Scientific paper

An Experimental Design Approach to Optimization of the Liquid Chromatographic Separation Conditions for the Determination of Metformin and Glibenclamide in Pharmaceutical Formulation

Ebru Çubuk Demiralay*

Süleyman Demirel University, Faculty of Science and Literature, Department of Chemistry, 32260 Isparta, Turkey

** Corresponding author: E-mail: ebrucubuk@sdu.edu.tr; Tel: 246 2114167; Fax:+90 246 2371106*

Received: 16-09-2011

Abstract

An optimization methodology is introduced for investigating the retention behavior and the separation factor of metformin, gliclazide (I.S.) and glibenclamide. This investigation has been focused on studying the influence of pH value of the mobile phase, concentration of acetonitrile and column temperature, which affect a complete separation of the chromatographic peaks of these compounds. The significant factors were optimized using full factorial design. Retention factor and separation factor were chosen as dependent variable. Optimum RP-LC chromatographic conditions for the separation of metformin, glibenclamide and gliclazide were obtained using X Terra column (150 mm × 4.6 mm I.D., 5 μm). The results show that the percentage of acetonitrile are the most important to investigate and ${}^{s}_{s}pH$ of the mobile phase and column temperature do not significantly affect the experimental results. The procedure was validated for linearity, accuracy, precision and recovery. Quantitation was accomplished using internal standard method.

Keywords: Metformin, glibenclamide, full factorial design, optimization, RP-LC, validation

1. Introduction

The main groups of oral hypoglycemic agents that lower blood sugar are the biguanides and the sulfonylureas and related compounds. The sulfonylureas are divided traditionally into to groups or generations of agents. All members of this class of drugs are substituted arylsulfonylureas. They differ by substitutions at the *para* position on the benzene ring and at one nitrogen residue of the urea moiety. Sulfonylureas cause hypoglycemia by stimulating insulin releases from pancreatic β cells. Their effects in the treatment of diabetes however are more complex. The acute administration of sulfonylureas to type II diabetes patients increases insulin release from the pancreas. The most commonly prescribed medications for type 2 diabetes are metformin and the second generation sulphonylureas, which include glipizide, gliclazide, glibenclamide, and glimperide.[1,2] Metformin is the only drug of biguanide. Main action of metformin is probably to increase glucose uptake across the cell membrane in skeletal muscle, although they also have minor effects on glucose absorption and hepatic glucose production. It can be combined with sulfonylurea drugs.[1]

Reversed phase high performance liquid chromatography (RP-LC) is a well-known technique for oral hypoglycaemic agents. Generally, a trial and error approach is adopted to select the optimum chromatographic conditions of metformin and any sulfonylureas (SU). Such trial and error approach is cost, time and labor intensive and should be substituted by more systematic approaches. In order to avoid trial and error, a 2^3 factorial design was used to choose an optimum chromatographic condition providing good separation in reasonable run time. Factorial design is a type of experimental design in which all the possible combinations of factors and levels are investigated.[3,4] Statistical experimental design is a useful tool to investigate chromatographic parameters such as percentage of organic modifier and pH of the mo-

bile phase and temperature of the chromatographic column in order to obtain an optimum response in a appropriate analysis time. In the novel literature there are many experimental design applications in development and optimization of RP-LC methods. The optimization of the investigated analytical factors can be carried out by applying different forms of experimental design, such as full factorial design, composition design, fraction factorial design, or artificial neural networking.[5–10]

Although, many methods have been reported in literature for the estimation of metformin, glibenclamide and gliclazide and other sulphonylureas individually, only a few methods are available for the simultaneous estimation of metformin and sulfonylureas.[11–14] There is no single RP-LC method reported for simultaneous determination of these compounds using experimental design. Because adequate separation between the solutes was not achieved by changing one factor at a time, optimization of the liquid chromatographic method had to be performed in a multivariate manner. The aim of optimization strategies is to obtain the largest quality information while carrying out a limited number of experiments. Hence, it was decided to apply an experimental design for it to get optimum chromatographic conditions for simultaneous estimation of metformin and glibenclamide from combined tablet dosage form by using the experimental design. For the statistical experimental design, the volume percentage of organic modifier, ${}^{s}_{s}pH$ of the mobile phase and temperature of the chromatographic column were chosen as factors.

