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Comparative study between UHPLC-UV and UPLC-MS/MS methods for determination of alogliptin and metformin in their pharmaceutical combination

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A new UPLC-MS/MS method (method A), for simultaneous determination of alogliptin (ALN) and metformin (MET) in their recently approved pharmaceutical combination Kazano® tablets, was developed and compared to a new UHPLC-UV method (method B). Concerning method A, separation was achieved on Hypersil gold 50 mm × 2.1 mm (1.9 µm) column, using acetonitrile and 0.2 % formic acid aqueous solution as the mobile phase with a gradient elution. Electrospray ionization (ESI) source was operated in positive ion mode. Selected reaction monitoring (SRM) mode on a triple quadropole mass spectrometer was used to quantify the drugs utilizing the transitions of 340.33 → 116.32 (*m*/*z*) and 130.12 → 71.32 (*m*/*z*) for ALN and MET, respectively. Concerning chromatographic separation using UV detection in method B, it was achieved on a Symmetry® C_{18} column 100 mm × 2.1 mm (2.2 µm) applying an isocratic elution based on methanol - water (10:90, *v*/*v*) at pH 3 as a mobile phase. The photodiode array detector was operated at 210 nm. Method A showed good linearity over the concentration ranges of 5-400 ng mL^{-1} and 25-2000 ng mL^{-1} for ALN and MET, respectively, while method B showed satisfactory results using ranges of 0.25-8 µg mL^{-1} and 5-50 µg mL^{-1} for ALN and MET, respectively. The optimized validated methods are suitable for QC labs but the UPLC-MS/MS method offered the advantage of shorter analytical times and higher sensitivity and selectivity.

1. Introduction

Alogliptin (ALN), (Fig. 1a) belongs to dipeptidyl-peptidase-4 inhibitor class which stimulates glucose-dependent insulin release (Yabe and Seino 2016). Metformin hydrochloride (MET), (Fig. 1b) is a biguanide which decreases gluconeogenesis and increases peripheral utilization of glucose (Alanazo 2015).

A literature review revealed that some chromatographic and spectrophotometric methods were reported for the assay of ALN alone in Nesina® tablets (El-Bagary et al. 2012; Zhou et al 2014; Rao et al. 2014; Yadav et al. 2014a,b; Zhang et al. 2015; Supriya et al. 2016). Moreover, some chromatographic methods (Deng et al. 2016; Chen et al. 2016; Ashutosh et al. 2015) were used for bio-analysis of ALN in biological fluids. On the other hand, few methods were described for simultaneous determination of ALN and MET in their pharmaceutical preparation including HPLC-UV (Thangabalan et al. 2014; Kumar et al. 2013; Sri et al. 2013; Swathi et al. 2015; Runja et al. 2016) and spectrophotometric methods (Sen et al. 2015; Chirag and Parle 2014; Nikalje et al. 2015; Patel and Mashru 2014).

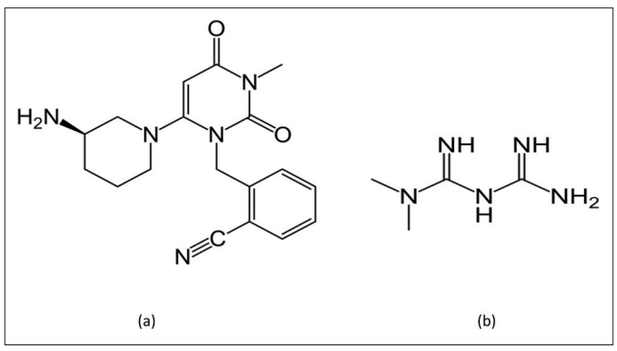


Fig. 1: Chemical structures of ALN (a) and MET (b).

