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Bioanalytical method development for dapagliflozin and saxagliptin by RP-HPLC method

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Abstract
The simultaneous quantification of Dapagliflozin and Saxagliptin in rat plasma, a straightforward, precise, quick, and accurate RP-HPLC method was created. By using methanol as a solvent and the protein precipitation method, the drug samples were extracted. The separation was achieved by using C-8 Eclipse plus(5µ particle size, 250×4.6mm,5µm internal diameter). The mobile phase comprises of 0.01% Trimethylamine in water and Methanol in the ratio of 40:60(v/v). The flow rate was 1.0ml/min and the effluents were monitored at 228 nm with a total run time of 15min. The retention time was found to be 4.243 and 11.304 respectively for Saxagliptin and Dapagliflozin. The detection concentration was linear over 25- 175ng/ml for Saxagliptin and Regression equation was found to be y =852.37x+3836with regression coefficients 0.9932 and percentage recovery of 99.96%.The detection concentration was linear over 100-700ng/ml for Dapagliflozin and Regression equation was found to be y=132.88x+322.9 with regression coefficients 0.9902 and percentage recovery of 99.80%. The liquid chromatography method was extensively validated for linearity, accuracy, precision and recovery. The developed approach was successfully shown for the simultaneous determination of Saxagliptin and Dapagliflozin in rat plasma and verified in accordance with ICH criteria. All these analytical validation parameters were found to be satisfactory. For the examination of rat plasma for use in pharmacokinetic studies, pharmacological interactions, bio availability, and bio equivalence, this method is therefore conveniently applicable.

Keywords: HPLC, method development, validation

Introduction
A set of processes used in the gathering, processing, storing, and analysis of a biological matrix for a chemical compound is known as a bio analytical method. A quantitative analytical method's suitability for biochemical applications is established through the process of bio analytical method validation. In the future, there will be many interesting chances to further advance the field of bio analysis, which is already making strides in terms of sensitivity, specificity, accuracy, efficiency, assay throughput, data quality, handling, and processing, analysis cost, and environmental effect. In forensic and clinical toxicology, the validity of analytical results is crucial because it is, of course, a requirement for accurate interpretation of toxicological data. [3]

Diabetes mellitus is a chronic disorder characterized by hyperglycaemia and the late development of vascular and neuropathic complications which are associated with a common hormonal defect namely insulin deficiency. Type 1 diabetes is a chronic illness characterized by the body's inability to produce insulin due to the autoimmune destruction of beta cells in pancreas. Type 2 diabetes is characterized by insulin resistance and relative insulin deficiency. It is a progressive and complex disorder that is difficult to treat effectively in long term. [1, 2]

Dapagliflozin is a drug of the gliflozin class and it can be used to treat type 2 diabetes mellitus. It inhibits subtype 2 of the sodium glucose transport proteins which are responsible for atleast 90% of the glucose reabsorption in the kidney. Blocking this transport mechanism causes blood glucose to be eliminated through urine. [4]

Saxagliptin is an oral hypoglycemic (antidiabetic) drug of the dipeptidyl peptidase-4 (DPP-4) inhibitor class of drug that works by affecting the action of natural hormones. It is used as a monotherapy or in combination with other drugs for the treatment of type 2 diabetes. [5]

High-performance liquid chromatography
High-performance liquid chromatography is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. It relies on a pump to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. [6]

HPLC instrument typically includes a degasser, sampler, pumps, and a detector. The sampler brings the mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportion to the amount of sample component emerging from the column. Hence, allowing for quantitative analysis of the sample components. [7]