2. Experimental

2. 1. Chemicals and Reagents

The compounds used in this study were kindly supplied as follows: Glibenclamide, Gliclazide and metformin were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade, Merck, Darmstadt, Germany) was used as organic modifier. Acetic acid (glacial) and ammonia (25–29%) (Riedel-de Haen, Germany) were used for pH adjustment of mobile phase.

2. 2. Apparatus

RP-LC analyses were performed using a Shimadzu chromatographic system, consisting of a Shimadzu HPLC system (Shimadzu Technologies, Kyoto, Japan), a pump (LC-10AD VP), a UV Visible detector (SPD-10AV VP), column oven (CTO-10AC VP) and a degasser system (DGU-14A). A X Terra C18 analytical column (150 mm × 4.6 mm I.D., 5 μm) provided by Waters® was used for all the determinations at different temperature (25, 30, 35 °C). Results were acquired and processed with the Shimadzu LC Solution data system software (Shimadzu Technologies, Kyoto, Japan).

pH measurements of the mobile phase were carried out with a Mettler Toledo MA 235 pH/ ion analyzer (GmbH; Schwerzenbach, Switzerland) using M–T combination pH electrode. pH measurements can be preferred in a manner similar to that in water, taking into account the pH values previously assigned to primary standard buffer solutions in these media. Potassium hydrogen phthalate was used as primary standard buffer reference solutions for the standardization of this apparatus in acetonitrile-water binary mixtures in accordance with IUPAC rules.[15–17]

2. 2. 1. Procedure

Throughout this study, the mobile phases assayed were acetonitrile-water at 45%, 50% and 55% (*v/v*), to determine the optimum chromatographic condition. The pH of the mobile phase containing 1×10^{-3} mol L^{-1} acetic acid was adjusted by the addition of ammonia. For each compound, the retention time values (t_R) were determined from three separate injections for every mobile phase composition The dead time (t_M) was measured by injecting uracil solution (Sigma, USA, 0.1%, in water). The flow rate of the mobile phase was kept constant at 0.8 mL min^{-1}. Based on the UV spectrum of the analytes the detector wavelength was set at 225 nm.

2. 2. 2. Preparation of Standard Solutions

Stock solutions of metformin, glibenclamide and gliclazide (I.S.) (100 μg mL^{-1}) were prepared by dissolving appropriate amounts of the compounds in the mobile phase. A series of working standard solutions were prepared by further diluting the stock solutions in the mobile phase. All solutions were protected from light and were used within 24 h to avoid decomposition. The concentration of metformin and glibenclamide were varied in the range of 5–100 μg mL^{-1} and 2.5–80 μg mL^{-1}, respectively and the concentration of I.S. was maintained at a constant level of 8 μg mL^{-1}. The peak area ratio of each anti-diabetic drug to that of the I.S. was plotted against the corresponding concentration to construct calibration graph.

2. 2. 3. Analysis of Tablets

To determine the content of metformin and glibenclamide simultaneously in conventional tablets (label claim: 500 mg of metformin per tablet; 2.5 mg of glibenclamide per tablet), 10 tablets were accurately weighed and they were ground to a fine powder. An equivalent to one tablet was accurately weighed and transferred into a volumetric flask (100 mL). Approximately 50 mL of acetonitrile was added and the solution was sonicated for 12–15 min. The volume was made up to 100 with acetonitrile. After filtration, suitable aliquot of clear filtrate was transferred to a volumetric flask. An aliquot of 1 mL I.S. solu-

tion was added this flask and volume was made up to 10 mL with mobile phase. The final solution was injected directly onto the X Terra column using the optimize condition. The amounts of metformin and glibenclamide were calculated from the corresponding regression equations.