The aim of the present work was to develop new more sensitive UPLC-MS/MS and UHPLC-UV methods for simultaneous determination of ALN and MET in bulk and in their pharmaceutical dosage form. The idea for a comparative study using two different detectors has been emerged from the need to find the most suitable analytical method in short time to be applicable in QC labs giving the analyst the choice of the preferable detector according to the underlying application. Furthermore, outputs from a comparative study using two columns with different dimensions will be useful for analysts working in the area of drug control as reported preliminary results. Using a Hypersil® gold C_{18} column (50×2.1 mm, 1.9 µm) showed better results than the commonly used Symmetry® C_{18} column (100×2.1 mm, 2.2 µm). When UPLC high pressure was combined with 1.9 µm particles (with 50 mm × 2.1 mm dimensions), high peak capacity was observed which is crucial for sensitive ALN determination due to its low contribution in the pharmaceutical combination with MET.

In addition, lack of UPLC-MS/MS methods for simultaneous analysis of ALN and MET in tablets has motivated us to propose and develop such a method (method A). Based on our planned future work, this comparative study is considered a crucial step to decide which method can be further applied for the analysis of the studied drugs in the presence of different degradation products or in biological fluids.

Furthermore, the developed UHPLC-UV method has many advantages over the HPLC-UV methods found in the literature (Thangabalan et al. 2014; Kumar et al. 2013; Sri et al. 2013; Swathi et al. 2015; Runja et al. 2016); including the use of a simple mobile phase, studying the validation parameters and taking in consid-

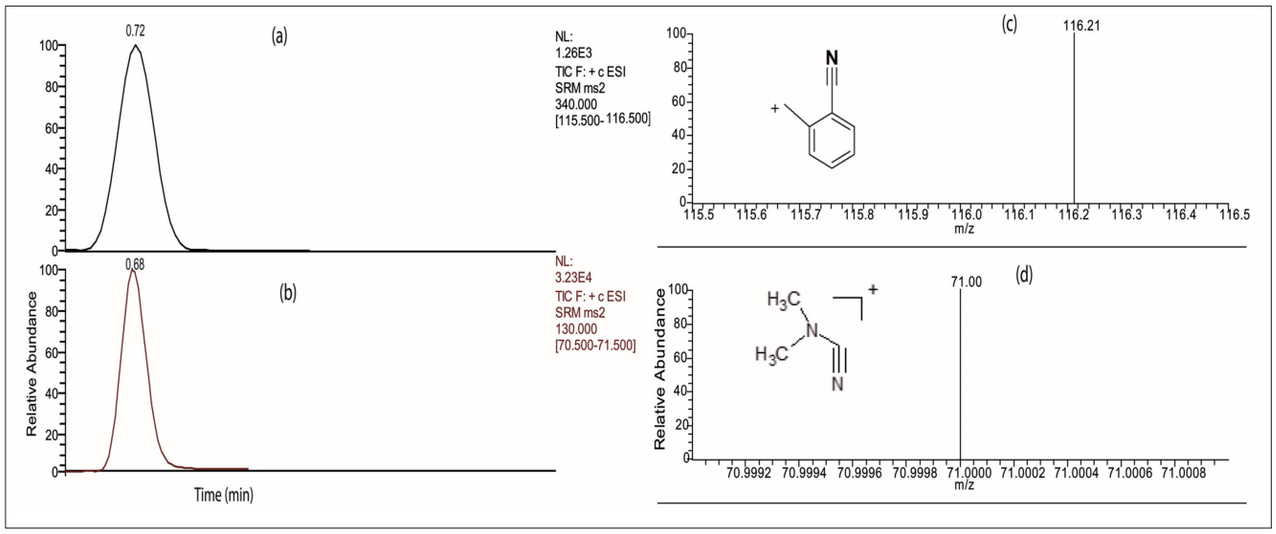
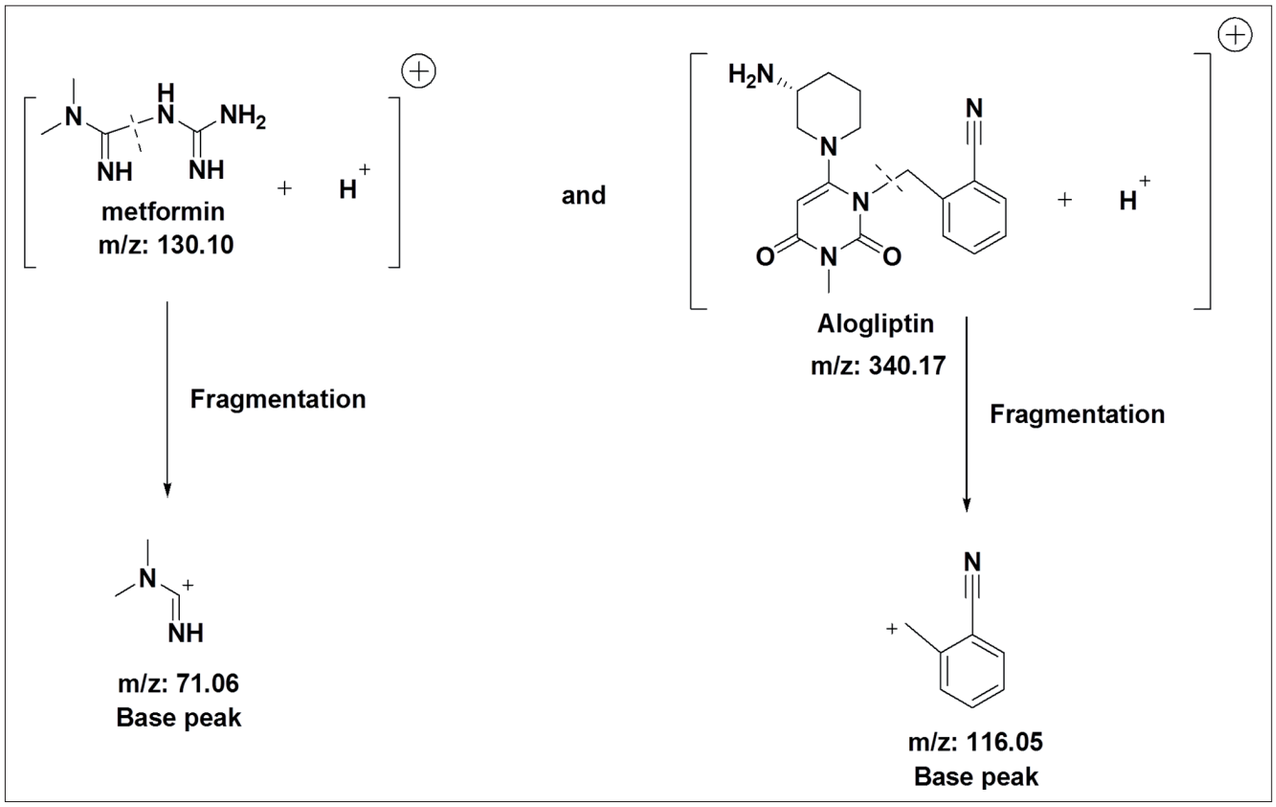


