https://doi.org/10.31925/farmacia.2021.2.8 **ORIGINAL ARTICLE**

DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS DETERMINATION OF GLIBENCLAMIDE AND LIPOIC ACID

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Abstract

Glibenclamide and lipoic acid are drugs frequently co-administered in the treatment of diabetes mellitus, this association targeting the glycaemic control and the management of chronic vascular complications. Considering that literature data do not provide analytical methods for simultaneous determination of these drugs, the aim of this study was to develop and validate a new HPLC method for the achievement of this objective. The developed method allowed the efficient separation of the drugs using acetonitrile and phosphate buffer pH = 2.7 as mobile phase, with gradient elution and UV detection at 201 nm. The method was validated, analysing the following parameters: selectivity, linearity, limit of detection and limit of quantification, precision, accuracy. All the validation parameters were within the specified limits, proving that the HPLC method was selective and linear in the defined concentration range, while the relative standard deviation for the analysis of precision and accuracy was found below ± 2% and ± 5%, respectively. Moreover the method is accessible and could be applied for the simultaneous separation and quantitative determination of the associated drugs in different therapeutic systems.

Rezumat

Glibenclamidul și acidul lipoic sunt medicamente co-administrate frecvent în tratamentul diabetului zaharat, această asociere țintind controlul glicemic și managementul complicațiilor vasculare cronice. Considerând faptul că literatura de specialitate nu oferă metode analitice pentru determinarea simultană a acestor medicamente, obiectivul acestui studiu a fost dezvoltarea și validarea unei noi metode HPLC în vederea realizării acestui deziderat. Metoda dezvoltată a permis o separare eficientă utilizând ca fază mobilă acetonitril și tampon fosfat pH = 2,7, cu eluare în gradient și detecție UV la 201 nm. Metoda a fost validată, analizând următorii parametri: selectivitatea, liniaritatea, limita de detecție și limita de cuantificare, precizia, acuratețea. Toți parametrii validării au fost în limitele specificate, dovedind faptul că metoda HPLC este selectivă și liniară pe domeniul de concentrații definit, în timp ce valoarea deviației standard relative la analiza preciziei și a acurateței a fost sub ± 2% și ± 5%, respectiv. Mai mult, metoda este accesibilă și ar putea fi aplicată pentru separarea și determinarea cantitativă simultană a substanțelor asociate din diferite sisteme terapeutice.

Keywords: glibenclamide, lipoic acid, HPLC, diabetes mellitus

Introduction

The management of diabetes mellitus is becoming more and more difficult, due to its chronic and progressive character and to the difficulty in maintaining a long-term optimal glycaemic control, being associated with the development of vascular complications as neuropathy, retinopathy, nephropathy [2, 16], stroke, myocardial infarction [8, 19], diabetic foot [5].

The treatment of diabetic patients is based on multiple associations of drugs, with different mechanisms of action, in order to maintain the glycaemic control and slow down the evolution of chronic complications, once appeared.

Glibenclamide and lipoic acid are frequently co-administered to diabetic patients, this association targeting lowering blood glucose values and the management of diabetic neuropathy [3, 18]. In addition, for lipoic acid the literature data reveals some important effects such as antioxidant [11, 17], increasing insulin sensitivity [10], antitumor and anti-inflammatory [9].

There are some analytical methods for the quantitative determination of each active substance. For determination of glibenclamide from pharmaceutical dosage forms, high performance liquid chromatography (HPLC) methods, using phosphate buffer:methanol = 60:40% (v/v), pH = 7 [12] or ethanol:methanol = 50:50% (v/v) [15], as mobile phase, with isocratic elution and UV-VIS detection, are applied. Another method using acetonitrile:water = 60:40% (v/v), with isocratic elution and UV-VIS detection was proposed for the determination of glibenclamide from pharmaceutical formulations and human plasma [6].

For the determination of lipoic acid from human plasma, a HPLC method using disodium hydrogen-phosphate:acetonitrile:methanol = 50:30:20, with UV-VIS detection was developed [7]. However, there are no analytical methods for the simultaneous determination of glibenclamide and lipoic acid, from the associated forms.

Taking these aspects into account, the aim of the present study was to develop and validate a new high performance liquid chromatography (HPLC) method, which allows the simultaneous assay of glibenclamide and lipoic acid from pharmaceutical dosage forms.

Materials and Methods

Materials

Glibenclamide (GB, min. 99%), lipoic acid (LA, min. 98%), LC-grade methanol, acetonitrile of chromatographic purity, ortho-phosphoric acid (min. 85%), disodium hydrogen phosphate (min. 99%) were purchased from Sigma Aldrich (Merck, Germany). Distilled water of chromatographic purity was purchased from Ircon (Romania).