2. 3. Recovery Studies from Tablets

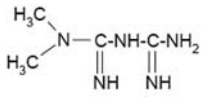
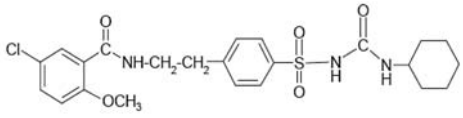
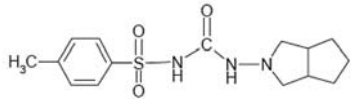
To study the accuracy for developed method, recovery studies were carried out by the addition of known amounts of the drug solution and a constant level of an internal standard to pre-analyzed tablet sample in the range of linearity for both the compounds. The percent recovery was calculated by using the area ratios of the targeted drugs to the internal standard. After five repeated experiments, the average recovery percentage of these compounds was calculated for each compound. Thus, the effect of common tablet formulation excipients on chromatograms (e.g., tail, broadening, etc.) was investigated.

3. Results and Discussion

3. 1. Optimization of HPLC Conditions

There are different retention pattern of glibenclamide and metformin because of their polarity and pK_a values. If the polarity range is too wide, it will be difficult to find a set of chromatographic conditions able to obtain an adequate separation power for the least retain solute and a reasonable elution time for the most retained ones. Factorial designs are widely used in this type complex separation involving several factors where it is necessary to study the joint effect of these factors on a response. This work was identified the influence of RP-LC conditions on the retention and the separation factor of each analyte. Based on experimental data analysis, the best conditions for simultaneous determination of metformin, glibenclamide and internal standard (gliclazide) in pharmaceutical formulation were obtained.

Table 1: Chemical structures of metformin, glibenclamide and gliclazide

Compounds	Structure
Metformin	
Glibenclamide	
Gliclazide	

In the present study, the data from 14 experiments, arranged in 2^3 full factorial design, were used to model the retention behavior of metformin, glibenclamide and gliclazide (I.S.) (Table 1). The effect of the percentage of organic modifier (x_1), the pH of the mobile phase (x_2) and column temperature (x_3) were investigated on the response factors selected (Table 2). The factors were applied into 3-factor-2-level (2^3) factorial experimental design, with their "high" (+) and "low" (–) values. The data of the full factorial design performed for optimization of the concentration of acetonitrile, the ${}^{s}_{s}pH$ value of mobile phase, and column temperature are described in Table 2.

Table 2: Factors and their corresponding levels as per 2^3 factorial design

Independent Factors	Low level (–)	High level (+)	Zero level (0)
(x_1) acetonitrile (%)	45	55	50
(x_2) pH of the mobile phase	4.5	5.5	5.0
(x_3) Temperature (^{o}C)	25	35	30

The method is based on logarithm of retention factor values using these factors. Fourteen experiments (including six zero-level experiments) were carried out and the logarithm of retention factor values for each peak was calculated. The results for logarithm of retention factor values for each experimental condition are presented in Table 3.

Original data collected under different experimental conditions were correlated to the three variables and their interactions and mathematical model was constructed. The mathematical models were estimated using a statistical program MINITAB 16 (Minitab Inc, USA). In such a design, the response variables are modeled by the following equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + \\ + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

where, y is the level of the measured response, b_0 is the intercept, b_1,b_2,b_3,b_{12},b_{13},b_{23},b_{123}, are the regression coefficients, x_1,x_2,x_3, stand for the main effects, x_1x_2,x_1x_3,x_2x_3, are the two- way interactions between the main effects and $x_1x_2x_3$ is the three-way interaction between the main effects.[18] MINITAB 16 programme was applied to process the data and to perform multivariate regression relating logarithm of retention factor values with independent parameters. These equations permit to predict the retention factor values at any point within the factor domain. The coefficient's values of mathematical model are given in Table 4.

There is significant effect percentage of acetonitrile on log *k* for these compounds. There is no significant inf-

Table 3: Coded factor levels for 2^3 full factorial design and logarithm of retention factor values of metformin, glibenclamide and gliclazide

Expt No.	x_1	x_2	x_3	Metformin	Glibenclamide	Gliclazide
1	–	–	–	0.028	0.763	1.014
2	+	–	–	–0.080	0.448	0.570
3	–	+	–	0.048	0.776	1.035
4	+	+	–	–0.076	0.469	0.592
5	–	–	+	0.043	0.779	1.037
6	+	–	+	–0.077	0.454	0.577
7	–	+	+	0.054	0.792	1.046
8	+	+	+	–0.076	0.470	0.594
9	0	0	0	0.020	0.600	0.777
10	0	0	0	0.022	0.598	0.777
11	0	0	0	0.024	0.601	0.779
12	0	0	0	0.023	0.601	0.779
13	0	0	0	0.026	0.602	0.779
14	0	0	0	0.023	0.601	0.778