Schematic diagram of HPLC

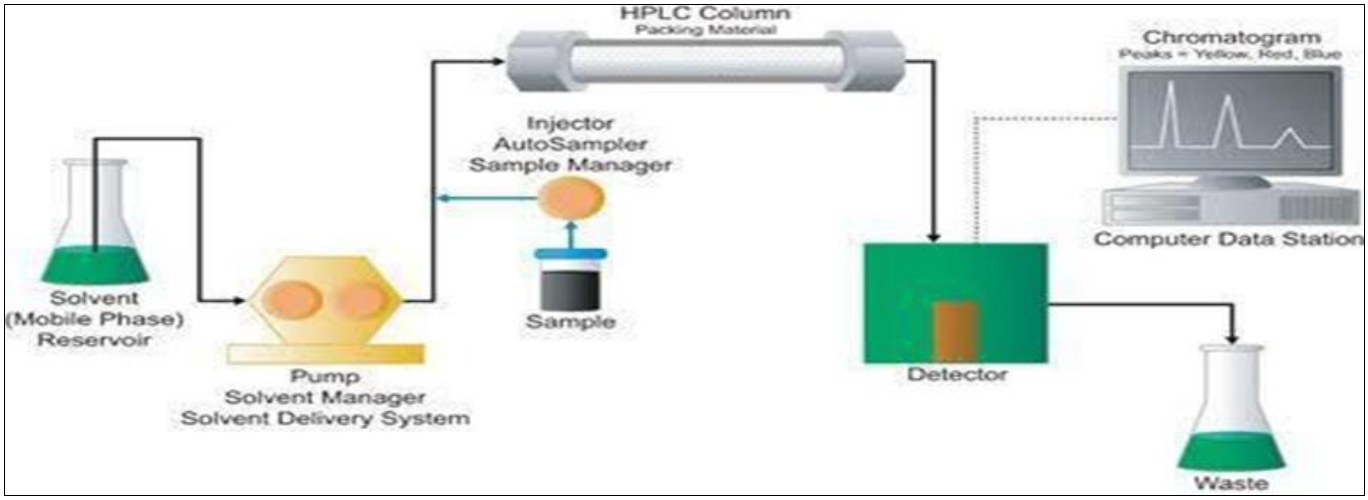

Fig 1

Method development

The determination of pharmaceutical dosage forms can be accomplished using the well-known method known as reverse-phase high performance liquid chromatography (RP-HPLC). Because HPLC makes use of a variety of chromatographic components. The process of optimising experimental settings involves considering factors like temperature, flow rate, molarity, pH, buffer, type and concentration of organic modifiers, among others. To maximise chromatographic separations, a systematic technique such as experimental design is hence more important.

1. **Solubility profile:** Dapagliflozin and Saxagliptin is freely soluble in Methanol.

2. **Selection of buffer:** Buffers are generally selected on the basis of greater stability for the drug under study and hence better peaks.0.01%triethylamine is added to water.

3. **Selection of mobile phase:** The selection of ideal mobile phase is done by altering the ratio of the buffers, composition of the mobile phase solvents in the different ratio and also by passing them through the column. Preparation of mobile phase is based on the following physic-chemical properties.
   - Solubility
   - Stability
   - pKa value

4. Based on the literature survey and other experimental parameters finally the suitable mobile phase for the drugs Dapagliflozin and Saxagliptin is found to be 0.01% Triethylamine in Water and methanol in the ratio 40:60% v/v.

Preparation of mobile phase

0.01 % triethylamine in Water and methanol were taken in separate mobile phase reservoir in the ratio of 40:60% v/v and sonicated for 15 min for degassing. The solution was filtered through 0.22 µm membrane filter.

Selection of extraction method

Protein precipitation

Protein precipitation is often used as the initial sample preparation scheme in the analysis of a new drug substance since it does not require any method development.

A volume of sample matrix (1 part) is diluted with a volume of organic solvent or other precipitating agent (3-4 parts), followed by vortex mixing and then centrifugation or filtration to isolate or remove the precipitated protein mass.

- Acids used as precipitating agents
- Organic solvents used as precipitating agents
- Saturated aqueous ammonium sulfate
- Heavy metal cation salts of zinc and copper coordinate.

Preparation of reagents

Dapagliflozin - Preparation of standard stock solution of API:

Stock- 1: About 100mg of Dapagliflozin is weighed accurately, and is transferred to a 100ml of clean glass volumetric flask. It is dissolved in Methanol and the volume is made up to 100ml with the same solvent (1000µg/ml). The above solution was filtered through 0.22 micron filter and Sonicate for 10-15 minutes.

Stock-2: From the above solution (1000µg/ml) pipette out 8ml and make up the volume with Methanol up to 100ml (800µg/ml). The solution was was filtered through 0.22micron filter and sonicated for 10-15 minutes.

Stock-3: From the above solution (800µg/ml) pipette out 1ml and make up the volume with methanol up to 100 ml (8µg/ml). The solution was filtered through 0.22micron filter and Sonicate for 10-15 minutes.

Preparation of working standard for APIs (Saxagliptin and Dapagliflozin)

The series of dilution were made to get concentration range 400, 800, 1200,1600,2000, 2400, 2800µg/ml of Dapagliflozin by taking 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5ml from the stock-3 solution of (8µg/ml)and 100,200,300,400,500,600,700µg/ml of Saxagliptin by taking 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5ml from the stock-3'of

(2µg/ml)to 10ml volumetric flask respectively, the volume is made up with methanol and name them as A,B,C,D, E, F,G. The solutions were sonicated for 10-15minutes.