A HPLC 1200 system (Agilent Technologies 1200, USA), equipped with a G1311A quaternary pump, G1322A degasser, Rheodyne injection loop (20 µL) and a G1315B diode array detector (DAD) was used. The chromatographic column used was ODS Hypersil C18, 250 x 4.6 mm, the size of the particles being of 5 µm (Thermo Fischer Scientific, USA).

Development and optimization of the HPLC method

The development and optimization of the analytical method aimed to establish the chromatographic conditions for the separation and quantitative determination of GB and LA. The selection of chromatographic conditions was based on the literature data for the analysis of each substance separately, with modifications and optimizations for their determination from the association GB-LA [1, 7, 13]. In order to achieve this purpose, 7 methods (M_1 - M_7) were experimentally studied, analysing the following parameters: the composition of the mobile phase, the mobile phase gradient, the run time and the percentage of each mobile phase. The parameters of the 7 analytical methods studied are summarized in Table I.

Table I

The chromatographic parameters of the analytical methods (M_1 - M_7) studied for the development and optimization of the HPLC method

Method	Mobile phase	Gradient	Rate (mL/min)	Run time (min)
M_1	CH_3OH:H_2O = 85:15, pH = 3.5	isocratic	1	30
M_2	CH_3OH:H_2O, pH = 3.5	0': 50% CH_3OH; 5': 75% CH_3OH; 10': 85% CH_3OH; 15': 100% CH_3OH 20': 50% CH_3OH	1	20
M_3	CH_3OH:H_2O, pH = 3.5	0': 80% CH_3OH; 5': 85% CH_3OH 7': 85% CH_3OH; 12': 90% CH_3OH 15': 80% CH_3OH	1	15
M_4	CH_3OH:H_2O, pH = 3.5	0': 80% CH_3OH; 10': 80% CH_3OH 25': 85% CH_3OH; 40': 85% CH_3OH 50': 90% CH_3OH; 55': 90% CH_3OH 60': 80% CH_3OH	1	60
M_5	phosphate buffer:acetonitrile:CH_3OH = 50:30:20, pH = 2.7	isocratic	1	5
M_6	acetonitrile:phosphate buffer = 1:1, pH = 2.7	isocratic	1	10
M_7	acetonitrile:phosphate buffer, pH = 2.7	0': 60% PB; 5': 60% PB; 25': 40% PB 30': 40% PB; 45': 10% PB; 55': 60% PB; 60': 60% PB	1	60

PB = phosphate buffer

Briefly, stock solutions of GB (1 mg/mL), LA (5 mg/mL) and a stock solution of both substances (GB 1 mg/mL and LA 5 mg/mL) were prepared by weighing the appropriate amount of substance and dissolving it in 50 mL methanol; the obtained solutions were filtered through Millipore filters (0.22 µm) and injected

in a volume of 20 µL. The column temperature was maintained at 25°C. The detection was performed at different wavelengths (201 nm, 230 nm, 245 nm, 254 nm, 300 nm), thus allowing to choose the wavelength that corresponds to the maximum absorbance peak. The chromatograms were recorded, the appearance of the peak was examined and the retention time (Rt) was calculated for each substance.

Validation of the HPLC method

After the establishment of the optimal conditions that allow the separation and quantitative determination of each substance from GB-LA association, the method was validated analysing the following parameters: selectivity, linearity, limit of detection and limit of quantification, precision, accuracy [4, 20].

Selectivity

The chromatograms of the following solutions were registered: the reference solution, represented by a stock solution containing the GB (1 mg/mL) – LA (2 mg/mL) association, the sample solution, represented by the solution obtained from the preparation of the therapeutic systems containing GB and LA, and the blank solution, obtained from the preparation of the therapeutic systems without GB and LA [4]. A volume of 20 µL of each solution was analysed following the chromatographic conditions corresponding to M_7. It were recorded the chromatograms, as well as the retention times and the shape of the peaks for each substance. The method is considered selective if the peak corresponding to GB and LA do not interfere with other peaks and has the same morphology both in the reference and in the sample solution [20]. Moreover, the selectivity factor (α) was calculated according to the formula:

$$\alpha = \frac{Rt2 - t0}{Rt1 - t0} \qquad (1),$$

where Rt_1 = retention time for LA in the reference solution; Rt_2 = retention time for GB in the reference solution; t_0 = retention time of the non-retained compound on the chromatogram.