Table 4: Regression coefficients and probability values for logarithm of retention factor values

Factor effect	Metformin (Coefficients/ P value)	Glibenclamide (Coefficients/ P value)	Gliclazide (Coefficients/ P value)
b_0	–0.017 (0.000)	0.619 (0.000)	0.808 (0.000)
b_1	–0.060 (0.000)	–0.159 (0.000)	–0.225 (0.000)
b_2	0.005 (0.001)	0.008 (0.000)	0.009 (0.000)
$b3_1$	0.003 (0.008)	0.005 (0.000)	0.005 (0.000)
b_{12}	–0.003 (0.006)	0.001 (0.000)	0.001 (0.023)
b_{13}	–0.002 (0.024)	–0.003 (0.037)	–0.003 (0.000)
b_{23}	–0.002 (0.087)	–0.0006 (0.001)	–0.002 (0.002)
b_{123}	0.0008 (0.337)	–0.0006 (0.256)	0.0009 (0.053)

luence of ${}^{s}_{s}pH$ of mobile phase and column temperature on log k values. The contour diagrams related with the change log k with x_1 and x_2 is shown in Fig. 1

coefficient's values of mathematical model are given in Table 6.

The values of coefficients b_1 for the all peak pairs de-

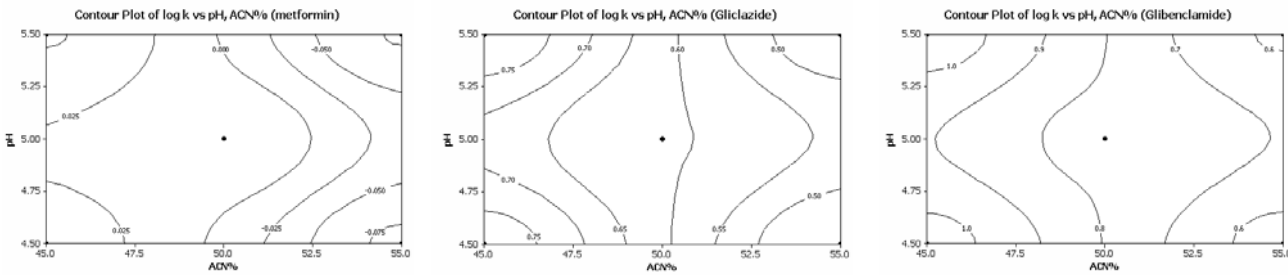


Figure 1: Contour plots showing the influence of percentage of acetonitrile and pH of the mobile phase on the logarithm of retention factor values

In addition, the resolution (R_s) between peak pairs was selected as a response(the elution order did not change). Percentage of organic modifier, the ${}^{s}_{s}pH$ of the mobile phase and the column temperature. For estimation of the influence of experimental conditions, fourteen experiments (including six zero-level experiments) were carried out and the R_s values for all consecutive peak pairs were calculated. These results are presented in Table 5. The monstrate that separation of the compounds as measured by the R_s values is most affected by the percentage of acetonitrile. The value of the coefficients for the two factor interaction, b_{12} for glibenclamide/metformin peak pair, showed that R_s value is most affected by the percentage of acetonitrile and ${}^{s}_{s}pH$ of the mobile phase. The column temperature had the lowest influence on R_s values. The combined influence of x_1 and x_2 on R_s and x_1 and x_3 on R_s

Table 5: Coded factor levels for 2^3 full factorial design and the R_s values of peak pairs

Expt No.	x_1	x_2	x_3	Eluted peak pairs	
				Glibenclamide/ metformin	Gliclazide/ glibenclamide
1	–	–	–	16.083	9.734
2	+	–	–	10.100	3.775
3	–	+	–	16.096	10.012
4	+	+	–	10.596	3.953
5	–	–	+	16.098	9.711
6	+	–	+	10.091	3.703
7	–	+	+	15.945	9.420
8	+	+	+	10.394	3.863
9	0	0	0	13.130	6.400
10	0	0	0	13.107	6.456
11	0	0	0	13.059	6.445
12	0	0	0	13.028	6.451
13	0	0	0	13.086	6.431
14	0	0	0	13.068	6.436