Extraction of plasma from rat blood (Procedure)

- Blood from rat was collected from retro orbital route using heparinised capillary tube.
- Around 2 ml of rat blood was collected in pre-coated EDTA blood collection tube.
- Collected blood is centrifuged at 5000 rpm for 5mins at 4ºC, plasma layer was separated.
- Separated plasma layer is collected in new eppendorf tubes.
- The separated plasma is deproteinated using Methanol

Preparation of serial dilution in plasma

Take eight Eppendorf tubes and mark each of them as 1,2,3,4,5,6,7,8. To the above tubes add 100µl of plasma and 50µl of methanol. To the tubes from 2-8 add 50µl of the drug solution from the series A, B, C, D, E, F, G respectively to get a concentration range of 100,200,300,400,500,600 and700ng/ml for Dapagliflozin and 25,50,75,100,125,150and175ng/ml for Saxagliptinin combination. The solutions were vortexed for 5 minutes and centrifuged at 5000rpm at 4ºC, filter through 0.22 micron filter and Sonicate the above solutions.

Preparation of Quality control levels in rat plasma solution

1. **Lower Limit of Quantification (LLOQ), (100ng Dapagliflozin/25ngSaxagliptin)/ml**: Take an Eppendorftube(1.5ml) and add 100µl of plasma, 50µl.methanol and 50µl of drug solution from dilution-A. The solution was vortexed for 5 minutes and centrifuged at 5000rpm at 4ºC, filter and sonicate the solution.

2. **Medium Quality Control (MQC), (500ngDapagliflozin/125ngSaxagliptin)/ml:** Take an Eppendorf tube(1.5ml) and add 100µl of plasma, 50µl.methanol and 50µl of drug solution from dilution-E. The solution was vortexed for 5 minutes and centrifuged at 5000rpm at 4ºC, filter and sonicate the solution.

3. **High Quality Control (HQC), (700ngDapagliflozin/175ngSaxagliptin)/ml**: Take an Eppendorf tube(1.5ml) and add 100µl of plasma, 50µl.methanol and 50µl of drug solution from dilution-G. The solution was vortexed for 5 minutes and centrifuged at 5000rpm at 4ºC, filter and sonicate the solution.

Bio analytical validation

The purpose of bioanalytical method development is to define the design, operating conditions, limitations, and suitability of the method for its intended purpose and to ensure that the method is optimized for validation.

1. Accurcy, Precision and recovery

Evaluating the accuracy and precision across the quantitation range during method development is essential to determine whether the method is ready for validation and involves analyzing replicate QCs at multiple concentrations across the assay range. Specifically, the sponsor should evaluate the performance at the LLOQ, low, mid and high QCsto determine if the method is suitable to analyze study samples. Method validation experiments for estimating accuracy and precision should include a minimum of three (for CCs)independent runs (i.e., accuracy and precision (A & P) runs; see Table 1) conducted over several days. Each A & P run should include a calibration curve and multiple QC concentrations that are analyzed in replicates. The sponsor should determine the accuracy and precision of the method based on the performance of the QC in the A & P runs. The specific validation requirements for accuracy and precision and A & P runs are listed in Table 07. The sponsor should use freshly prepared **c**alibrators and QCs in all A & P runs. Use of freshly prepared QCs in all A & P runs is preferred; however, if this is not possible, the sponsor should use freshly prepared QCs in one or more A & P runs.

2. Stability studies

- **Bench-top stability**: The sponsor should determine the stability of samples under the laboratory handling conditions that are expected for the study samples (e.g., the stability of samples maintained at room temperature or stored in an ice bucket).

- **Stock solution stability**: Stock solutions should not be made from reference materials that are about to expire unless the purity of the analyte in the stock solutions is re-established. When the stock solution exists in a different state (e.g., solution versus solid) or in a different buffer composition (which is generally the case for macromolecules) from the certified reference standard, the sponsor should generate stability data on stock solutions to justify the duration of stock solution storage stability.

- **Short term temperature stability:** Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 0 to 12 hours (based on expected duration that samples will be maintained at room temperature in the intended study) And analysd.