Fig. 2: Representative SRM chromatograms of: (a) ALN, (b) MET and the corresponding SRM spectra of $[M + H]^+$ of: (c) ALN and (d) MET with the proposed structures of the main productions.



Scheme: Suggested fragmentation pathways of ALN and MET.

eration the ratio of the drugs in their pharmaceutical preparation (17 ALN : 500 MET). Moreover, UHPLC is more economic than HPLC consuming less organic solvent, less time and resulting in relatively sharp peaks (Ayoub 2015).

In addition, the proposed method (method B) has another major outcomes over the reported methods (Thangabalan et al. 2014; Kumar et al. 2013; Sri et al. 2013; Swathi et al. 2015; Runja et al. 2016) which include detection at most sensitive wavelength for the two studied drugs after preliminary investigation, using simple mobile phase as 10 % methanol without buffer or TEA so it may be adopted for the mass detector, better resolution between peaks, adjusted pH less than the pka of the considered drugs by more than two units, Lower LOD and LOQ values and high throughput analysis. Finally, referring to the reported methods; detection of MET at 254 nm (Kumar et al. 2013) or 290 nm (Swathi et al. 2016) was not linear in the range applied and not accurate according to MET spectra and was not applicable for simultaneous determination of the drugs with satisfactory validation parameters. Also, using pH more than three showed deformed peaks with low resolution (Kumar et al. 2013; Sri et al. 2013; Runja et al. 2016) and linearity range of