(x_1) acetonitrile (%); (x_2) pH of the mobile phase and (x_3) column temperature (°C)

Table 6: Regression coefficients and probability values for R_s values

Factor effect	Glibenclamide/ metformin (Coefficients/ P value)	Gliclazide/ glibenclamide (Coefficients/ P value)
b_0	13.175 (0.000)	6.771(0.000)
b_1	–2.880 (0.000)	–2.948 (0.000)
b_2	0.082 (0.001)	0.041 (0.002)
b_3	–0.043 (0.019)	–0.097 (0.000)
b_{12}	0.117 (0.000)	0.044(0.002)
b_{13}	–0.009 (0.497)	0.057 (0.001)
b_{23}	–0.045 (0.017)	–0.073 (0.000)
b_{123}	0.003 (0.803)	0.069 (0.000)

are shown by the contour diagrams (Fig. 2). The plots clearly indicate that the goals set for R_s value can be achieved at any point in the experimental domain.

As a result, the optimum chromatographic conditions were predicted using the log k equations. The data clearly indicate that log k within the experimental limitation can be obtained using lower and intermediate percentage of acetonitrile in the mobile phase. The retention factor (k) is thought to be best between 1 and 5.[19] The retention time of metformin was found also to be sufficiently high (t_R = 2.49 min, k = 1.06) allowing complete separation of metformin from the solvent peak. The retention time of the last separation compound was less than 9 min. The results showed that all peaks were symmetric (tailing factors ≈ 1.0). This is the first study for simultaneous separation method of these compounds with 2^3 full factorial designs. It is shown that the best conditions were acetonitrile-water (50:50, *v/v*) pH 5.0 at 30 °C for these compounds.

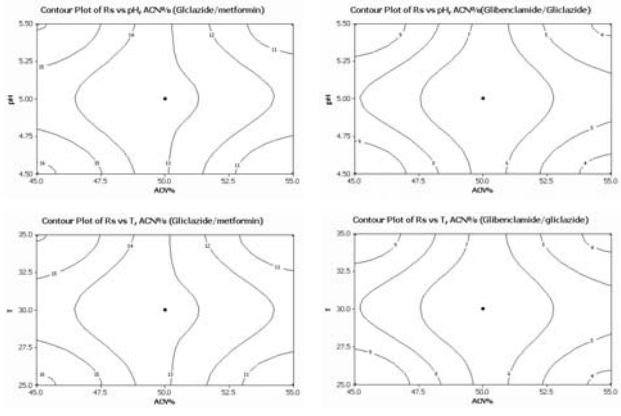


Figure 2: Contour plots showing the influence of percentage of acetonitrile, pH of the mobile phase and column temperature on the R_s values

3. 2. Validation of Analytical Method

After establishing the optimum chromatographic conditions for the separation, linearity, precision, accuracy, limit of detection and limit of quantification were determined for metformin and glibenclamide. Representative chromatograms of the compounds in the optimized condition are presented in Fig. 3. Finally, using the condi-

tions above, a satisfactory chromatographic peak resolution was obtained in a short analysis time as can be seen in Fig. 3. For all compounds, sharp and symmetrical single peaks were obtained with good resolution.