Result

Ultra-fast liquid chromatography

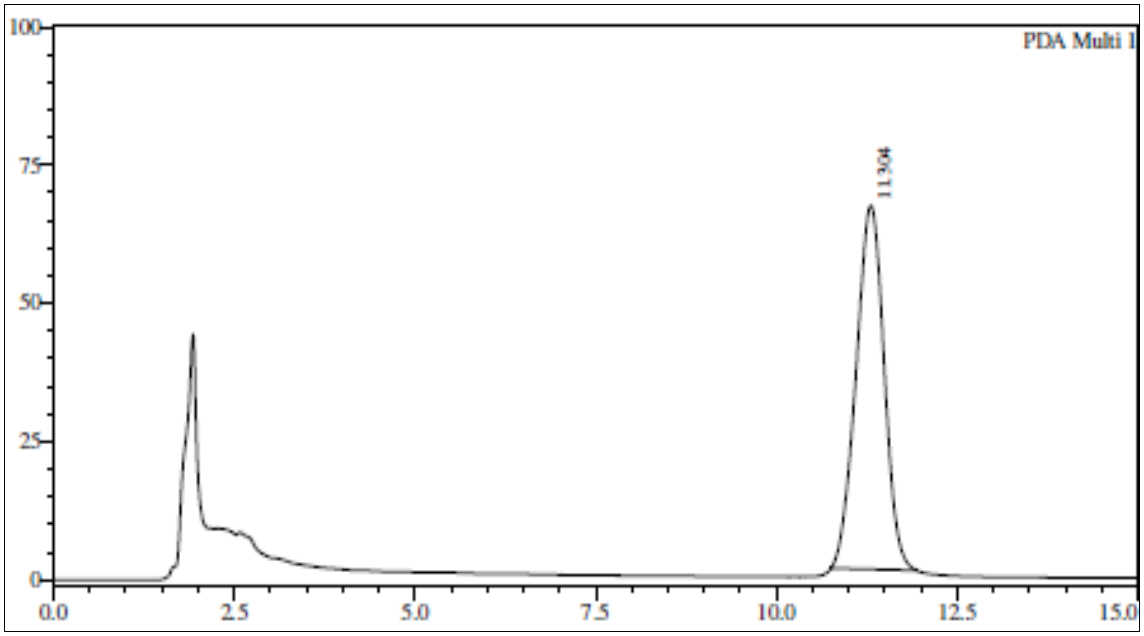

Fig 1: Peak obtained for Dapagliflozin

Table 1

Chromatographic conditions	Report
1. M/P-water: Methanol (40:60)	Peak obtained for dapagliflozin Satisfactory
2. F/R: 1ml/minute	
3.Column: Eclipse C_8	
4. wavelength :228nm	

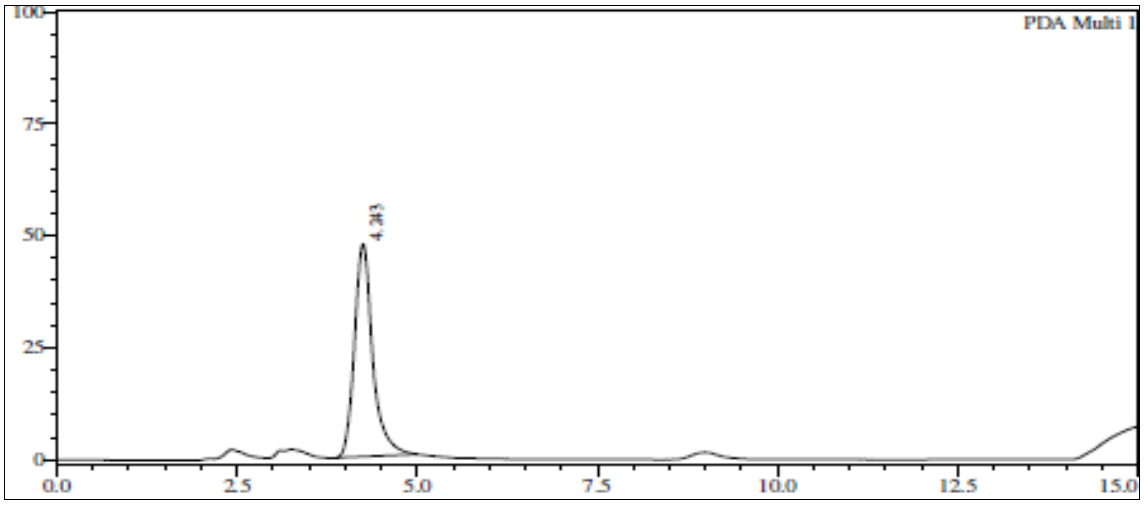

Fig 2: Peak obtained for saxagliptin

Table 2

Chromatographic conditions	Report
1. M/P- 0.01% Tri ethyl amine in water: Methanol (40:60)	Peak obtained for Saxagliptin Satisfactory
2. F/R: 1ml/minute	
3. Column: Eclipse C_8	
4. wavelength :228nm	
5.Resolution: 7.06	
6.Peak purity:0.99	

