-drug interaction

EASD: European Association for the Study of Diabetes

EIS: Electrochemical Impedance Spectroscopy

ESI: Electrospray Ionization

FDA: Food and Drug Administration

GIP: Glucose Dependent Insulinotropic Polypeptide

GLP-1: Glucagon-like Peptide- 1

LLE: Liquid –Liquid –Extraction

LOQ: limit of Quantification

LOD: Limit of Detection

MET: Metformin

HPLC/LC: High Performance Liquid Chromatography

MS: Mass Spectrometry

RAM: Restricted Access Material

SAX: Saxagliptin

SITA: Sitagliptin

SPE: Solid Phase Extraction

SPE-LC: Solid Phase Extraction Liquid Chromatography

SPME: Solid Phase Micro Extraction

T2DM: Type2 Diabetes Mellitus

VIL: Vildagliptin

ÖZET

Mohammad, A.M. (2018). Yeni Nesil Antidiyabetiklerin Biyolojik Sıvılardan HPLC ile Yanyana Analizi. İstanbul Üniversitesi Sağlık Bilimleri Enstitüsü, Analitik Kimya AD. Yüksek Lisans Tezi. İstanbul.

Anahtar Kelimeler: Gliptin, İdrar, Tayin, HPLC, Diyabet

Bu çalışmada Sitagliptin, Saksagliptin, Vildagliptin ve Metforminin insan idrarında yanyana tayini için katılmış idrar örneklerinin tekrarlı ve doğrudan enjeksiyonuna imkan veren tam otomatik bir on-line SPE-LC-UV metodu ilk defa geliştirilmiştir. Tip 2 Diyabet dünyada en çok görülen hastalıklardan biridir. Bu hastalığın tedavisinde kullanılan gliptin grubu ilaçlar inkretin hormonunun yıkımının inhibe edilmesinde rol oynarlar. Tabletlerde ya tek başlarına ya da metformin ile kombine halde bulunurlar. Bu ilaçların idrarla atılım oranları yüksek olduğu için geliştirilen metot insan idrar örneklerine uygulanmıştır. Çalışmada kromatografik ayrılma için bir Gemini C18 kolon 10 mM o-fosforik asit-metanol mobil faz karışımının gradiyent elüsyonu ile ve idrar örneklerinin fraksiyonlanması için LiChrospher® ADS SPE-kolon kullanılmıştır. Katılmış idrar örneklerinin fraksiyonlanma, transfer, elüsyon ve ayrılması için geçen toplam analiz süresi sadece 9.6 dakikadır. Geliştirilen metot ICH kılavuzlarına göre valide edilmiştir. Kalibrasyon eğrileri, tatmin edici regresyon katsayıları ile beraber 50 ng/mL ile 100 μg/mL gibi geniş bir aralıkta doğrusaldır. İdrar atılımları gözönüne alındığında LOD ve LOQ değerleri tatmin edicidir. Doğruluk, gün içi ve günler arası tekrarlanabilirlik değerleri düşük, orta ve yüksek konsantrasyonlarda ANOVA testi ile hesaplanmıştır. Geliştirilen metot klinik laboratuvarlarda pratikliği, kolayca bulunabilen detektörü ve güvenilir sonuçları bakımından uygulanabilirdir.

ABSTRACT

Mohammad, A.M. (2018). Simultaneous Determination of New Generation Antidiabetics in Bio-Fluids by HPLC. İstanbul University, Institute of Health Sciences, Department of Analytical Chemistry. Master Thesis. İstanbul.

Key Words: Gliptin, Urine, Determination, HPLC, Diabetes

To the best of our knowledge a fully automated on-line SPE-LC-UV method for the simultaneous determination of Sitagliptin, Saxagliptin, Vildagliptin and Metformin in human urine samples allowing the repetitive direct injection of biological fluid was developed and validated for the first time in this study. Type 2 diabetes is one of the most common diseases in the world. For the treatment of this disease gliptin drugs act by inhibiting the destruction of incretin hormones. They are either used alone in tablets or in combination with metformin. As their amount of excretion is high in urine the developed method was applied to the urine samples. For the chromatographic separation a Gemini C18 analytical column with a mobile phase mixture of 10 mM o-phosphoric acid-methanol in gradient elution and for the fractionation of urine samples LiChrospher® ADS SPE-column was used through the study. Fractionation, transfer, elution and separation of the spiked urine samples were achieved in just 9.6 min runtime. The developed method was validated according to ICH Guidelines. The calibration curves were found to be linear in a wide the range between 10 ng/mL and 100 μg/mL with satisfactorily regression coefficients. LOD and LOQ values were found to be satisfactory as the urine excretion taken into account. Accuracy, inter-day and intra-day precision were studied on low, medium and high level of concentrations by using Excel software to perform ANOVA test. The developed method could be applied in clinical laboratories in terms of its practical use, easily found detection and reliable results.

1. INTRODUCTION AND AIM

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disease resulting from functional insulin deficiency or insulin resistance which may cause organ or function loss due to possible complications. As the current treatment methods for T2DM have some side effects (i.e. gain weight, hypoglycemia and gastrointestinal problems) and new treatment options are needed. Dipeptidyl peptidase-4 inhibitors (DPP-4), in other words the gliptins, are a fairly new class of oral hypoglycemic agents and used for the treatment of T2DM. Sitagliptin, Saxagliptin and Vildagliptin are the most known and newest members of this group of drugs. These medicines are available as stand-alone medicines or as combined preparations with metformin.

Developing new chromatographic methods for analyses of active substances of drugs in pharmaceutical preparations and biological liquids is an important and essential field in analytical chemistry. For this purpose, different techniques and sample preparation methods are developed day by day so that more reliable, fast and precise chromatographic analyses can be carried out.

In this study, an automated sample preparation method, on-line solid phase extraction high-performance liquid chromatography method was developed for the simultaneous determination of sitagliptin, saxagliptin and vildagliptin in the presence of metformin, in human urine for the first time and the developed method was validated according to ICH guidelines.

2. GENERAL INFORMATION

2.1. Diabetes and New Generation Antidiabetics

Hyperglycemia or uncontrolled glucose level is a metabolic disease referred to diabetes mellitus which is classified into two groups; Type 1 and Type 2. This disease if stayed uncontrolled will lead to serious different complications within different organs. (Filippatos, 2013). Untreated and progressive type 2 diabetes (T2DM) is associated with different serious complications that affect different organs; neuropathy, retinopathy, nephropathy and arteriosclerosis. These complications caused by high glucose level in plasma because of impaired insulin secretion and insulin resistance. (Liu, Y., et al., 2012)

By 2030, the incidence of diabetes will be doubled to be around 366 million which is considered as a protest in different economic and social points to health care providers in the world. (Wu, D., et al.,2012).

A lot of the different oral anti hyperglycemic agents are now available in the market with different mechanism of action to treat T2DM; i.e. biguanides and thiazolidinediones, sulfonylureas and glinides and glucosidase inhibitors, which considered as insulin sensitizers, insulin secretagogues and carbohydrate modulators, respectively. (Mizuno, C, et al. , 2008) (Srinivasan, B. T et al., 2008)

Till now, all available antidiabetic drugs are not able to stop the progression of the disease or at least cure it, unlike most of these drugs have the unwanted adverse effects such as stomach side effect, hypoglycemic effect and edema. (Liu, Y., et al., 2012)

Gliptins which inhibit dipeptidyl peptidase-4 (DPP-4), are incretin-based therapies used in diabetic patients T2DM to control glucose level. Gut endocrine cells produce two incretin hormones related to food intake; glucose-dependent insulinotropic polypeptide and Glucagon-like peptide–1 (GLP-1), both have a meaningful role in keeping glucose level within normal range by activating β-cells to produce insulin.(Vilsboll T, et al,2001)

Drugs that inhibit dipeptidyl peptidase-4 include linagliptin, vildagliptin, saxagliptin, sitagliptin which decrease glucose levels in blood by GLP-1 and gastric inhibitory polypeptide (GIP) degradation inhibiting, therefore, increasing plasma levels of the intact (active) forms of these peptides. Currently, all of the group members are approved for use in patients with T2DM. (Covington, P,2008)

Dipeptidyl peptidase-4 is a ubiquitous trans membrane serine protease highly selective for peptides, which degrade both (GLP-1) and (GIP) in a highly specific mechanism and as mentioned before both incretin hormone have a major role in maintaining glocuse level within normal range by own mechanism. (Y. Liu, 2012).

Depends on that inhibition, more GLP-1 and GIP will be in the blood leads to more insulin release with higher β-cells work and restrains glucagon release. All of these effects summed to decrease glucose level in blood with low side effect and considered as beneficial drugs for diabetic patients with T2DM. Depends on the structure, the drugs have been classified into peptide and non-peptide class. (Y. Liu, 2012) (Wiedeman, P. E., 2007).

The peptidomimetics class was discovered first for that a huge number of applications and publications have been completely examined and because of metabolic rate due to cleavage effect of the enzymes and low oral stability a new class (non-peptidomimetic) has to be developed. (Ferraris D., 2007) (Y. Liu, 2012).

Over the most recent years, creating the DPP-4 inhibitors non-peptidomimetic class achieved a finish, after that the dispatch of linagliptin, sitagliptin, alogliptin , and a few other medicines as anti diabetic. (Gallwitz, B,2011).

For that, nonpeptidomimetics turned out to be more appealing class of gliptins or dipeptidyl peptidase-4 inhibitors.

2.2. Pharmacokinetics (PK) of DPP-4 inhibitors and Metformin:

Sitagliptin is available in pharmacies under the brand name (Januvia®) as a single drug or as (Janumet®) combined with metformin.

Since the in vitro studies shows that sitagliptin has no inhibition effect of CYP isoenzyme and not the inducer of CYP3A4 (A. J. Scheen,2010), depends on that the drug-drug interaction (DDI) is uncommon related to the drugs undergoes these isoenzymes. (Zerilli T, 2007).

Also, Sitagliptin has no significant role in DDI because of protein binding, since this drug isn't bound to proteins in blood widely. Sitagliptin has no inhibition role in p-glycoprotein (Pgp) mediated transport of digoxin even it is a substrate of p-glycoprotein. (Scheen AJ, 2010).

After taking 83 mg/193 muCi orally of 14C-sitagliptin, the metabolism and excretion were assessed. The result demonstrates that the drug excreted mainly by renal route within 87 % of the dose without extensive metabolism and the remaining percentage excreted by urine. As metabolite only 16 % of the administered dose was excreted, divided into two routes; 13 % renal and the remaining were in feces. All of these data conclude that the kidney is the major elimination route for the drug. (Vincent SH, 2007).