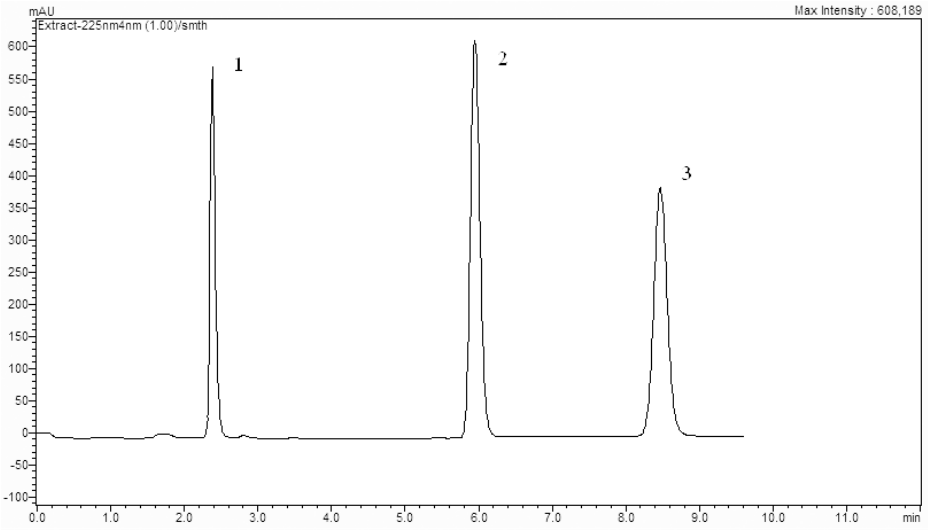


Figure 3: Chromatogram of standard mixture. 1. Metformin (60 μg mL^{-1}), 2. Gliclazide (170 μg mL^{-1}), 3. Glibenclamide (280 μg mL^{-1}). Experimental conditions as in chromatographic procedure.

Development and optimization are important and essential steps when developing new analytical procedures. In this study, the quantitative determination of metformin, glibenclamide and gliclazide (I.S.) was also carried out under the optimized conditions. A widely used technique of quantitation involves the addition of an internal standard to compensate for errors in the analytical measurements. As an internal standard, gliclazide was chosen taking into account that it was a compound belonging to the same sulfonylurea family of drugs. Gliclazide has structural similarity with glibenclamide but is markedly different from metformin.

System suitability tests are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. System suitability parameters like retention factor, theoretical plate number, retention time, asymmetry factor, selectivity and RSD% of peak height or area for repetitive injections. The values for system suitability parameters showed feasibility of this method for routine pharmaceutical application. System suitability tests were carried out on freshly prepared standard stock solutions of metformin and glibenclamide. Selectivity factors were 3.776 and 1.506, for metformin and glibenclamide under optimized conditions, respectively. The asymmetry and retention factors were 1.12 and 1.058 for metformin; 1.07 and 6.014 for glibenclamide, respectively. Number of theoretical plates (N) was 3348 and 7989 for metformin and glibenclamide, respectively.

The calibration curves and equations for metformin and glibenclamide were calculated by plotting the peak area ratios of these compounds to I.S. *versus* concentration of the compounds in the range of 5–100 μg mL^{-1} for metformin and 2.5–80 μg mL^{-1} for glibenclamide (Table 7). The linear regression analysis indicated that the response of the RP-LC system was linear for these compounds. The low values of SE of slope and intercept and higher than 0.999 correlation coefficient values for all compounds were established the precision of the proposed methods. The LOD and LOQ were calculated from the following equations by using the standard deviation (s) of response and the slope (m) of the corresponding calibration curve.[20]

$$LOD = 3.3\frac{s}{m} \quad (2)$$

$$LOQ = 10\frac{s}{m} \quad (3)$$

The precision of the proposed method was performed by five replicate injections of metformin and glibenc-

Table 7: Statistical evalution of the calibration data of metformin and glibenclamide by RP-LC

Sample	Linearity Range (μg mL^{-1})	Slope	Intercept	S.E. of Slope	S.E. of Intercept	Correl Coeff.	Detection Limit (μg mL^{-1})	Quantitation Limit (μg mL^{-1})
Metformin	5–100	0.299	0.067	0.001	0.069	0.999	1.289	3.906
Glibenclamide	2.5–80	0.589	0.075	0.001	0.053	0.999	0.566	1.715

lamide at different concentrations on different days. The obtained intra-day and inter- day precision results are shown in Table 8. The results indicated sufficient precision of the developed RPLC method.

Table 8: Intra-Day and Inter-Day Precision of metformin and glibenclamide

Compound	Theoretical Concentration (μg mL^{-1})	Intra-Day measured Concentration Mean	RSD %	Inter-Day measured Concentration Mean	RSD %
Metformin	10	10.015	1.066	9.948	1.354
	80	80.154	0.334	80.409	0.505
Glibenclamide	5	5.025	0.475	5.004	0.965
	60	60.113	0.338	60.110	0.743

* Mean values represent five replicate / for each concentration.