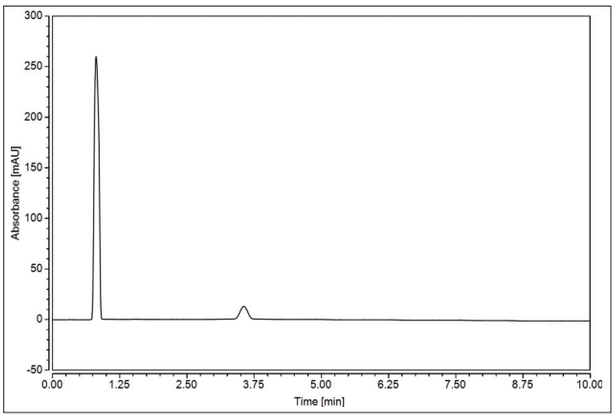


Fig. 3: UPLC chromatogram of Kazano® tablet extract containing (a) MET (29.4 µg mL^{-1}) at 0.8 min and (b) ALN (1 µg mL^{-1}) at 3.6 min.

ALN starting from 3 µg mL^{-1} to 25 µg mL^{-1} in the reported methods (Thangabalan et al. 2014; Kumar et al. 2013; Sri et al. 2013; Swathi et al. 2015; Runja et al. 2016), was not applicable for sensitive tablet analysis with the ratio of (17 ALN: 500 MET) that lead to increasing the required MET concentration with extremely peak deformation. To the best of our knowledge there is no UPLC-MS/MS reported method for simultaneous determination of ALN and MET in tablets.

2. Investigations and results

It is worth to mention that the proposed methods are the first methods apply UPLC techniques for the analysis of the studied drugs rather than HPLC technique. UPLC is advantageous in terms of withstanding the high system back pressure, improved resolution, shorter run times, and fewer consumables (Ayoub 2015). Also the proposed methods were developed with simple mobile phase without buffer to enable its further application on different detectors. The obtained results confirmed the validity of the methods with better resolution and sharp peaks.

2.1. Optimum results obtained by method development

Concerning method A, chromatographic analysis of the drugs was firstly tried using various combination of acetonitrile, methanol and 0.2 % formic acid aqueous solution with varying ratios. However, acetonitrile and 0.2 % formic acid was the mobile phase of choice where a gradient system was developed. The ratio of acetonitrile was gradually increased from 10 % till 90 % within 0.7 min, then maintained at the final ratio for 1.3 min, finally, the gradient was returned to the starting conditions; (10: 90, *v/v*) acetonitrile: formic acid (0.2% aqueous solution) in 1 min, thus, the total run time was 3 min. Formic acid solution has assisted in accomplishing good response for MS detection functioning in the positive mode. The described gradient system produced the best peak shape, where the analytes were protonated and well separated within 3 min at a flow rate of 250 µL min^{-1} (Fig. 2).

Regarding the mass spectrometric parameters, the precursor ions and product ions were adjusted by infusion of 1.0 µg mL^{-1} standard solutions into the mass spectrometer which was operated in positive polarity mode using electrospray ionization technique. As ALN and MET are basic in nature, thus, they are having the ability to gain protons, so the intensity of their precursor ions and product ions was found to be ideal in the positive mode. The protonated molecular ions $[M + H]^+$ of 340.33 for ALN and 130.12 for MET were observed on the full scan mass spectra. The collision energy employed in Q2 has resulted in the production of characteristic ions. Upon utilization of sufficient collision activated dissociation gas and collision energy, the following MS/MS transitions were carefully chosen, 340.33 → 116.32 and 130.12 → 71.32 for ALN and MET, respectively. These represented transitions are the most abundant products ions (Fig. 2) and illustrated in the Scheme revealing the suggested fragmentation pathways of ALN and MET. Moreover, as both the capillary temperature and sheath gas flow play an important role in altering the sensitivity, thus, they were optimized. Adjustment of capillary temperature at 270 °C and sheath gas at 15 psi, has improved the intensity of the analytes. On the other hand, slight changes in ion spray voltage showed no obvious effect on the signal intensity and it was maintained at 3500 V.