The plasma concentration - time curve demonstrate that around 74 % of (AUC) of aggregate radioactivity was represented by parent medicine. All inactive metabolites which are six were seen in a range between (<1 – 5%), (<1 – 8%) of the urine and plasma radioactivity, respectively.(A. J. Scheen,2010)

There is a similarity in excretion profile of the drug in urine and feces which is same as plasma profile except that feces has no glucuronide metabolite. Sitagliptin undergoes oxidative metabolism via CYP3A4 as a major isoenzyme with little effect of CYP2C8. (A. J. Scheen, 2010). The renal excretion of the drug depends on glomerular filtration and active tubular secretion. (Chu XY, 2007).

Saxagliptin is available in the market under the brand name of Onglyza® and is recently approved by FDA and EMEA as an anti-diabetic drug. (Scheen AJ,2010)

CYP 450 3A4/5 is the isoenzyme responsible for the metabolism of saxagliptin. Its major metabolite has lesser selectivity to inhibit DPP-4 comparing to saxagliptin, so using drugs that induce or inhibit this isoenzyme at the same time with saxagliptin like rifampicin and ketoconazole, will lead to change pharmacokinetic parameters of the drug and it is the primary metabolite. (Fura A, et al.,2009) (Scheen AJ, 2010) Saxagliptin and its metabolite have no inducing or inhibiting effect on CYP isoenzymes compared to other members (Scheen AJ, 2010).

50 non-diabetic participants recruit and randomized into two groups as the placebo group and different doses of saxagliptin group. The aim was to predict and

measure the pharmacokinetics parameters of saxagliptin and it is the major metabolite. The result of the study showed that the bioavailability of the drug was 67 % (Boulton DW, Geraldes M., 2007). The half life of saxagliptin and the primary metabolite was between 2.2 to 3.8 h, and 3–7.4 h, respectively. While the time to reach the maximum serum concentration for the drug in and the metabolite was ≤ 2 h and ≤ 4 h, respectively.

Saxagliptin like other gliptins orally absorbed and excreted by renal system. Active tubular secretion has a role in the clearance of the drug since the predicted glomerular filtration rate was higher than kidney clearance of the drug within the mean of 120 and 320 ml/min, respectively. The recovery of the drug and it is metabolite in the urine was 12-29 % and 21-52%, respectively and the percentage of urine excretion for saxagliptin was 75%. (Dhillon S, Weber J,2009) (Scheen, A. J. 2010)

There is a DDI between the drug and the drugs that strongly induce CYP3A4, depends on that using these drugs at the time must be followed by precise control of glucose level, also the data demonstrate that saxagliptin dose must be changed when taking with drugs that inhibit the isoenzyme CYP3A4/5 at the same time. (Scheen, A. J. 2010)

The pharmacokinetic parameters were affected by different factors such as the age and gender of the patients. To asses these factors affect 56 non-diabetic subject administered 10 mg of the drug once. (Boulton DW,2008)

The result showed that the elimination and metabolism of the drug affected by the age of the subject. Younger participants with age between 18 to 40 years have higher Vd, higher kidney clearance and metabolism when compared to geriatric subjects. This difference mainly occurred because of the low function of the renal system in the elderly. Although male has lower exposure than females the data showed that there is no effect of the gender on the pharmacokinetic profile of the drug. All of these data leads to no need for changing dose of the drug for patients depending on gender or age without changing in parameters among races. (Scheen, A. J. 2010)

Vildagliptin is available in the market under brand name of Galvus® as single and Eucreas® in combination with metformin.

Since vildagliptin does not depends on cytochrome P450 and not inhibit or induce cytochrome P450 enzymes, the DDI of vildagliptin with other drugs that induce or inhibit this enzyme CYP 450 considered as uncommon interaction. (Croxtall JD, Keam SJ,2008), which means drugs metabolized by these enzymes CYP3A4/5, CYP2C8, CYP2C9, CYP1A2, CYP2D6 and CYP2E1 will not be influenced.

The pharmacokinetic behavior of vildagliptin was measured using 100 mg of drug orally on four different healthy participants. (He H, et al.,2009)

The result demonstrated that vildagliptin was rapidly absorbed, and Cmax was measured at 1.1 h after the dose. The result showed that 85.4 % of the drug entered the systemic circulation. Area under the curve showed that 55 % of it was related to the major metabolite which is known as M20.7, while 25.7 % for the unchanged drug .

The $t_{1/2}$ of the drug was measured to be 2.8 h. Within 7 days the dose of the drug was complete recovered, the urine and feces recovery was 22.6% and 4.5 % as unchanged drug, respectively. And higher percentage of recovered drug was in urine 85.4%.

Before excretion, vildagliptin undergoes with four different metabolism pathway, the major one is M20.7 which is the result of hydrolysis of cyano group, while the minor metabolites such as; M15.3 coming from hydrolysis of amide group then glucuronidation or oxidation of pyrrolidine moiety which is referred to (M15.3) (M20.2) (M20.9 and M21.6), respectively.

All of the mentioned metabolites lead to conclude that CYP P450 enzymes system has no role or at least uncommon role in DDI because of inactivity of all of these metabolites. (Scheen AJ,2010)

Vildagliptin bioavailability was measured by using IV and oral form of the drug on 11 different non diabetic participants with the dose 25 mg and 50 mg, respectively. The urine elimination of the drug was measured as 33 % of the oral dose and 21 % of the intravenous dose as unchanged drug. Like other gliptin drugs, the major way for elimination was by kidney. The active tubular secretion mechanism has an increment role in the drug renal excretion as the drug clearance overreach glomerular filtration rate.

Metformin is a drug used to treat patients with T2DM wich related to a group name biguanide. Metformin has different mechanism of action to improve glucose tolerance in patients with T2DM. It lowers the intestinal absorption of glocuse due to lowering liver secretion of it. The other mechanism for improving glucose tolerance is incresing the insulin sensitivity and incresing glucose uptaking and using.

Metformin is not recommended and should not be used in patients with renal failure CrCl < 30 ml/min, and heart failure. The drug is excreted in urine at high renal clearance rate of about 450 mL/min.The initial elimination of metformin is rapid with a half-life varying between 1.7 and 3 hours. The terminal elimination phase accounting for about 4 to 5 % of the absorbed doseis slow with a half-life between 9 and 17 hours.

2.3. Physical and Chemical Properties of Sitagliptin

Chemical name of Sitagliptin is (2R)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6 dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-il]butan-2-amine and its closed formula is C15H15F6N5O. The molecular weight of the compound is 407.32 g/mole while the pKa value is 8.78.

Chemical formula of Sitagliptin is given in Figure 2-1.

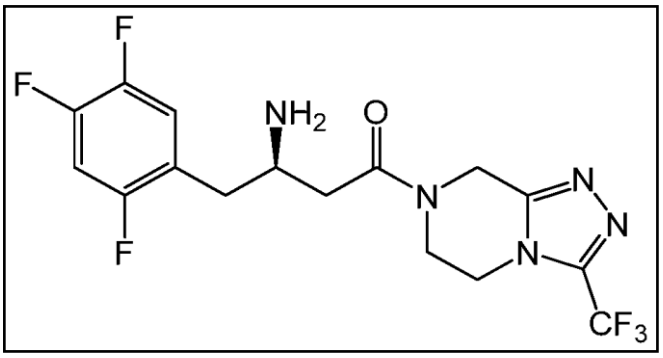

Figure 2- 1 Chemical Formula of Sitagliptin

Sitagliptin phosphate monohydrate is white or whitish, crystalized and non-hygroscopic powder. It is soluble in water and N,N-dimethylformamide, mildly soluble in methanol, poorly soluble in ethanol, acetone and acetonitrile and it is not soluble in isopropanol and isopropyl acetate (The Merck Index Thirteenth edition Whitehouse Station, NJ, M9960)

2.4. Physical and Chemical Properties of Saxagliptin

Saxagliptin is poorly soluble in 24 degrees of water. It is mildly soluble in ethyl acetate and soluble properly in methanol, ethanol, isopropyl alcohol, acetonitrile, acetone and PEG 400. Chemical formula of Saxagliptin is given in Figure 2-2

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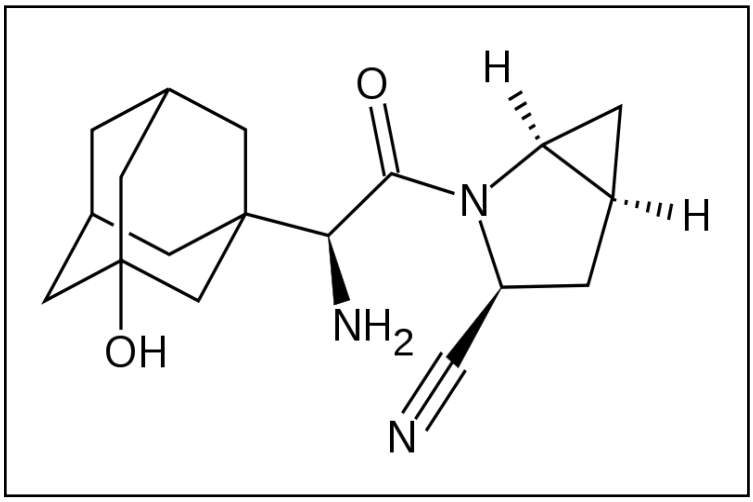


Figure 2- 2 Chemical Formula of Saxagliptin

Chemical name of saxagliptin is (2S,4S,5R)-2-((2S)-2-Amino-2-(3-hydroxy adamantane-1-il)acetyl)-2- azabicyclo(3.1.0) hexane-3-carbonitrile. Closed formula is shown as C18H26N3O2. Its molecular weight is 351,87 with a pKa value of 7.9

2.5. Physical and Chemical Properties of Vildagliptin

Vildagliptin is a white and yellowish or greyish crystalized powder. It is soluble in water and organic polar solvents. The chemical formula of vildagliptin is (S)-(((3-Hydroxyadamantan-1-il)amino)acetil)pyrolidin-2-carbonitrile and its closed formula is C17H25N3O2 while molecular weight is 303.399 with pKa value of 9,03. Vildagliptin must be stored at room temperature and away from humidity. The medicine's chemical formula is shown in Figure 2-3.

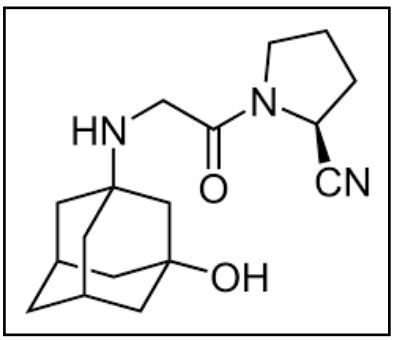


Figure 2- 3 Chemical Formula of Vildagliptin

2.6. Physical and Chemical Properties of Metformin

Metformin is a white crystalized powder. It is soluble in water and organic polar solvents. The chemical formula of metformin is 1-carbamimidamido-N,N-dimethylmethanimidamide and its closed formula is C4H11N5 while molecular weight is 129.1636. Metformin is a basic durg with a pKa value of 12.3 The chemical formula is shown in Figure 2-4.