Chromatograms of standard calibration curve for APIs with Rat plasma

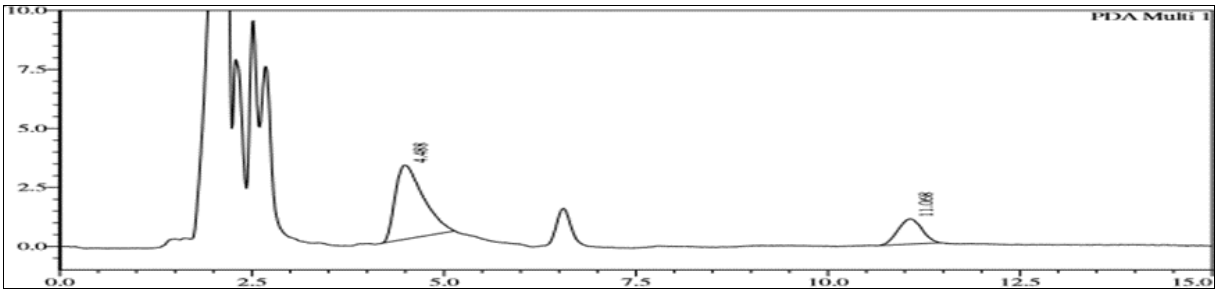

Fig 3: Chromatogram of Saxagliptin and Dapagliflozin with plasma

Precision

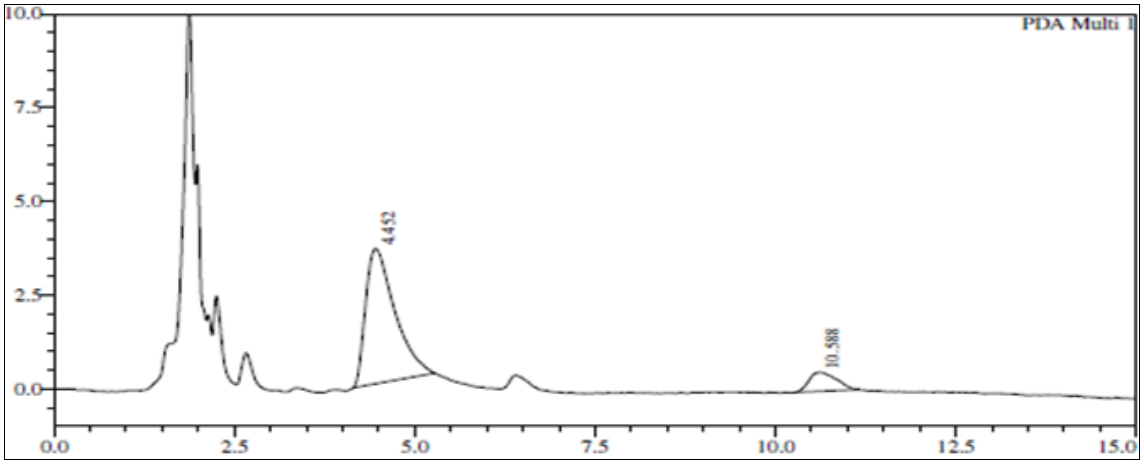

Fig 4: Intraday precision chromatogram of Saxagliptin and Dapagliflozin with plasma.