Aiming for the optimum detection of the studied drugs using method B, several columns had been described in the literature for determination of gliptins and metformin. Cyano column was used with sharp peaks and good results for single gliptins (El-Bagary et al. 2012a,b) but C_{18} column was used for the simultaneous determination of MET in mixture with gliptins (El-Bagary et al. 2013) so a C_{18} column was selected for the present method. Moreover, optimization of the mobile phase pH was studied. It was expected to be in the acidic region (2.5 and 3.5) to ensure its value below the pka of the studied drugs by more than two units as the studied drugs are hydrochloride and benzoate salts therefore adjusting pH to 3 using acetic acid showed the optimum results. UV detection at wavelength 210 nm was selected for the simultaneous determination of the two drugs with high sensitivity. In addition, the methanol percent was crucial for the proposed method to enhance the resolution between the two eluted peaks. Adjusting column temperature to 50 °C improved the shape of peaks and their corresponding area under the peaks values. The flow rate was selected to be 0.5 mL min^{-1} and the injection volume was 10 µL to keep the back pressure below 400 bar during the run.

2.2. System suitability tests for method B

System suitability tests were used to verify the reproducibility of the analysis performed; including column efficiency (number of theoretical plates), tailing of chromatographic peak and resolution between peaks. The results of these tests are listed in Table 1.

2.3. Discussion of the validation parameters according to ICH guidelines

2.3.1. Linearity

Under the optimum conditions, ALN and MET were evaluated by analyzing different concentrations of each drug. Linear relationship between area under the peak (AUP) and the concentration of each drug (C) was obtained, regression equations were computed and the linearity of the calibration curves were validated by the high value of correlation coefficient as listed in (Tables 2 and 3). Method A showed good linearity over the concentration ranges of 5-400 ng mL^{-1} and 25-2000 ng mL^{-1} for ALN and MET, respectively, while method B showed satisfactory results using ranges of 0.25-8 µg mL^{-1} and 5-50 µg mL^{-1} for ALN and MET, respectively.

2.3.2. Accuracy and precision

Accuracy of the results was verified through calculation of % recovery of five concentrations of each drug by each method (A and B). Besides, calculation of % recovery of each drug in laboratory prepared mixture was carried out. The results include the mean of the recovery and standard deviations are mentioned in (Tables 2, 3 and 4). Precision was verified by analyzing three concentrations of

Table 1: System suitability tests

Item	MET	ALN
N (Number of theoretical plates)	1084	2877
T (Tailing factor)	1.05	1.20
R (Resolution between two consecutive peaks)	6.1	
RSD% of 6 injections		
Peak area	0.21	0.33
Retention time	0.28	0.19

N: Number of theoretical plates, T: Tailing factor, R: Resolution between two consecutive peaks, RSD: Relative standard deviation

Table 2: Results obtained by the proposed LC-UV method

Item	Alogliptin benzoate	Metformin hydrochloride
UPLC-UV detection	210 nm	210 nm
Retention time (min)	3.6	0.8
Linearity	0.25-8 µg.ml^{-1}	5-50 µg.ml^{-1}
Regression equation	AUP = 4.6145 $C_{µg/ml}$ + 0.2596	AUP = 1.6914 $C_{µg/ml}$ + 0.4059
Regression coefficient (r^2)	0.9998	0.9996
Accuracy (mean ± SD)	100.63 ± 1.47	100.42 ± 1.87
Lab prepared mixtures	99.45 ± 0.93	99.96 ± 0.72
Dosage form (mean ± SD)	100.56 ± 1.21	100.16 ± 0.98
Drug added (Standard addition)	99.89 ± 1.97	100.16 ± 1.73
LOD µg.ml^{-1}	0.07	1.35
LOQ µg.ml^{-1}	0.20	4.10
Intraday %RSD	0.21-0.29	0.18-0.29
Interday %RSD	0.10-0.28	0.11-0.19
*S_b	0.014	0.018
*S_a	0.064	0.661
Confidence limit of the slope	4.61 ± 0.064	1.69 ± 0.03
Confidence limit of the intercept	0.26 ± 0.02	0.41 ± 0.27
Standard error of the estimation	0.09	0.69

*S_b: Standard error of slope, S_a: Standard error of intercept.