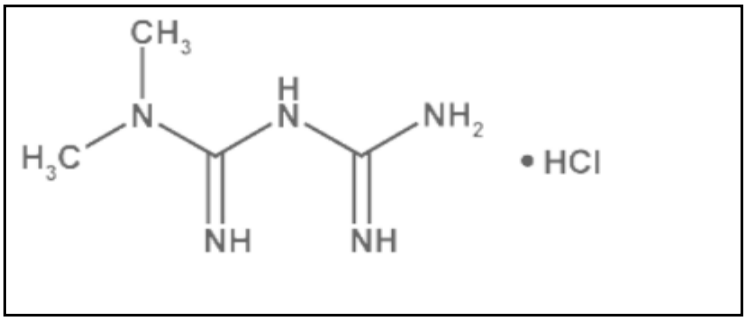


Figure 2- 4 Structure of metformin hydrochloride

2.7. Determination of Gliptins in Biological Fluids

Although there are several analytical methods available for the determination of gliptins in biological fluids in the literature as single drug or in combination with metformin, there is no simultaneous determination method available for sitagliptin, saxagliptin, vildagliptin and metformin in urine with LC-UV. There are two chromatographic methods developed with MS/MS detection which were discussed below. In one method there is no quantification for gliptins, just identification was performed, and in the other one saxagliptin was not studied. In this dissertation the quantification and validation were performed for the simultaneous determination of three gliptins and metformin in urine with LC-UV detection for the first time with no need for sample preparation. Sample preparation was achieved automatically by on-line SPE method coupled to LC by direct injection of the urine samples to the chromatograhy system.

A liquid chromatography-mass spectrometry method with electrospray ionization was developed by (Hess *et al.*,2010) for quantification of 11 oral hypoglycaemic drugs in plasma and simultaneous identification and validated. In the

study glimepiride, glibenclamide, gliquidone, glibornuride, glisoxepide, glipizide and gliclazide (sulfonylurea type), nateglinide and repaglinide (glinide type), rosiglitazone and pioglitazone (thiazolidinedione type) and the dipeptidyl peptidase inhibitors vildagliptin, sitagliptin and saxagliptin were identified. After a liquid–liquid extraction with tertbutyl methyl ether at two pHs, the oral antidiabetics were separated with fast gradient elution over a C8 column. Luna® RP C8 analytical column with 3 μm particle size and 150 mm×2 mm (Phenomenex, Aschaffenburg, Germany) dimensions and a Kromasil® C18 guard column, 4 mm×2 mm (Phenomenex, Aschaffenburg, Germany) were used for separation. The eluents were 90:10 ammonium formate (0.005 M, adjusted to pH 3 with formic acid)/acetonitrile (eluent A) and acetonitrile (eluent B) which were used in gradient elution. The ion source was operated in the positive mode at 400 °C. In the ESI mode in a single run, the simultaneous identification of 14 oral antidiabetics has been allowed by the LC-MS/MS assay, also a quantification of eleven oral anti- diabetics in plasma. Linearity has been shown up for purpose of over dosing condensations. Sitagliptin, saxagliptin and vildagliptin were not quantified, only identified with this method. For all analytes, with a signal-noise-ratio, the detection limit bigger than 3 has been below 1 ng/ml. Whereas recoveries have been ranged from 78% to 105%; while the recoveries of vildagliptin and saxagliptin, they have been worse (45%) due to their hydrophilic feature. For 11 drugs at three concentrations, accuracy and intraday precision were found below 20%. With regard to quantation of gliptins this method is inconvenient due to the fact that several validation parameters are out of range.

Regarding ICH/USFDA guidelines, to assess the plasma concentration of anti diabetic drugs; sitagliptin, vildagliptin, linagliptin and metformin a new liquid chromatography tandem mass spectrometry method was developed and validated. A Chromolith High Resolution RP-18 HPLC column (100 mm x 4.6 mm, macropores 1.15 um) was used to achive chromatographic separations with 0.01 M ammonium formate buffer (pH 3.0): acetonitrile (80:20 v/v) mobile phase mixture using isocratic elution mode. A flow rate of 0.4 mL/min was maintained throughout the analysis. Detection was performed by triple quadrupole MS fitted with ESI probe functioning in the positive ion MRM mode. Acetonitrile crash method was utilized to extract the drugs from the plasma, to decrease the errors that may occur during the analysis and alogliptin was used as an internal standard. The new technique considered as a sensitive and

selective technique since the data coming from validation research showed that the limits of the detection were in ng/mL for metformin, linagliptin, sitagliptin and vildagliptin 1.76, 1.94, 0.17 and 3.08, respectively. The method considered as very precise and accurate since the % CV and % RE were less than 1% for most of the data which is in accepted limit. A linear calibration curve (correlation coefficient, r (2) > 0.999) was obtained at the concentration range of 0.5-400.0; 5.0-400.0; 10.0-500.0 and 0.5-40.0 ng/mL for metformin, linagliptin, sitagliptin and vildagliptin, respectively. The extraction efficacy was evidenced from recovery study and all four analytes were found to be stable in plasma. The new LC-MS/MS technique can be used to determine the plasma concentration of anti-hyperglycemic drugs that is used in both pre and clinical PK research (Mohammed, B& H 2017).

Some examples were given below for the single drug determination of gliptins in biological fluids by liquid chromatographic methods.

Xu et al. (2012) determined saxagliptin and its active metabolite from plasma with LC-MS/MS method and prepared plasma samples with protein sedimentation method. Although the detector used in the study allowed a level of precision which is sufficient for pharmacokinetic studies, this sample preparation method is a low-efficiency and time-consuming method.(Xu *et al.*, 2012)

Gao et al. (2012) studied to develope a fast UPLC-MS/MS method to determine saxagliptin from rat plasma and apply this method to the pharmacokinetic study. Plasma samples which are prepared by liquid-liquid extraction is treated with liquid-liquid extraction with ethyl acetate and separated in a C18 column (2.1 x 50 mm id, 1.7 μm). Mobile phase consists of methanol and 0.1% formic acid (40:60, v / v). Determination of the samples are made in C18 column at 0.5 ng/ml lower limit.(Gao *et al*,. 2012)

In this study, the effect of a liquid-liquid extraction system on the formation of an in-situ DPP-4 inhibitor, sitagliptin, was investigated and a sensitive liquid chromatography tandem mass spectrometry developed. Dispatching the analysts have been implemented by following a mobile phase of an isocratic elution on a reverse-phase column. MS/MS analyse has been conducted through the multiple reaction monitoring mode by applying the respective ions of [M + H](+), m/z 408-235, also m/z 310-148 has been for the internal standard. The chromatography was performed using Symmetry C18, 150 × 4.6 mm, 5 μm at 30°C temperature. The isocratic mobile phase

composition was a mixture of 0.03% formic acid–acetonitrile (30:70, v/v), which was pumped at a flow-rate of 1.0 mL/min with a split ratio of load to waste of 10:90. Plasma samples were extracted using tertbutyl methyl ether:dichloromethane (80:20, v/v). A linear dynamic range of 0.1-250 ng/ml has been exhibited by the assay for sitagliptin in plasma of human. It has been made probable for analyzing more than three hundred samples of human plasma daily by a run time of 2.0 min/per sample. The validated method has been used to analyze plasma samples for application in pharmacokinetic studies. (Nirogi Ramakrishna, *et al.,* 2008).

High turbulence liquid chromatography-tandem mass spectrometry (MS/MS) method with turbulent flow online extraction was developed aiming to determine sitagliptin in human urine and hemodialysate and validated for supporting clinical studies (Wei Zeng, *et. al*.,2008). There had been a large particle size for the column of reversed-phase (Cyclone, 50 mm × 1.0 mm, 60 μm), also a column of BDS Hypersil C18 (30 mm × 2.1 mm, 3 μm) have been used as analytical columns and extraction, respectively. Mobile phase-A was 2.5mM ethylamine, 0.1% formic acid (FA) aques solution; mobile phase-B was 0.1% FA acetonitrile (ACN) solution. The injection volume was 5 and 40 μml for the urine and hemodialysate assay, respectively. The LLOQ was found as 0.1 μg/ml concerning the urine assay, the range of the linear calibration was 0.1 to 50 μg/ml, the precision of interday was (R.S.D.%, n = 5) was 2.3–6.5%, whereas the accuracy was 96.9–106% for nominal values. Regarding the quality of urine control samples (QCs), the precision of the intraday (R.S.D.%, n = 5) and accuracy were found as 1.8%–2.6% and 96.2%–106% concerning the nominal value, consecutively.

Rezaee et al. (2015) successfully developed high-performance liquid chromatography and liquid phase micro-extraction (HF-LPME) method for determination of anti-diabetic medicine sitagliptin (STG). With urine samples, Sitagliptin was extracted from 15 mL of the basic sample solution with a pH of 8.5 into an organic extracting solvent (n-octanol) impregnated in the pores of a hollow fiber and then back extracted into an acidified aqueous solution in the lumen of the hollow fiber. After extraction, it was injected as 20 uL to HPLC system. In order to obtain a high extraction efficiency, the parameters affecting HF-LPME such as pH, type of the organic phase, ionic strength, stirring rate, extraction time, volume ratio of donor phase

to acceptor phase and temperature were studied and optimized. Enrichment factors of up to 88 percent were obtained under optimized conditions and the relative standard deviation of the method is determined to be 3% to 6%. The results showed that the HL-LPME method was an excellent clean-up capacity and a high pre-concentration factor as well as it could serve as a simple and sensitive method for monitoring the medicine in urine samples. (Rezaee, R. *et al,* 2015)

Rao et al. (2013) developed and validated a reversed-phase high-performance liquid chromatographic method with fluorescence detection for the determination of sitagliptin enantiomers in rat plasma. Deproteinized rat plasma containing racemic sitagliptin was derivatized with o-phthalaldehyde and N-acetyl-L-cysteine under alkaline conditions, converted to diastereomers, and separated on a Lichrospher 100 RP-18e column using 20 mM phosphate buffer and methanol (45:55 v/v) as a mobile phase under isocratic mode of elution at a flow rate of 1.0 mL/min. Fluorescence detection was performed at 330 and 450 nm as excitation and emission wavelengths, respectively. The method was linear in the range of 50-5000 ng/ mL for both enantiomers. The intra- and interday accuracy and precision were within the predefined limits of ≤15% at all concentrations. The method was successfully applied to a pharmacokinetic study of sitagliptin after 5 mg/kg oral administration to Wistar rats. Robustness of the method was evaluated using design of experiments. (Nageswara Rao, R *et al.,* 2013)

2.8. Sample Preparation Methods for the Biological Fluid Analysis

Analytical methods are used to determine and separate drugs especially at low concentrations in biological fluids. Preparing sample before analyzing needs several steps before to achieve different goals, such as; decreasing any undesirable material that may decrease the detection of the drug, discharge of the analytes from matrix, decreasing of fluids to improve sensitivity and selectivity of the method (Andersson, L. I., 2001).