Linearity

A series of standard solutions containing the GB-LA association, of known concentration, obtained by successive dilutions from a stock solution, were analysed. The concentration of standard solution ranged between 0.03125 and 1 mg/mL for GB and between 0.15625 and 5 mg/mL for LA. The chromatograms obtained for each concentration were integrated and the peak area for each substance was calculated. The linearity of the response function was determined by representing the peak area as function of concentration. Thus, the linear regression curve and its corresponding equation were obtained and further the correlation coefficient (R^2) and the standard deviation of the slope of the regression curve (SD) were calculated. From the equation of the linear regression curve the concentration was calculated, which was represented as function of the theoretical concentration. The determinations were performed in triplicate for each concentration, the peak area representing the arithmetic mean of those 3 determinations.

The calibration curve is considered linear if the correlation coefficient value (R^2) is over 0.98 [20].

Limit of detection and limit of quantification

The limit of detection (LOD), representing the minimum amount of the analyte that can be evidenced, and the limit of quantification (LOQ), representing the minimum amount of the analyte that can be quantitatively determined, were calculated based on the standard deviation (SD) and on the slope of the regression curve, using the following formulae:

$$LOD = \frac{3.3 \times SD}{\text{slope of the regression curve}};$$

$$LOQ = \frac{10 \times SD}{\text{slope of the regression curve}} \qquad (2).$$

Precision of the system (repeatability of the injection)

From stock solution of GB (1 mg/mL) - LA (5 mg/mL) association, prepared as described above, it was prepared a standard solution of GB (0.25 mg/mL) – LA (1.25 mg/mL), which was filtered and injected in volume of 20 µL in the chromatographic conditions corresponding to M_7. The injection was repeated for 6 times with the same solution, determining Rt and calculating the average value, standard deviation (SD) and the relative standard deviation (RSD%). The chromatographic system used is considered precise if the RSD% is ± 2% [20].

Precision of the method (repeatability of the analysis)

The intra-day and inter-day variations using 3 determinations for 3 levels of concentration were analysed. From the stock solution previously prepared, standard solutions for 3 levels of concentration were obtained as follows: low level (0.0625 mg/mL GB – 0.3125 mg/mL LA), medium level (0.25 mg/mL GB – 1.25 mg/mL LA) and high level (0.8 mg/mL GB – 4 mg/mL LA). Intra-day variations: for each level of concentration, three samples were analysed, determining their experimental peak area. Inter-day variations: for each level of concentration, a sample was analysed in three different days, determining the experimental peak area. Based on the equation of the regression curve and on the peak area values, the concentrations as percent of the theoretical concentration values were determined; then the average, SD and RSD% were calculated. The precision of the method is considered within acceptable limits if the RSD% value is ± 5% [20].

Signal to noise ratio

The signal to noise ratio (S/N) was determined for each peak, corresponding to GB and to LA, respectively. This parameter was calculated using ChemStation software of HPLC apparatus, using the six times the standard deviation of the linear regression of the drift. The S/N was calculated based on the formula:

$$S/N = \frac{\text{Height of the peak}}{\text{Noise of closest range}} \qquad (3).$$

Accuracy

In a stock solution containing 0.25 mg/mL GB and 1.25 mg/mL LA, determined volumes of the solution containing the substances of interest were added, in order to obtain spikes of 80%, 100% and 120% of the initial concentration. For each level of concentration, three samples were prepared and analysed in the mentioned conditions, determining the peak area. Based on the equation of the regression curve, the concentration of each sample and, then, the percentage of recovery, expressed as percent of the theoretical concentration, the mean recovery and RSD% were calculated. The accuracy of the method is acceptable if RSD% value is ± 5% [20].

Results and Discussion

Development and optimization of the HPLC method

The separation of the substances is considered efficient if the Rt for GB and LA are different enough and the corresponding peaks are not overlapped, in order to allow the quantitative determination based on the peak area.

Table II

Development and optimization of the HPLC method: retention time (Rt) for GB and LA and observations for each method studied (M_1 - M_7)

Method	Rt LA (min)	Rt GB (min)	Observations
M_1	5.15	6.19	Partial overlap of peaks for GB and LA
M_2	-	16.03	Wide peak for lipoic acid
M_3	4.55	5.8	Additional peaks
M_4	4.53	4.93	Common peak for GB and LA; additional peaks
M_5	3.4	3.02	Common peak for GB and LA
M_6	-	-	Simultaneous elution of GB and LA
M_7	9.09	19.05	Individual peaks; efficient separation

The results (Table II) evidenced that the most efficient separation was achieved using a mixture of acetonitrile and phosphate buffer (pH = 2.7) as mobile phase, with gradient elution, the concentration of phosphate buffer ranging between 60% and 10%. Using this method (M_7), GB was able to interact more than LA with the stationary phase of the column. Consequently, individual and narrow peaks were obtained and the Rt were enough different to allow a complete separation (Rt was 19.05' for GB and 9.09' for LA). The maximum of absorption was registered at 201 nm for both substances, and this wavelength was chosen for all determinations.