Table 3: Results obtained by the proposed LC-MS/MS method

Item	Alogliptin benzoate	Metformin hydrochloride
Retention time (min)	0.72	0.68
Linearity	5.0-400 ng.ml^{-1}	25-2000 ng.ml^{-1}
Regression equation	AUP = 0.0005 $C_{ng/mL}$ + 0.5255	AUP = 0.0007 $C_{ng/mL}$ + 0.1313
Regression coefficient (r^2)	0.9999	0.9998
Accuracy (mean ± SD)	99.98 ± 0.44	100.07 ± 0.523
Lab prepared mixtures	100.06 ± 0.63	100.13 ± 0.60
Dosage form (mean ± SD)	100.12 ± 0.78	100.35 ± 0.98
Drug added (Standard addition)	99.63 ± 1.47	98.15 ± 1.04
LOD ng.ml^{-1}	1.44	7.75
LOQ ng.ml^{-1}	4.36	23.50
Intraday %RSD	0.23	0.31
Interday %RSD	0.33	0.48
*S_b	$3.54x10^{-5}$	$1.1x10^{-4}$
*S_a	0.009	0.13
Confidence limit of the slope	$5x10^{-4} \pm 1.77x10^{-8}$	$7x10^{-4} \pm 7.92x10^{-8}$
Confidence limit of the intercept	0.52 ± 0.004	0.13 ± 0.017
Standard error of the estimation	0.013	0.19

*S_b: Standard error of slope, S_a: Standard error of intercept.

both ALN and MET by each method three times, within the same day and on three successive days, using the procedures mentioned under (section 3.5). The % RSD was calculated and found to be less than 2 % in the three concentrations, as shown in (Tables 2 and 3).

2.3.3. Robustness

Robustness of method A was verified by the uniformity of the peak area of the analytes with the intentional minor changes performed in the mass spectrometric parameters and chromatographic conditions, e.g., the capillary temperature or turbo ion spray temperature (± 5 °C), sheath gas flow (± 5 psi), collision energy (± 5 V) and the flow rate (± 10 µL).

However, the robustness of method (B) was ascertained by studying the effect of minor changes in experimental parameters on the resolution factor between the two peaks of MET and ALN. The flow rate of the mobile phase was changed from 0.5 mL min^{-1} to 0.48 mL min^{-1} and 0.52 mL min^{-1}, the organic strength was changed by % ± 1 and the value of pH of the mobile phase was varied from 3 to 2.9 and 3.1, there was no significant difference in the results, indicating good robustness of the proposed method.

2.3.4. Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences. In the present work, specificity was checked by analyzing five different concentrations of each drug using methods A and B in the presence of the excipients of the pharmaceutical dosage form as shown in (Table 5). It is worthy to shed light on one of the advantages of coupling LC with MS/MS detection in the SRM mode is of high specificity, since only the ions resulting from the analytes of concern are observed.

Table 4: Results for determination of ALN and MET in bulk by the proposed methods