In toxicology laboratories, the most common sample preparation technique used is solvent extraction. It is named liquid-liquid extraction (LLE) and it needs two immiscible liquid phases. To enhance the separation in LLE, the separation takes place

between two phases; organic and the sample. Generally, the extraction based on; pH of the aqueous phase (sample), the analytes lipophilic and hydrophilic and the solvent used for the extraction. (McDowall, R. D. 1989)

Solid phase extraction (SPE) is also a common technique to prepare sample, this technique depends on using adsorbent with the biological fluids, isolating the solid phase and evading the analytes with a solvent or more than one solvent at once. The desire for solid phase should be higher than the sample regarding the analytes. A convenient solvent used to wash the interfering compounds and desorbe the analyte. The isolation concept based on the active site of solid phase and the sample also the intermolecular forces within the analytes (Walker, V., & Mills, G. A., 2002).

Currently, solid-phase microextraction (SPME) which is a new method, is used for determination of drugs in biofluids. The importance and usage of this method increases due to different reasons such as; less sample usage and no solvent used for extraction in addition to its simplicity and rapid performance. Two different ways are available for the exposure of the sample to fiber coated the stationary phase; headspace and direct immersion. In either case, the needle securing the fiber is withdrawn and the fiber is presented to the earth of the sample the space over the heated solution or the aqueous solution. The polymer coating (SPME fiber) concentrates the analytes by absorption or adsorption process. The efficiency of extraction depends on the temperature, agitation, pH, ion strength and salting out effect. the fiber is put in the infusion port of the chromatograph keeping in mind the end goal to strip the adsorbed substances.

One of the most used sample preparation method for biofluids is protein precipitation. By changing the solvation strength of the solvent, precipitation of proteins alter. The electrostatic forces between proteins are manipulated if specific reagents added thus the solubility of solute is lowered. The mechanism of precipitation for proteins is to alter the solvation potential of the solvent. Although it is one of the most practical sample preparation method, its low recovery values makes it less attractive.

The sample preparation technique choice based on several factors such as; chemical properties of the analytes and biological sample complexity. These sample

preparation techniques will enhance the effectiveness of the method in term of identification and quantification of the drug.

2.8.1. On-line Solid Phase Extraction

Unluckily, because of the the complexity and content of protein of biological fluids, direct injection of these samples are not congruent with the majority of chromatographic systems. Biological samples are regarded as problematic due to the irreversible adsorption of proteins on the stationary stage, a matter that leads to a an essential loss of column competence and a rise in back pressure (Souverain S, et al., 2004). In addition, it has become broadly recognized that samples of a considerable complexity can lead to matrix influences in mass spectrometry, which is resulted in under/over-estimation of concentrations for analyte. (Matuszewski BK et al., 2003).

So, a convenient sample preparation is regarded as a critical and a major consideration for developing methods of quantitative HPLC as well as for drugs measurements in biological fluids. Furthermore, the role of sample preparation carries on to be a considerable and a critical area for purpose of development, this is due to the fact that the rising acceptance for instrumentation of a high-throughput as for instance the LC/MS/MS, has transferred the bottleneck analysis backward in directions of sample preparation (Ma M, Cantwell FF,1998)

Commonly employed techniques such as liquid/liquid (LLE) or solid phase extraction (SPE) can enhance sensitivity, selectivity, and sample clean-up (Cantwell FF & Losier M,2003). Such processes are typically conducted off-line, it is performed either semi-automatically or manually, this may need huge hazardous solvents volumes, cost and labor. Despite the fact that requirements of solvent have been strongly lowered by micro-extraction processes, like solid-phase micro-extraction (SPME) (Theodoridis G &de Jong GJ,2005) or liquid/liquid micro-extraction (Ma M & Cantwell FF,1998) automation of such methods regarding systems of liquid-chromatographic is not mutual. Methods of direct on-line injection possess the reduction of steps regarding preparation enabling efficient clean-up and preconcentration of biological fluids, thereby lowering the need for handling possibility of dangerous material, cost, labor and time. On the other hand, additional errors and interferences from disposable plastic labware can also be introduced by additional sample manipulations for off-line and manual procedures (Boos K-S,et al.,1995)

Restricted-access materials are the one class of biocompatible materials of sample preparation where the direct injection of biological fluids is enabled. A special class of materials are represented by these sorbents and can make a partition of a biological sample into the matrix of protein as well as the analyte fraction, this is by relying on a molecular-weight cut-off. Coinciding with this procedure low molecular-weight compounds are enriched and extracted throughout a partition by size-exclusion, into the interior of the phase, as shown in Figure 2-5. Applying such materials for the repeated and direct analysis of pharmaceutical compounds within biological fluids has been well made, particularly with the supports of the commercialization, this is in accordance with phases of hydrophobic extraction (C4, C8 or C18) like alkyl-diol silica (ADS) and Biotrap. (Boos K-S,et al.,1995) (Hermansson J, Grahn A.1994)

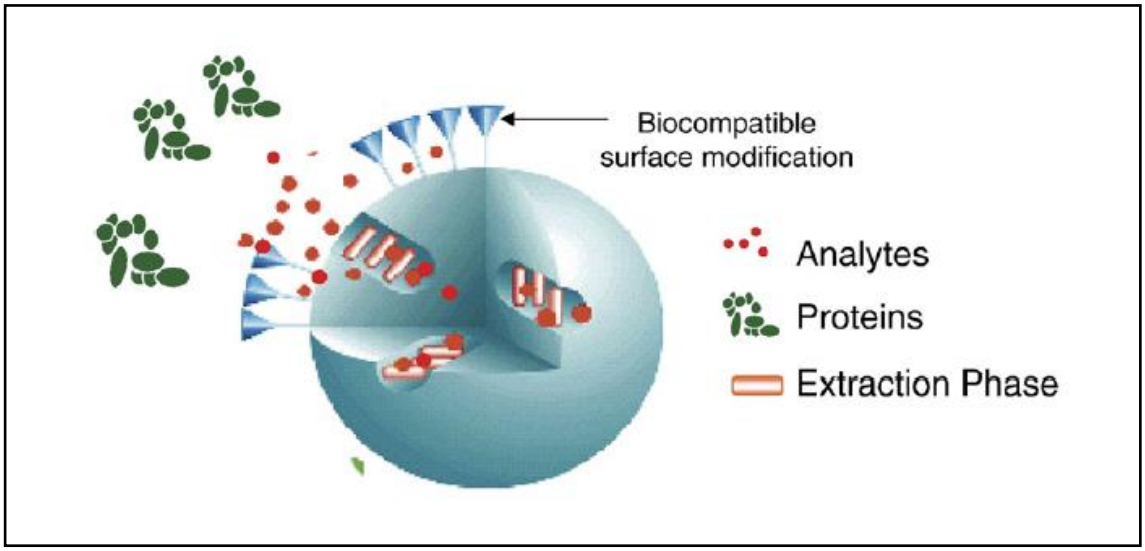


Figure 2- 5 Schematic representation of a RAM particle

In the technique of coupled-column, the RAM column acts as a pre-column for the analytical column. As illustrated in Figure 2-8, a a pump controls a sample loading onto the pre-column along with the one applied to condition the analytical column, Under such set-up, the protein component of the sample can be fractionated by the RAM pre-column, while enabling coinciding the analyte pre-concentration. Furthermore, additional selectivity is provided by the pre-column, grounded on the extraction phase, which is located in the center of the RAM particle. After flushing the

unretained matrix from the column, the valve is switched, the analytes are desorbed by the mobile phase from the second pump onto the analytical column for separation and detection. Before completing the analytical separation, the valve can be switched back towards the load position for the injection of the next sample for clean-up and reconditioning of the pre-column. (Mullett, W. M. ,2007).

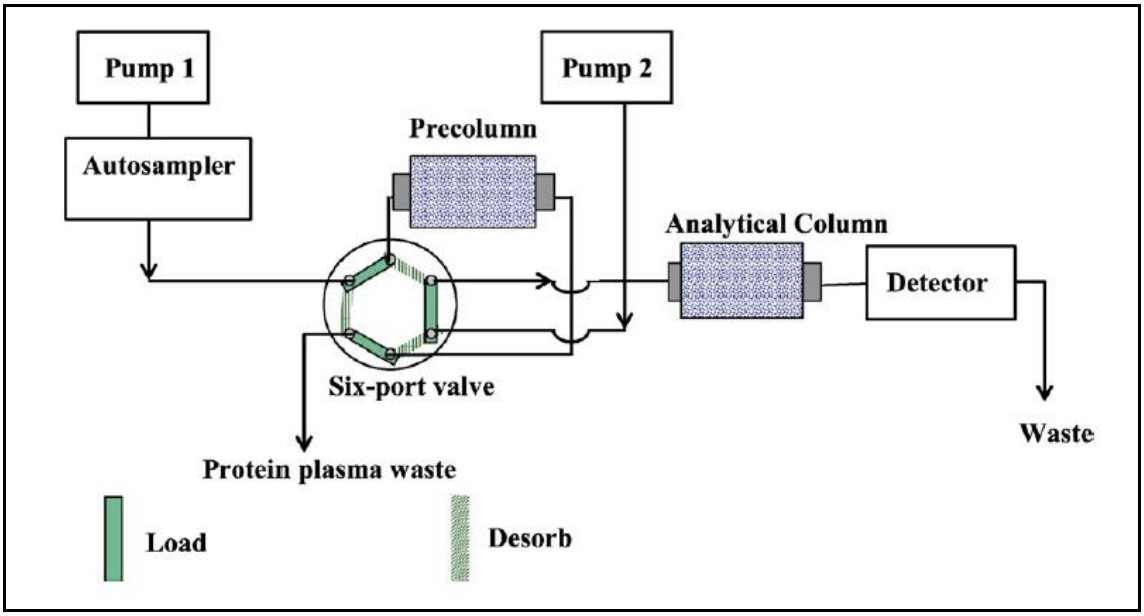


Figure 2- 6 Experimental set up of coupled-column for loading of sample and desorption of analysis.

3. MATERIAL AND METHODS

This thesis study was carried out in Istanbul University Faculty of Pharmacy, Research Laboratory of the Department of Analytical Chemistry between the years of 2017-2018.