Validation of the HPLC method

Selectivity. In order to study the selectivity of the method, the Rt values of GB and LA recorded in the chromatograms of reference solution and sample solution were compared. A maximum difference of ± 5% between values was accepted. The chromatograms registered for reference solution, sample solution and blank solution are presented in Figures 1, 2 and 3. The results evidenced that the Rt value for GB is 19.43' in the chromatogram of reference solution and 19.27' in that of sample solution; for LA, the Rt value is 8.84' in the chromatogram of reference solution and 8.74' in that of sample solution; also, the chromatogram of blank solution proved no interference with the substances of interest. The calculated selectivity factor was higher than 1 ($\alpha = 2.75$). Based on these results, it is appreciated that the developed method is selective and allows the optimal separation of the studied substances.

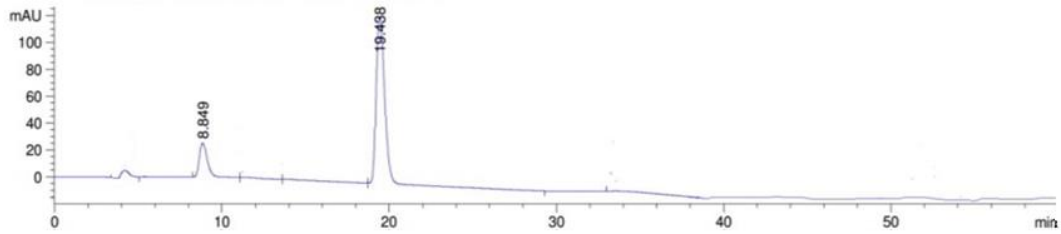

Figure 1.
The chromatogram registered for reference solution

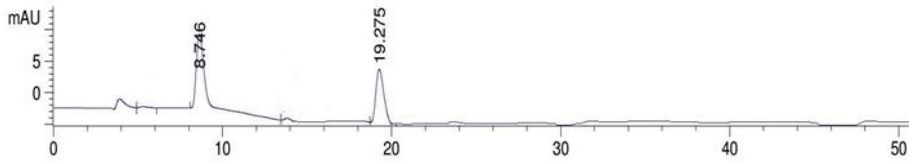

Figure 2.
The chromatogram registered for sample of GB-LA solution

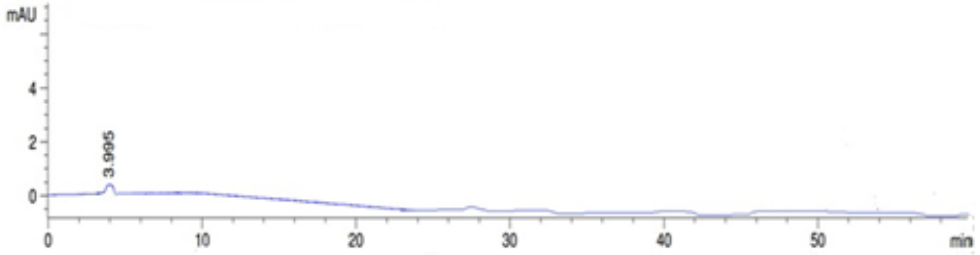

Figure 3.
The chromatogram registered for blank solution

Linearity. The linearity of the response function was obtained through the graphical representation of the mean peak area versus the concentration, drawing the calibration curve of the defined concentrations range and determining the equation of the linear regression curve and the correlation coefficient (R^2). The results prove that the linearity of the answer function is within the acceptable limits, the R^2 value being above 0.98 (R^2 = 0.9984 for GB and R^2 = 0.9978 for LA).

The results of the linearity prove that between the theoretical concentration and the calculated concentration there is a linear correlation, the slope of the regression curve value is close to 1 (0.9996 for GB and 1.0001 for LA) and the intercept value is close to 0 (0.0004 for GB and 0.0001 for LA); therefore, it can be considered that the regression curve passes through origin.