LC-UV					
ALN			MET		
Pure (µg mL^{-1})	Found (µg mL^{-1})	*Recovery %	Pure (µg mL^{-1})	Found (µg mL^{-1})	*Recovery %
1.50	1.48	98.67	7.50	7.43	99.07
3.00	3.05	101.67	15.00	14.68	97.87
4.50	4.56	101.33	25.00	25.39	101.56
6.00	6.12	102.00	35.00	35.44	101.26
7.50	7.46	99.47	45.00	46.05	102.33
Mean ± S.D.		100.63 1.47	Mean ± S.D.		100.42 1.87
LC-MS/MS					
Pure (ng mL^{-1})	Found (ng mL^{-1})	*Recovery %	Pure (ng mL^{-1})	Found (ng mL^{-1})	*Recovery %
5.00	5.02	100.50	45.50	45.57	100.15
30.00	30.07	100.23	340.00	339.15	99.75
105.00	104.84	99.85	750.00	744.45	99.26
200.50	198.89	99.20	1500.50	1507.25	100.45
350.50	350.88	100.11	2000.00	2015.00	100.75
Mean ± S.D.		99.98 0.44	Mean ± S.D.		100.07 0.523

*Mean of three determinations.

2.3.5. *Limit of detection and limit of quantification for the proposed methods*

Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3.3 and limit of quantification (LOQ) at which S/N is 10 were determined for the proposed methods and results are given in (Tables 2 and 3).

2.4. *Pharmaceutical dosage forms & standard addition technique*

The proposed chromatographic methods were successfully applied to the analysis of a pharmaceutical dosage form (Fig. 3). Moreover, the standard addition technique was applied by adding different known concentrations of the pure drug to different known concentrations of the drug product and the procedure mentioned above was adopted. The concentrations were calculated using the corresponding regression equations as in Table 5.

2.5. *Conclusion*

The proposed comparative chromatographic study has revealed that UPLC-MS/MS method is more sensitive. Moreover, it offers more advantage as complete separation is not necessary comparing to the LC-UV method which was reflected in shorter time of development and analysis. Linearity at nano gram range using method A has supported our future intention for the analysis of the same drugs in the presence of degradation products and in biological fluids. Furthermore, the developed methods can be conveniently used by quality control laboratories.

3. Experimental

3.1. *Instrumentation*

3.1.1. *Method A*

The analysis was achieved using a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer, Thermo Scientific, New York, USA, equipped with an electrospray ionization (ESI) source and Hypersil® gold C_{18} column (50 mm × 2.1 mm, 1.9 µm). The control of the UPLC-MS/MS system, collection and analysis of the data was performed utilizing Xcalibur software version 2.2. Chromatography was carried on Accela UPLC system which was composed of Accela 1250 quaternary pump and Accela open autosampler, New York, USA (operated at 25 °C).

3.1.2. *Method B*

The liquid chromatography consisted of a Thermo Fisher UPLC Model Ultimate 3000 (USA). For the UPLC system, a Symmetry® Acclaim RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 µm) was used. The system was equipped with a Diode Array detector (DAD-3000RS, USA) and an autosampler (WPS-3000TRS, Thermo scientific, USA). An Elmasonic S 60 H (Germany) was used for the degassing of the mobile phases. Jenway (UK) digital pH meter was used to adjust and determine the hydrogen ion concentration (pH) of the mobile phase.

3.2. *Reagents and reference samples*

Pharmaceutical grade samples of ALN, MET and Kazano® tablets containing 17 mg ALN and 500 mg MET per tablet were supplied by Takeda Pharmaceutical Ltd. Co. (Japan). ALN and MET purity were found to be 99.64 % and 100.48 %, respectively according to reported methods (El-Bagary et al. 2012a; Ayoub 2016). Deionized water, formic acid, acetic acid, HPLC grade methanol and acetonitrile were purchased from Sigma Aldrich (Germany). Standard stock solutions of ALN and MET (1 mg mL^{-1}) were prepared in methanol. Working standard solutions were prepared by diluting the corresponding stock solutions with methanol (method A) or with the mobile phase (method B) to the required concentrations. The stock and working solutions were preserved at 4 °C and discarded within 30 days.

3.3. *Chromatographic and mass spectrometric conditions*

3.3.1. *Method A*

Chromatographic separation was accomplished on Hypersil-Gold column, (C_{18}-bonded ultrapure silica based column), 50×2.1 mm (1.9 µm) from Thermo Scientific, New York, USA. Gradient elution was achieved using the binary mobile phase consisting of 0.2 % formic acid aqueous solution (A) and acetonitrile (B) using a flow rate of 250 µL min^{-1}, where elution was performed at room temperature. A gradient program was conducted as follows: 10 % B from 0-0.2 min, ramp to 90 % B from 0.2-0.7 min, hold at 90 % B till 2 min, back to 10 % B from 2.0-3.0 min. The injection volume was 10 µL and the total run time for each sample was 3 min.