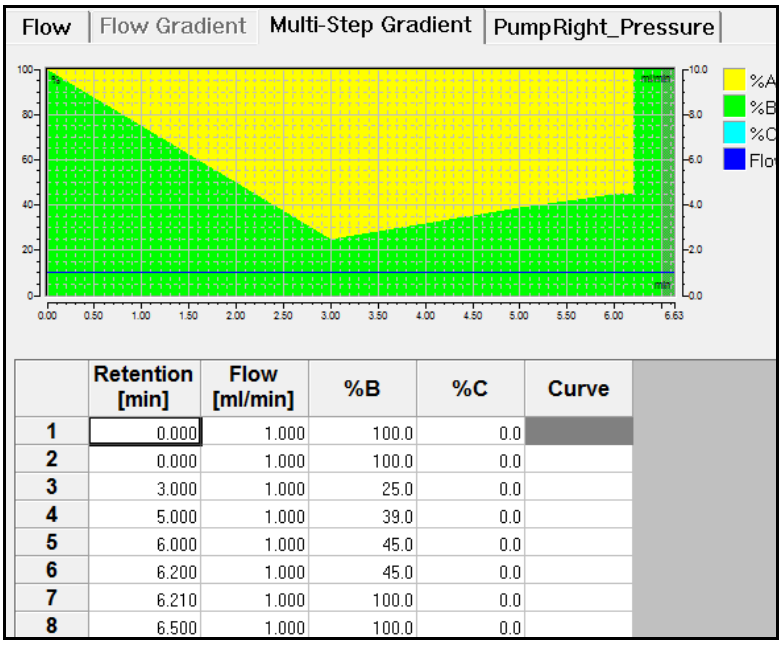
3.1. Reagents and Solutions:

Sitagliptin phosphate monohydrate (SITA) was kindly supplied by Merck Sharp and Dohme Pharmaceuticals (NJ, USA). Metformin and Vildagliptin (VIL), were kindly supplied by Abdi Ibrahim Ilac (Istanbul, Turkey) Saxagliptin (SAX) was purchased from Richem International Co., Ltd. (Shanghai, China) and internal standard (IS; Doxazosin) was obtained from Sigma-Aldrich (Steinheim, Germany). Ortho-phosphoric acid (≥98%) and methanol (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Deionized water up to a resistivity of 18.2 MΩ was purified with an Elga water purification system (London, UK). Stock solutions containing the SITA, SAX, VIL, MET and IS were prepared in water and further diluted with water. All stock solutions were kept at +4°C.

3.1.2. Solutions

Mobile Phase:

HPLC-grade methanol and 10 mM orthophosphoric acid solution were used as Mobile Phase A and B, respectively. 10 mM orthophosphoric acid solution was prepared by solving 70 µL of orthophosphoric acid solution in 100 mL of ultra-pure water. Both mobile phases were degassed in ultrasonic bath. The gradient program for mobile phase elution is given in Fig.3.1.



	Retention [min]	Flow [ml/min]	%B	%C	Curve
1	0.000	1.000	100.0	0.0	
2	0.000	1.000	100.0	0.0	
3	3.000	1.000	25.0	0.0	
4	5.000	1.000	39.0	0.0	
5	6.000	1.000	45.0	0.0	
6	6.200	1.000	45.0	0.0	
7	6.210	1.000	100.0	0.0	
8	6.500	1.000	100.0	0.0	

Figure 3- 1 Gradient program for mobile phase A: Methanol B: 10 mM o-phosphoric acid and C:water

Preparation of standard solutions

SITA, SAX, VIL and MET were prepared in 1.0 mg/mL concentration as a mixture to obtain the main stock solution. Diluted stock solutions were prepared by diluting main stock solution with water; the obtained concentrations were as followed, respectively; 100 µg/ml, 10 ug/ml and 1 ug/mL. IS stock solution was prepared in 1.0 mg/mL concentration and diluted to 100 µg/ml to obtain stock solution and 25 µl of this solution was spiked to all of the sample solutions.

3.2. Instruments and Other Appliances:

- Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC
- Analytical Column: Gemini C18, 110 A0, 5µ, 250 x 4.6 mm i.d
- Analytical balance (Denver Instrument)
- Vortex Mixer (Velp)
- Automatic pipettes (Eppendorf 1-10 µL, 10-100 µL and 100-1000 µL)
- Filters (Millipore 0.45 µm Nylon filter)
- Ultrasonic Bath (Ultrasonic LC 30H)
- Volumetric flasks (Isolab, 5, 10, 100 mL)

● Glass tubes (Screw cap, round-bottom, 12 cm)

3.3. Sample preparation and analysis

The study was approved by Local Ethics Committee of Istanbul University, Institute of Cardiology with an approval number of B.08.06. YOK. 2. I.U. E. 50. 0. 05. 00/02 on 5thMar 2014. After collecting the raw urine sample from a healthy volunteer, samples were diluted 10 times with water. During the analyses 20 µL of the sample was introduced to on-line SPE-LC-PDA system.

Drug-free urine samples were prepared as ten calibration standards for SITA, SAX, VIL and MET to give concentrations between 0.05-100.0 µg/mL for SITA, SAX, VIL and MET, respectively. Three levels of urine quality controls (0.25 – 2.50 –50.0 µg/mL) were studied to assess inter-day and intra-day precision and accuracy. To prepare the solutions with 100.0-50.0-10.0-5.0 µg/mL concentrations 100 µl of urine test was spiked first with 100.0-50.0-10.0-5.0 µL the volume of 1.0 mg/mL main stock solution, respectively and 25.0 µg/mL of IS stock solution, vortexed and made up to 1000 µL volume with water. Same procedure was used to prepare samples with 2.5-1.0-0.5 µg/mL concentration by spiking 25.0-10.0-5.0 µL of 100.0 µg/mL stock solution, respectively and 25.0 µg/mL of IS stock solution to 100 µl of urine sample, vortexed and made up to 1000 µL volume with water. To prepare the solutions with 0.25-0.1 µg/mL concentrations, 100 µl of urine test was spiked first with 25.0-10.0 µL of 10.0 µg/mL main stock solution, respectively and 25.0 µg/mL of IS stock solution, vortexed and made up to 1000 µL volume with water. Finally, to prepare samples with 0.05-0.01 µg/mL concentration by spiking 50.0-10.0 µL of 1.0 µg/mL stock solution, respectively and 25.0 µg/mL of IS stock solution to 100 µl of urine sample, vortexed and made up to 1000 µL volume with water. Each sample was prepared in 3 replicates and injected 3 times directly to online SPE-LC-PDA system as 20 µl. The spiked urine sample was compared with blank urine sample.

3.4. Chromatographic conditions and On-line SPE-LC set up

Tests were examined utilizing a completely automated on-line SPE-LC-PDA framework, and the design of the system was indicated schematically in Figure 3-2. A Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC framework with Chromeleon 6.8 programming was utilized. The framework comprises of a LPG 3600SD Dionex Dual Gradient Pump, a Ultimate 3000 Solvent Rack (6 channel), a

WPS 3000 SL Dionex Autosampler, a 10-Port exchanging valve, a PDA 3000 Dionex Photo Diode Array Detector (190– 800 nm) and a TCC 3000SD Dionex Column broiler. The PDA locator was set at 250 nm and 212 nm. 1 mL/min of flow rate was used for all the fractination, conditioning, separation and elution steps. A Gemini C18, 110 A^0, 5µ, 250 x 4.6 mm analytical column was utilized for separation. A 10 mM o-phosphoric acid-methanol mixture was used as mobile phase with gradient elution. The gradient elution program (Fig. 3-1) was run for 9.6 min. The on-line SPE was executed on a tailor-made LiChrospher® ADS RP4 RAM SPE-column (20 mm × 2 mm ID, 25 µm; Merck KGaA, Darmstadt, Germany) for fractionation of urine samples. SPE and LC section were connected by a 10-port switching valve and the schematic configuration of fully automated on-line SPE-LC-PDA system is presented in Fig 3-2.

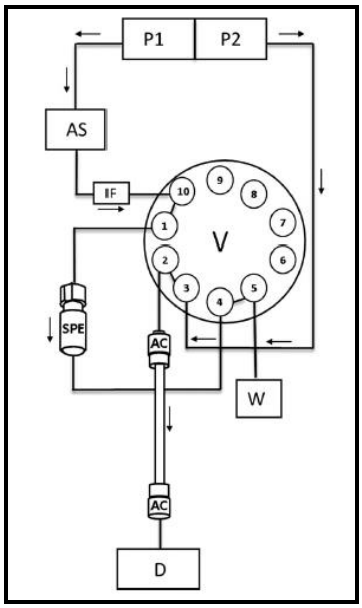


Figure 3- 2 Schematic configuration of fully automated online SPE-LC-PDA system. P1-P2, pumps; AS, autosampler; IF, inline filter; V, valve; AC, analytical column; D, detector; W, waste. (Andac S.C., 2016)

Pump right was used for sample fractionation, SPE wash and conditioning while Pump left was used for transfer and elution. The time program for pump left and right were shown in Fig. 3-3 where A: Methanol, B: 10 mM o-phosphoric acid.