When working on standard solutions and according to the validation parameters, results encourage the use of the proposed method described for the assay of metformin and glibenclamide in their pharmaceutical dosage form. On the basis of above results, the proposed method was applied to the direct determination of these compounds in their tablet dosage form, using the related calibration straight lines without any sample extraction or evaporation other than filtration and adequate dilution steps. The results obtained from the analysis of tablet dosage form are summarized in Table 9. The quantities found were in conformity with the values claimed by the manufacturers.

Table 9: Results of the assay and the recovery analysis of metformin and glibenclamide in pharmaceutical dosage form

	Metformin	Glibenclamide
Labeled claim (mg)	500	2.50
Amount found (mg)[a]	499.51	2.52
RSD %	0.383	1.608
Bias %	0.099	–0.0084
Added (mg)	500	2.50
Found (mg)[a]	501.88	2.51
Recovery %	100.38	100.22
RSD % of recovery	0.172	1.174
Bias %	–0.375	–0.217

[a] Each value of the mean five experiments

For checking the accuracy, precision and selectivity of the proposed method and in order to know whether the excipients in pharmaceutical dosage form show any interference with the analysis, the proposed method was evaluated by recovery tests after addition of known amounts of pure drug compound to various pre-analyzed formulation of these compounds. These results showed that the proposed method had adequate precision and accuracy and consequently can be applied to the determination of metformin and glibenclamide without any interference from inactive ingredients used in the selected formulation (Table 9). Hence, the accuracy was determined by using the difference between nominal and measured concentration (% Bias). It is concluded that the method is sufficiently accurate and precise in order to be applied to tablet dosage form. Chromatograms obtained from pharmaceutical dosage form sample (with I.S.) are shown in Fig. 4.

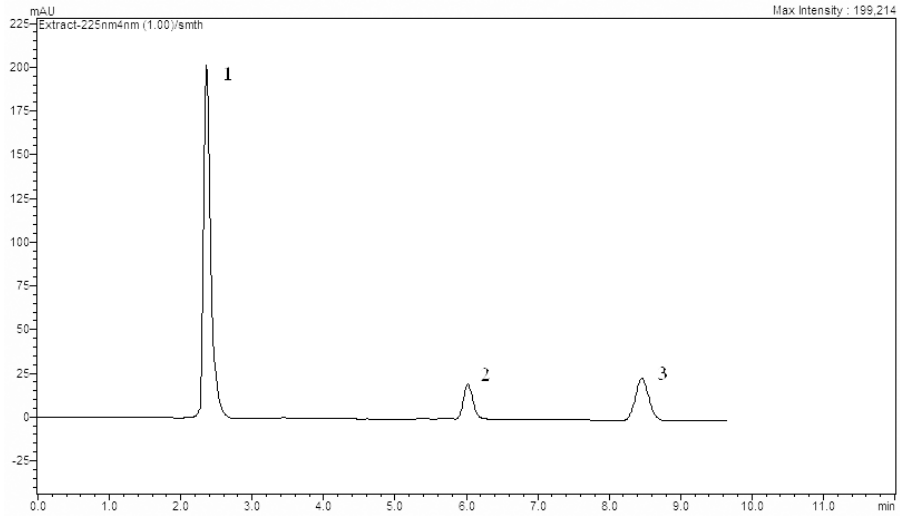


Figure 4: Chromatogram of metformin, glibenclamide and gliclazide (I.S.) in pharmaceutical dosage form. 1. metformin, 2. gliclazide (I.S), 3. glibenclamide, Experimental conditions as in chromatographic procedure.

4. Conclusion

This paper presents the first study dealing with the optimization of chromatographic separation of metformin and glibenclamide using a 2^3 full factorial design. Because of different polarity of metformin from glibenclamide, metformin tends to have different retention pattern than glibenclamide. Optimum conditions for separation are predicted and models presented to describe the retention behavior of metformin, glibenclamide and gliclazide on X Terra column are built. Chromatographic peak separation is very sensitive to mobile phase acetonitrile content. Chemometric approach allowed us to reduce the number of experiments needed for optimization of chromatographic separation.

5. Acknowledgements

Suleyman Demirel University financially supported the project with the Grant Number 2589-M-10.