Accuracy

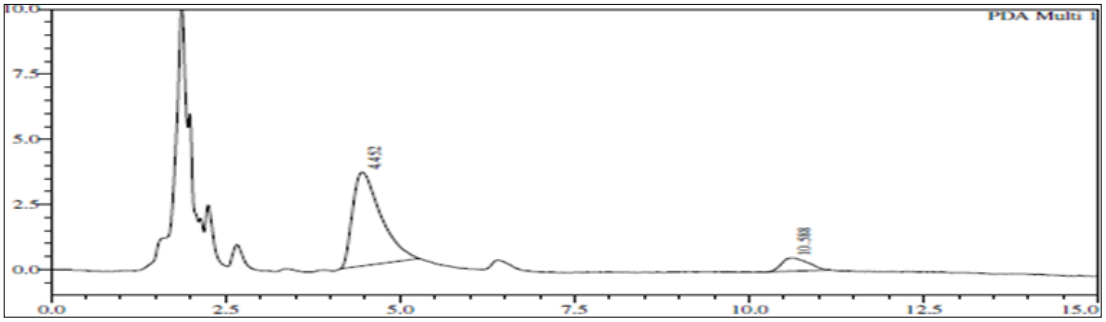

Fig 5: Accuracy chromatogram of Saxagliptin and Dapagliflozin with plasma

Linear regression data for the calibration of Saxagliptin & Dapaglifloxin

Table 3: Linear regression data for the calibration of saxagliptin & dapaglifloxin

Parameters	Value
Calibration range(µg/ml)	25-175 µg/ml
Regression equation(Y)	Y=852.37x+3836, R^2 =0.9932
Slope(b)	852.37
Intercept(a)	3836
Correlation coefficient(R^2)	0.9932

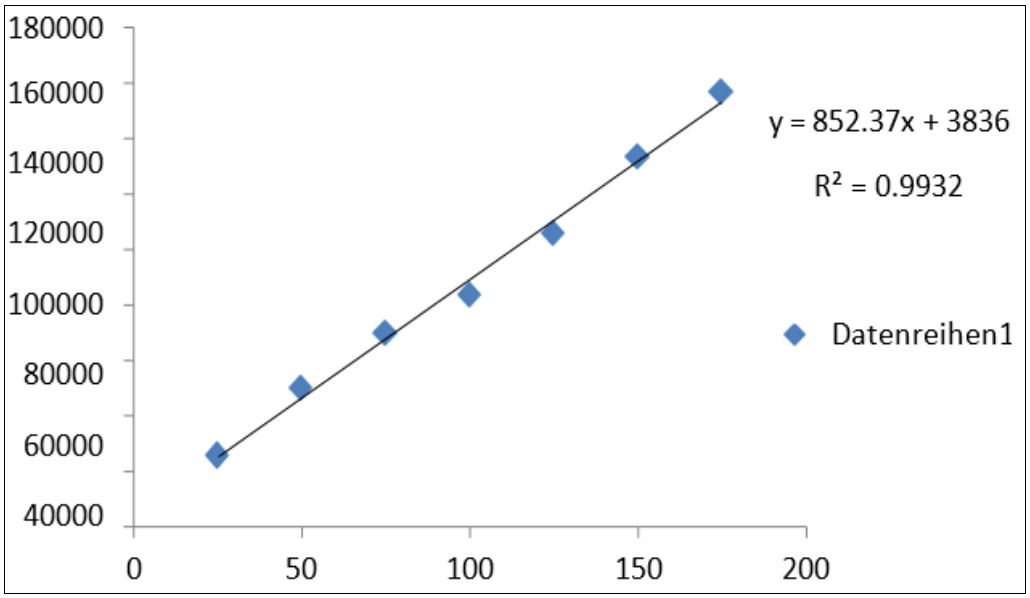

Fig 6

Table 2: Result of validation parameter of Saxagliptin and Dapaglifloxin

Parameters	Results
Accuracy (%Mean, %RSD) of Saxagliptin and Dapagliflozin	0.259 and 0.359
Precision (% RSD) of Saxagliptin and Dapagliflozin	0.207 and 0.216
Intraday (% RSD) of Saxagliptin and Dapagliflozin	0.207 and 0.207
Interday (% RSD) of Saxagliptin and Dapagliflozin	0.216 and 0.216
Linearity and rage (r^2) of Saxagliptin and Dapagliflozin	0.9932 and 0.9902

Conclusion

In this method C8 column was used. The selected mobile phase was 0.01% Triethylamine in water and: Methanol in the ratio of 40:60%, v/v. The drugs were injected and retention time was found to be time was found to be 4.243 for Saxagliptin and 11.304 Dapagliflozin at flow rate 1.0 ml/min. After developing method, it is validated by using different parameters such as linearity, selectivity, accuracy, precision, recovery and stability studies. The developed method showed linearity with correlation coefficient of 0.9932 for Saxagliptin and 0.9902 for Dapagliflozin. In interday and intraday precision, the %RSD values are in good precise in the developed method. Based on the obtained results the proposed RP-HPLC method is proven to be suitable as well as found to be simple, precise and economical for the determination and can be routinely adopted technique for the quantification of Saxagliptin and Dapagliflozin.

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