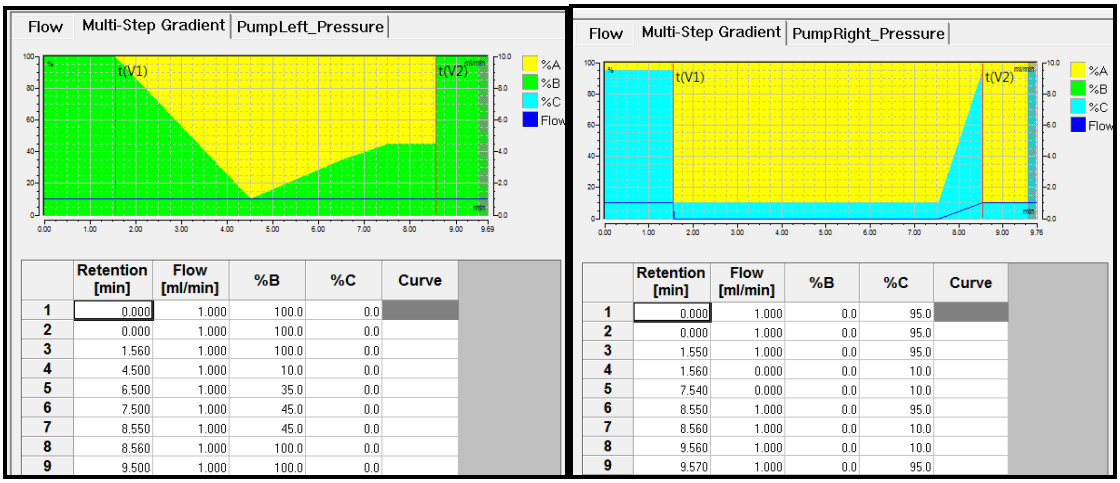


Figure 3- 3 The time program for pump left and right

The valve switching parameters tM (time for complete depletion of sample matrix), tA (breakthrough time of analyte) and tT (desorption/ transfer time of analyte from SPE-column to analytical column) were determined and calculated according to Majors et al. (30). (Figure 3-4)

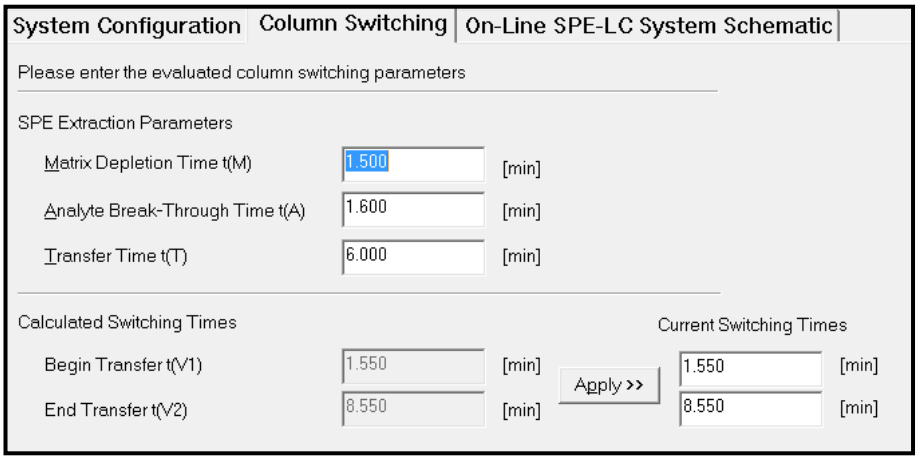


Figure 3- 4 Column switching parameters

3.5. Method validation

To confirm the performance of the developed method which meets the requirements for intended use it was validated according to widely accepted protocols and international regulations. Here the ICH Guideline was followed for the validation study. For method validation linearity and range, accuracy, precision, selectivity, limit of detection (LOD) and limit of quantification (LOQ) parameters were studied.

Linearity and range were studied by constructing the calibration curves with 10 plots in spiked urine samples between the concentrations and 0.05-100.0 µg/mL for

SITA, SAX, VIL and MET, respectively. The preparation of samples were described in Section 3.3 in detailed. The calibration curves were constructed from the samples prepared in 3 replicates for each plot and injected 3 times. The peak area ratios of the analyte to the IS were plotted against the concentration of the analyte.

To assess the accuracy and inter-day and intra-day precision studies, the concentrations of 0.25–2.50–50.0 μg/mL sitagliptin, saxagliptin, vildagliptin and metformin in urine corresponding the low, medium and high concentrations were analyzed. Interday precision was studied in three different days in the same pattern. One way ANOVA was carried out to measure the inter-day, intra-day precision (repeatability) at three different levels. Excel sofware was used to perform ANOVA test. At 95% confidence level, F experimental and critical F were compared.

Accuracy was carried out by standard addition method, at three different concentration levels, as low, medium, high.

Recovery was studied by comparing the on-line SPE-LC analysis of a spiked urine sample with the calibration standard prepared in water at 50.0 μg/mL concentration level. Also the off-line recovery of the drugs was achieved at the same concentration level by direct injection of the samples onto the analytical column.

The formula x × s/slope of the calibration curve were used to find LOD and LOQ, for LOD x=10 and x=3 for LOQ where s value is the SD of the regression lines.

The carry-over of the on-line sample preparation system was evaluated by injecting blank urine sample after a high concentration spiked urine sample.

4. RESULTS

The fully automated online SPE-LC-PDA system allows the direct injection of biological samples repetitively to the chromatographic system without a need for further sample pretreatment. For this purpose, RAM SPE-column packed with LiChrospher® ADS RP4 material was utilized for size-selective fractionation of urine samples through the study.

Following direct injection of 20 µL of urine sample to the SPE-column, the high molecular weight matrix was depleted during the fractionation step. H_2O/Methanol: 95/5, v/v mixture was used to remove the drugs from the protein binding sites for sample fractionation which takes only 1.5 minutes. 7 minutes required for transfer and elution of drugs from SPE to analytical column with the gradient program shown in Figure 3-1. After elution of all the analytes the valve was switched back to the original position to clean and condition the SPE column.

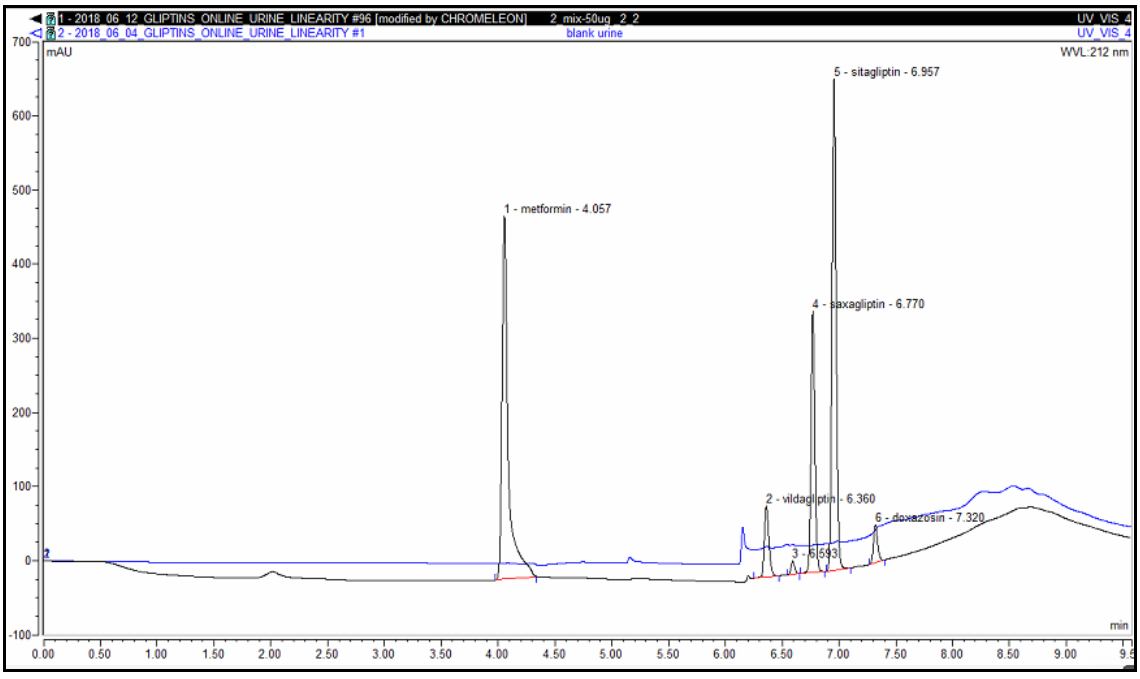


Figure 4- 1 A chromatogram of a spiked urine sample with 50 µg/mL concentration of SITA, SAXA, VIL, MET and 25 µg/mL of IS compared to blank urine chromatogram

As could be seen in Fig. 4-1, no carry-over was observed on the on-line SPE-LC system. Well resolved analyte peaks were eluted between the minutes of 4.0-7.35 where the retention times were 4.057; 6.36; 6.77; 6.95 and 7.32 for MET; VIL; SAX; SIT and

IS, respectively. Also in Fig.4-2 a chromatogram for spiked urine sample with 50 ng/mL concentration could be seen.

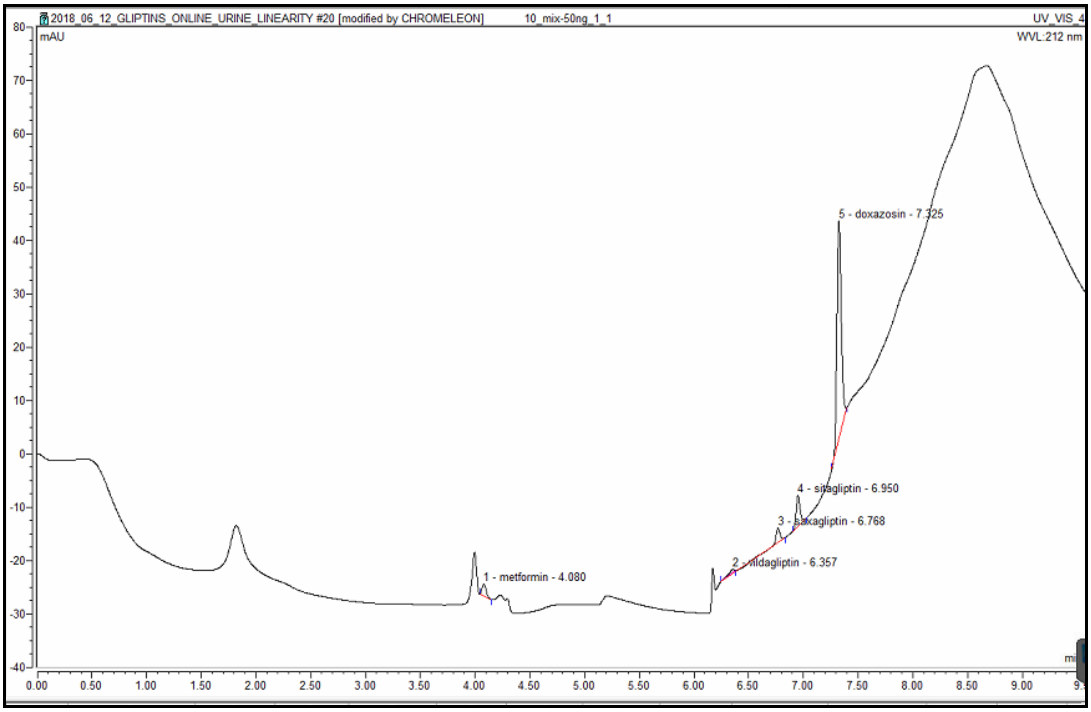


Figure 4- 2 A chromatogram of a spiked urine sample with 50 ng/mL concentration of SITA, SAXA, VIL, MET and 25 µg/mL of IS

The calibration curves were found to be linear in the range of 0.05-100.0 µg/mL for SITA, SAX, VIL and MET, respectively. The concentration range, calibration curve equation, regression coefficient, LOD and LOQ values for SITA, SAX, VIL and MET were shown on Table 4-1.