Table 5: Simultaneous determination of ALN and MET in pharmaceutical dosage form and standard addition technique

Pharmaceutical dosage form	*% found ± S.D.		Claimed taken (µgmL^{-1})		Standard addition technique					
	ALN	MET	ALN	MET	Pure added (µg mL^{-1})		Found added		* % R pure added	
					ALN	MET	ALN	MET	ALN	MET
Kazano® tablets (alogliptin benzoate and metformin hydrochloride) 17 mg/ 500 mg	100.56 ± 1.21	100.16 ± 0.98	1.00	29.40	1	2.5	1.02	2.46	102.00	98.40
					2	5	1.97	4.96	98.50	99.20
					3	10	3.03	10.21	101.00	102.10
					4	15	4.03	15.29	100.75	101.93
					5	20	4.86	19.83	97.20	99.15
			Mean ± S.D.						99.89 ± 1.97	100.16 ± 1.73

*Mean of three determinations.

The mass spectrometric detection method was carried out in the positive-ion utilizing electrospray ionization (ESI) and selected reaction monitoring (SRM) mode. The optimized parameters are: Auxiliary gas of 2 psi, Sheath gas of 15 psi, Capillary temperature of 270 °C, Turbo ion spray temperature of 400 °C, Ion spray voltage of 3500 V and Capillary offset of 35 V. The quadrupole mass spectrometer was operated at the SRM mode, monitoring the transition of molecular ions to the product ions for ALN 340.33 → 116.32 and MET 130.12 → 71.32 using collision energy of 25 eV for each.

3.3.2. Method B

Chromatographic separation was achieved on a Symmetry® Acclaim™ RSLC 120 C_{18} column (100×2.1 mm, 2.2 µm) applying an isocratic elution based on a mixture of methanol - water in the ratio of (10:90, *v/v*), adjusted to pH 3 using 1N acetic acid, as a mobile phase. The ultraviolet detector was operated at 210 nm. The mobile phase was filtered through 0.2 µm membrane filter and degassed for 30 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 0.5 mL min^{-1}. Column temperature was adjusted to 50 °C and the injection volume was 10 µL.

3.4. Sample preparation

Twenty Kazano® tablets were weighed, powdered and mixed in a mortar. An accurately weighed amount of the finely powdered Kazano® tablets equivalent to 1.7 mg of ALN and 50 mg of MET were made up to 50 mL with methanol and sonicated to dissolve. The solutions were filtered followed by serial dilutions using methanol for method A, or using the mobile phase for method B, to the required concentrations for each experiment including the standard addition technique.

3.5. Procedure

3.5.1. Method A

Calibration curves were constructed by preparing five solutions of each drug using methanol in the concentration ranges of 5-400 ng mL^{-1} for ALN and 25-2000 ng mL^{-1} for MET. The solutions were filtered through a nylon membrane filter (0.45 µm) followed by injection of 10 µL aliquots of each solution onto the LC-MS system. The calibration curve was then constructed for each drug by plotting of the peak area of each drug against the corresponding concentrations.

3.5.2. Method B

Calibration curves were constructed by preparing five solutions of each drug using the mobile phase in the concentration ranges of 0.25-8 µg mL^{-1} for ALN and 5-50 µg mL^{-1} for MET and only 10 µL was injected. A calibration curve was obtained by plotting Area under the peak (AUP) of the corresponding drug against its concentration (C).

3.6. Assay of ALN and MET in lab prepared mixtures and Kazano® tablets

The procedures mentioned under (section 3.5) were applied for different ratios of ALN and MET and for the sample solutions prepared under (section 3.4). The concentrations of the mentioned drugs were calculated using the calculated regression equations.

Conflicts of interest: None declared.

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