6. References

1. H. P. Rang, M. M. Dale, J. M. Ritter, Pharmacology, Third Edition, Churchill Livingstone, **1995**, pp. 413–415.
2. J. Hardman and L. Limbird, eds., Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, Pergamon Press, **1995**, pp.1507–1510.
3. T. Lundsted, E. Seifert, L. Abramo, B. Thelin, A. Nystrom, J. Pettersen, R. Bergamn,. *Chemom. Intell. Lab. Syst.* **1998**, *42,* 3–40.
4. S. Bolton, C. Bon, Pharmaceutical Statistics Practical and Clinical Application. In: Optimization techniques and screening designs, 4th ed. Marcel Dekker, Inc; **2004**, pp. 506–523.
5. V. Harang, A. Karlsson, M. Josefson, *Chromatographia*, **2001**, *54(11),* 703–709.
6. N. Ferreirós, G. Iriarte, R. M. Alonso, R. M. Jiménez, *Talanta*, **2006**, *69,* 747–756.
7. K. Vučićević, G. Popović, K. Nikolic, I. Vovk, D. Agbaba, *J. Liq. Chromatogr. & Rel. Tech.,* **2009**, *32,* 656–667.
8. Y. Wang, M. Harrison, B. J. Clark, *J. Chromatogr. A*, **2006**, *1105,* 199–207.
9. N. M. Aragão, M. C. C. Veloso, M. S. Bispo, S. L. C. Ferreira, J. B. Andrade, *Talanta*, **2005,** *67,* 1007–1013.
10. K. Novotná, J. Havliš, J. Havel, *J. Chromatogr. A*, **2005**, *1096,* 50–57.
11. S. AbuRuz, J. Millership, J. McElnay, *J. Chromatogr. B*, **2005,** *817,* 277–286.
12. P. Venkatesh, T. Harisudhan, H. Choudhury, R. Mullangi, N. R. Srinivas, *Biomed. Chromatogr.,* **2006**, *20,* 1043–1048.
13. H. N. Mistri, A. G. Jangid, P. S. Shrivastav, *J. Pharm. Biomed. Anal.,* **2007**, *45,* 97–106.
14. F. S. Bandarkar, I. S. Khattab, *J. Liq. Chromatogr. & Rel. Tech*., **2010**, *33,* 1814–1830.
15. T. Mussini, A. K. Covington, P. Longhi, S. Rondinini, *Pure Appl. Chem.,* **1985**, *57,* 865–876.
16. S. Rondinini, P. R. Mussini, T. Mussini, *Pure Appl. Chem.*, **1987**, *59,* 1549–1560.
17. J. Barbosa, I. Marquès, D. Barron, V. Sanz-Nebot, *Trends Anal. Chem*., **1999**, *18,* 543–549.
18. P. W. Araujo, R. G. Brereton, *Trends Anal. Chem*., **1996**, *15,* 63–68.
19. L. R. Snyder, J. L. Glajch, J. J. Kirkland, Practical HPLC method development. Wiley-Interscience, New York, **1988**.
20. C. M. Riley, T. W. Rosanske, Development and Validation of Analytical Methods, Elsevier, New York, **1996**.

Povzetek

Uvedli smo optimizacijsko metodologijo za raziskave retencije ter separacijskih faktorjev metformina, gliklazida (I.S.) in glibenklamida. Raziskava se osredotoča na študij vpliva pH mobilne faze, koncentracije acetonitrila in temperature kolone, ki vplivajo na popolno ločbo kromatografskih vrhov za te spojine. Signifikantne faktorje smo optimizirali z uporabo popolnega faktorskega načrta. Retencijski faktor in separacijski faktor smo izbrali kot odvisni spremenljivki. Optimalne RP-LC kromatografske pogoje za ločbo metformina, glibenklamida in gliklazida smo dosegli s kolono X Terra (150 mm × 4,6 mm I. D., 5 μm). Rezultati kažejo, da je delež acetonitrila najbolj pomemben, medtem ko pH mobilne faze in temperatura kolone bistveno ne vplivata na rezultate eksperimenta. Postopek smo validirali za linearnost, točnost, natančnost in izkoristek. Kvantifikacijo smo izvedli z metodo internega standarda.