	Concentration range (µg/mL)	Calibration curve y = ax + b	Regression coefficient (R2)	LOD (µg/mL)	LOQ (µg/mL)
SITA	0.05-100.0	y=0.4952x-0.4149	0.9989	0.01744	0.05814
SAX	0.05-100.0	y=0.2388x+0.1008	0.9993	0.02497	0.08324
VIL	0.05-100.0	y=0.1043x+0.0855	0.9988	0.03543	0.1181
MET	0.05-100.0	y=0.5578x-0.9749	0.9946	0.02477	0.08258

Table 4- 1 The range, calibration curve equation, regression coefficient, LOD and LOQ values for SITA, SAX, VIL and MET

The developed method was linear in a wide range of concentration with satisfactorily regression coefficients for mentioned drugs. The concentration values maximum 15 % RSD values were used for calculations through the validation study.

LOD and LOQ values were found to be as 0.02497- 0.08324 µg/mL for SAX, 0.03543-0.1181 µg/mL for VIL, 0.01744- 0.05814 µg/mL for SITA and 0.02477-0.08258 µg/mL for MET, respectively.

The calibration curves for SITA, SAX, VIL and MET were given respectively in Fig 4-3, 4-4, 4-5 and 4-6.

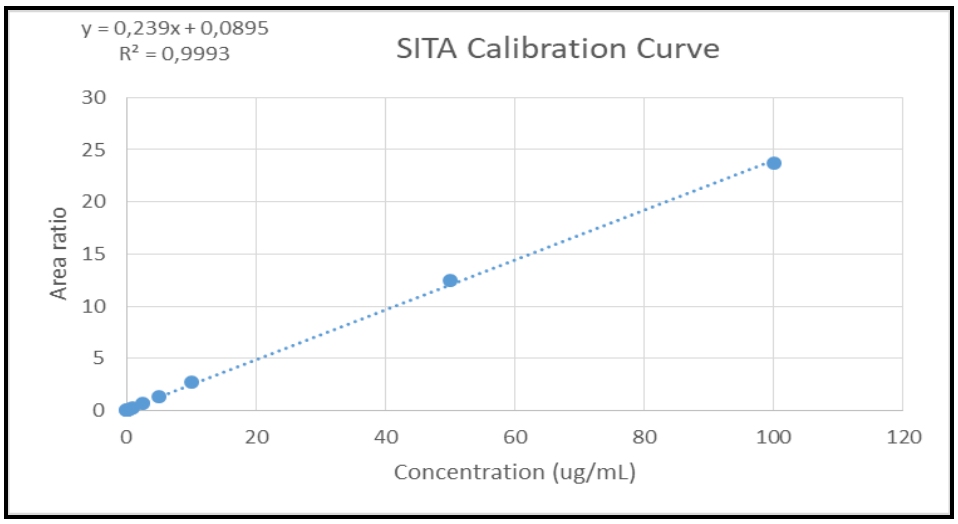


Figure 4- 3 Calibration curve for SITA

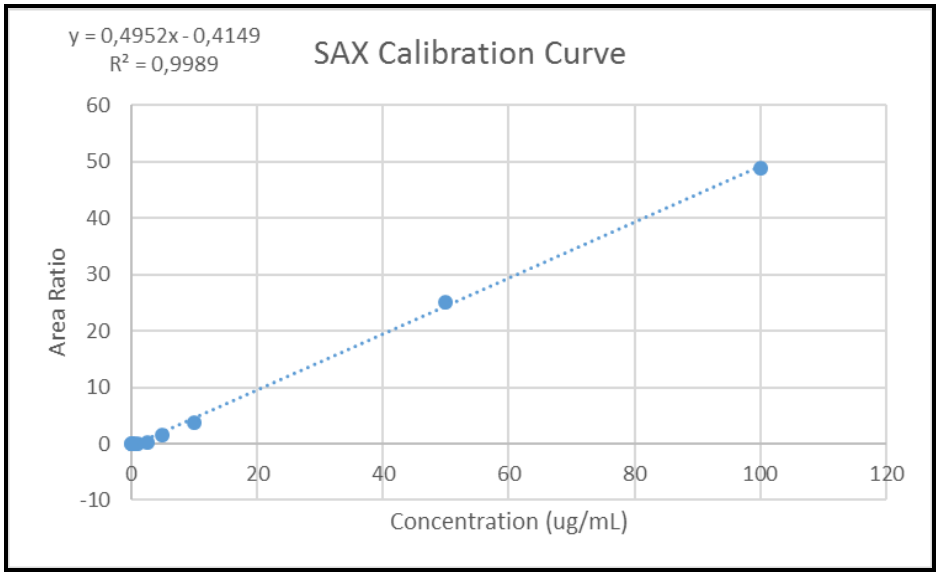


Figure 4- 4 Calibration curve for SAX

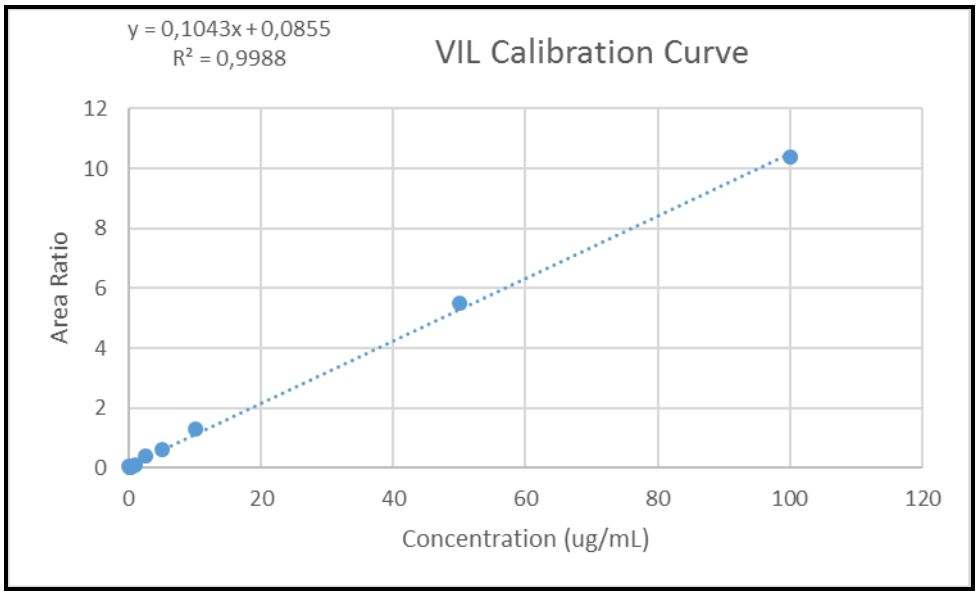


Figure 4- 5 Calibration curve for VIL

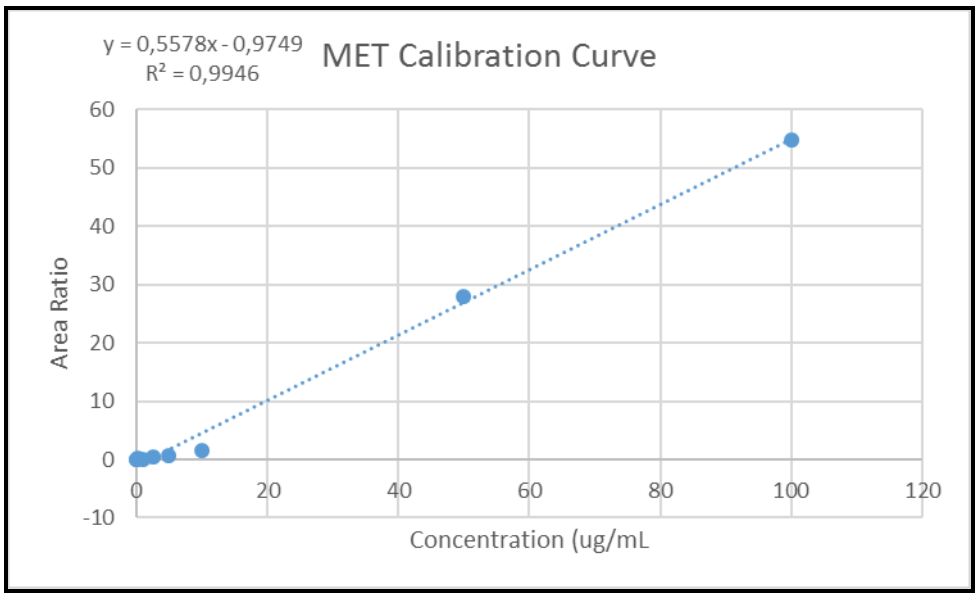


Figure 4- 6 Calibration curve for MET

Inter-day and intra-day precision were studied on low, medium and high level of concentrations which were 0.25–2.50–50.0 µg/mL for SITA, SAX, VIL and MET. Interday precision was studied in three different days. One-way ANOVA was carried out to measure the inter-day, intra-day precision (repeatability) at three different levels

(n=3). Excel software was used to perform ANOVA test. At 95% confidence level, F experimental and critical F were compared in Table 4-2.

	Spiked Concentration	Calculated Concentration (µg/mL) ± SD	Repeatability (for inter-day) RSD %	Intermediate precision (for intra-day) RSD %	F values from ANOVA analysis (F_{EXP} < $F_{CRITICAL}$, %95)
SITA	LOW (0.25 µg/mL)	0.26±0.02	7.93	8.42	1.12 < 5.14
	MID (2.50 µg/mL)	2.49±0.03	1.13	1.74	1.38 < 5.14
	HIGH (50.0 µg/mL)	49.98±2.92	5.98	5.99	0.01 < 5.14
SAX	LOW (0.25 µg/mL)	0.25±0.01	3.46	7.09	3.20 < 5.14
	MID (2.50 µg/mL)	2.45±0.15	0.27	0.40	1.12 < 5.14
	HIGH (50.0 µg/mL)	50.01±0.17	3.37	6.16	2.33 < 5.14
VIL	LOW (0.25 µg/mL)	0.24±0.01	3.55	4.54	0.63 < 5.14
	MID (2.50 µg/mL)	2.49±0.01	7.29	10.26	0.98 < 5.14
	HIGH (50.0 µg/mL)	50.72±1.05	0.38	0.38	0.01 < 5.14
MET	LOW (0.25 µg/mL)	0.25±0.02	7.37	7.41	0.01 < 5.14
	MID (2.50 µg/mL)	2.51±0.25	9.61	9.67	0.01 < 5.14
	HIGH (50.0 µg/mL)	49.97±0.35	0.67	0.73	0.17 < 5.14

Table 4- 2 Intra-day and Inter-day accuracy and precision results at three different concentration levels for SITA, SAX, VIL and MET in spiked urine

The recoveries of the On-line SPE-LC-PDA method were compared with the result of the same level standard solution injection to the analytical column and founded as 96.73 %; 99.78%; 98.83 % and 96.26 % for SITA, SAX, VIL and MET, respectively.

5. DISCUSSION

Type 2 diabetes is a disease that increases day by day in Turkey and in all over the world. Dipeptidyl peptidase-4 inhibitors so called gliptins are a fairly new class of oral hypoglycemic which are used in the treatment of Type 2 diabetes. Sitagliptin, Saxagliptin and Vildagliptin are the members of this group acting by inhibiting the destruction of incretin hormones. They are either used alone in tablets or in combination with metformin. Here in this study we are aimed to develop a new chromatographic method for the determination of these three gliptins simultaneously and due to their availability in combination with metformin, we also added metformin to our method development. Since their amount of excretion is high in urine, we applied our method to urine samples.

There are not much analytical methods available in the literature for the determination of gliptins in biological fluids, especially in urine samples. The developed methods are mostly based on single drug determination in biofluids. The are only two literatures present for simultaneous gliptin determination and they are based on tandem mass spectometric detection which is not commonly available in every research laboratory due to its high cost.

Since we have developed a chromatographic method for the determination of sitagliptin, saxagliptin, vildagliptin, metformin simultaneously in human urine samples by using UV detection, and used an on-line sample preparation method requiring no sample preparation and made the direct injection of urine samples repeitetively to chromatographic system possible. Our method will be the first method in the literature with advantages of being fast, reliable and time-labor saving.

In detailed, chromatographic method development was based on reversed phase chromatographic method development with an acidic mobile phase due to the basic feature of these four drugs. A Gemini C18, 110 A0, 5µ, 250 x 4.6 mm analytical column was used for chromatographic separation with a mobile phase mixture of 10 mM o-phosphoric acid-methanol in gradient elution.

On-line SPE-LC is a fully automated sample preparation method which combines the sample preparation and separation in one. In this method it is possible to

inject the biological fluid directly and repetitively to the system due to the use of switching valves which send the high molecular weight matrix in the bio-fluid to the waste after fractionation on SPE. For this purpose, a tailor-made LiChrospher® ADS RP4 restricted access material SPE-column (20 mm × 2 mm ID, 25 µm; Merck KGaA, Darmstadt, Germany) was used in the study for fractionation of urine samples with a mobile phase mixture of H_2O/Methanol: 95/5, v/v for 1.5 minutes. Transfer of the drugs from SPE to LC column and subsequently separation and elution on LC column were achieved with the gradient elution in total of 7 minutes.

Well-separated peaks were observed in a 9.6 min runtime, with retention times of 4.057; 6.36; 6.77; 6.95 and 7.32 for MET; VIL; SAX; SIT and IS, respectively.

The repetitive injection of urine sample was allowed to the system with no carry-over issue which was proved by injecting blank urine sample at the end of the sequences.

The developed method was validated according to ICH Guidelines by means of linearity, range, selectivity, accuracy, precision, limit of detection and limit of quantitation. The calibration curves were found to be linear in a wide the range between 50 ng/mL and 100 µg/mL with satisfactorily regression coefficients. Maximum 15 % RSD values were used for calculations through the validation study. LOD and LOQ values were found to be satisfactory as the urine excretion taken into account. Inter-day and intra-day precision were studied on low, medium and high level of concentrations by using Excel software to perform ANOVA test. The recoveries of the On-line SPE-LC-PDA method were compared with the result of the same level standard solution injection to the analytical column and founded between 99.26 and 99.78 %.

In conclusion, to the best of our knowledge the developed and validated method will be the first fully automated on-line SPE-LC-UV method for the determination of Sitagliptin, Saxagliptin, Vildagliptin and Metformin simultaneously in human urine samples allowing the repetitive direct injection of spiked human urine samples. It could be applied in clinical laboratories in terms of its practical use, easily found detection and reliable results.

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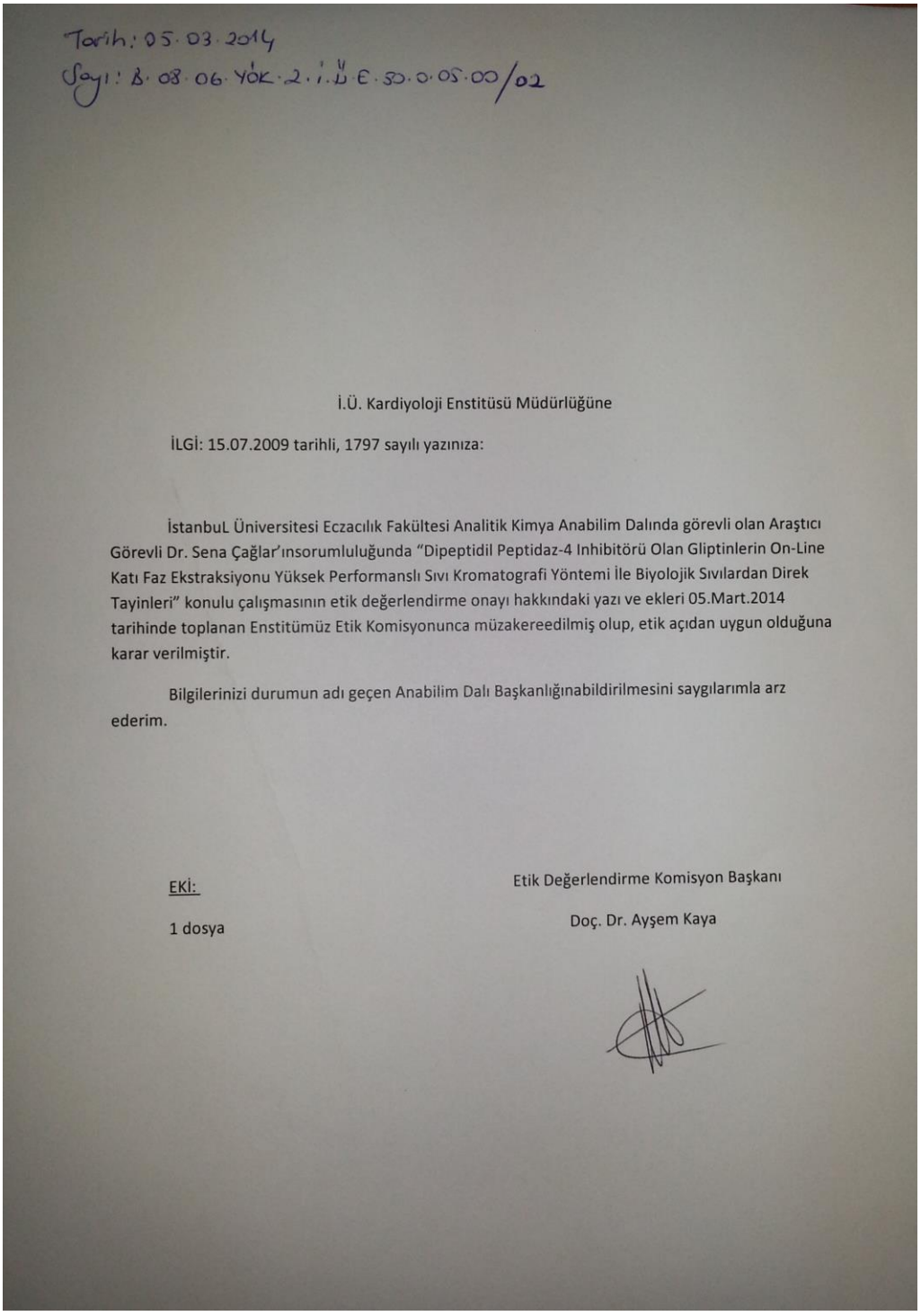
ETHICAL COMMITTEE DECISION



Tarih: 05.03.2014
Sayı: B.08.06.YÖK.2.İ.Ü.E.50.0.05.00/02

İ.Ü. Kardiyoloji Enstitüsü Müdürlüğüne

İLGİ: 15.07.2009 tarihli, 1797 sayılı yazınıza:

İstanbuL Üniversitesi Eczacılık Fakültesi Analitik Kimya Anabilim Dalında görevli olan Araştıcı Görevli Dr. Sena Çağlar'ınsorumluluğunda "Dipeptidil Peptidaz-4 Inhibitörü Olan Gliptinlerin On-Line Katı Faz Ekstraksiyonu Yüksek Performanslı Sıvı Kromatografi Yöntemi İle Biyolojik Sıvılardan Direk Tayinleri" konulu çalışmasının etik değerlendirme onayı hakkındaki yazı ve ekleri 05.Mart.2014 tarihinde toplanan Enstitümüz Etik Komisyonunca müzakereedilmiş olup, etik açıdan uygun olduğuna karar verilmiştir.

Bilgilerinizi durumun adı geçen Anabilim Dalı Başkanlığınabildirilmesini saygılarımla arz ederim.

EKİ:

1 dosya

Etik Değerlendirme Komisyon Başkanı

Doç. Dr. Ayşem Kaya

6. FIRST PAGE OF PLAGIARISM REPORT

SIMULTANEOUS DETERMINATION OF NEW GENERATION ANTIDIABETICS IN BIO- FLUIDS BY HPLC (YENİ NESİL ANTİDİYABETİKLERİN BİYOLOJİK SIVILARDAN HPLC İLE YANYANA ANALİZİ

ORİJİNALLİK RAPORU

%20	%12	%17	%3
BENZERLİK ENDEKSİ	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCİ ÖDEVLERİ

BİRİNCİL KAYNAKLAR

1	Sena Caglar Andac. "Determination of Drugs by Online Column-Switching Liquid Chromatography", Journal of Chromatographic Science, 2016 Yayın	%4
2	Wayne M. Mullett. "Determination of drugs in biological fluids by direct injection of samples for liquid-chromatographic analysis", Journal of Biochemical and Biophysical Methods, 2007 Yayın	%2
3	www.shd.org.rs İnternet Kaynağı	%1
4	waesearch.kobv.de İnternet Kaynağı	%1
5	edoc.ub.uni-muenchen.de İnternet Kaynağı	%1

CURRICULUM VITAE

Personal Date

Name	AMAL	**Last name**	MOHAMAD
Place of birth	Libya AL- Marj	**Date of birth**	09/07/1989
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Master		
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Work experience (type from past to future)

	Mission	**Corporation**	**Duration(year-year)**
1.	Teacher Assistant	Higher Institute of Medical Vocation – El-marj	2011-2014
2.	Pharmacist	AL- Marj Hospital	2011-2013
3.			-

Foreign languages	**Reading**	**speaking**	**Writing**	**TOEFL**	**Categories**
English	Good	**Moderate**	Good		

Computer Information

Program	**Using skill**
Excel	Good
Photo shop	Good

Publications / Certificate Notifications / Awards

Special interests.