Limit of detection and limit of quantification. The limit of detection and the limit of quantification have the following values: for glibenclamide, LOD = 0.05 mg/mL and LOQ = 0.16 mg/mL; for lipoic acid, LOD = 0.06 mg/mL and LOQ = 0.18 mg/mL.

Precision. The results referring to the precision of the system are presented in Table III. From the statistical analysis of the data, it can be concluded that the system is precise, the relative standard deviation (RSD%) being below the accepted limit of ± 2% for both substances.

According to experimental data, the precision of the method when studying intra-day variations is within the acceptable limits, the RSD% value being 3.56% for GB and 4% for LA, below the accepted limit of ± 5% (Table IV).

Concerning inter-day variations, the precision of the method is also within the acceptable limits, the RSD% value being 3.68% for GB and 4.16% for LA, respectively (Table V).

Table III
Precision of the system: retention time for each substance and statistical analysis

No. det.	Rt GB (min)	Statistical data	Rt LA (min)	Statistical data
1	19.512	Average = 19.52 SD = 0.02 RSD% = 0.14%	8.993	Average = 8.98 SD = 0.02 RSD% = 0.27%
2	19.577		8.999	
3	19.537		9.011	
4	19.513		8.983	
5	19.538		8.949	
6	19.497		8.954	

Table IV
Intra-day variations: mean recovery for each substance and statistical analysis

Level of conc.	Theoretical conc. (mg/mL)	GB			LA		
		Peak area (mUA*s)	Calculated conc. (mg/mL)	Recovery (%)	Peak area (mUA*s)	Calculated conc. (mg/mL)	Recovery (%)
superior	0.8 mg/mL GB 4 mg/mL LA	3924.1	0.82	102.5	1995.5	3.92	98
		4378.7	0.90	112.5	2162	4.25	106.2
		3835.2	0.803	100.3	2051.7	4.03	100.7
medium	0.25 mg/mL GB 1.25 mg/mL LA	1273.8	0.260	104	633.7	1.226	98.08
		1265.1	0.258	103.2	635.1	1.223	97.84
		1235.8	0.252	100.8	601.3	1.156	92.48
inferior	0.0625 mg/mL GB 0.3125 mg/mL LA	341.2	0.063	100.8	177.9	0.317	101.44
		346.3	0.064	102.4	180.7	0.322	103.04
		353.6	0.065	104	180.5	0.322	103.04
Statistical data		Mean recovery = 103.38% SD = 3.68 RSD% = 3.56%			Mean recovery = 100.09% SD = 4.00 RSD% = 4%		

Table V

Inter-day variations: mean recovery for each substance and statistical analysis

Level of conc.	Theoretical conc. (mg/mL)	GB			LA		
		Peak area (mUA*s)	Calculated conc. (mg/mL)	Recovery (%)	Peak area (mUA*s)	Calculated conc. (mg/mL)	Recovery (%)
superior	0.8 mg/mL GB 4 mg/mL LA	3829.9	0.802	100.2	2002.33	3.93	98.25
		3755.63	0.786	98.25	2013.66	3.95	98.75
		3811.81	0.798	99.85	2045.11	4.01	100.47
medium	0.25 mg/mL GB 1.25 mg/mL LA	1194.47	0.243	97.2	629.61	1.21	96.8
		1239.88	0.253	101.2	646.73	1.246	99.68
		1208.77	0.247	98.8	614.27	1.181	94.56
inferior	0.0625 mg/mL GB 0.3125 mg/mL LA	357.6	0.067	107.6	180.69	0.322	104.4
		341.45	0.063	100.8	189.92	0.341	109.1
		356.97	0.067	107.2	175.46	0.312	99.84
Statistical data		Mean recovery = 101.24% SD = 3.73 RSD% = 3.68%			Mean recovery = 100.094% SD = 4.16 RSD% = 4.16%		

Signal to noise ratio (S/N). The S/N value for GB was 584.5 (time range for noise calculation 17.5 - 18.5 min) and 529.8 (time range 7 - 8 min). For LA, the S/N value was 315.5 (time range 7 - 8 min) and 348.2 (time range 17.5 - 18.5 min). The S/N values determined are above 50, the limit considered to provide an acceptable precision of the HPLC methods [14].

Accuracy. Accuracy is an important parameter that reflects the approximation of the experimental results with the real ones, measuring the deviation from the real value of the average value obtained following the experimental analysis [7]. The results (Table VI) prove that the accuracy of the HPLC method is within the acceptable limits; the mean recovery was 102.32% for GB and 94.27% for LA, the RSD% value being below ± 5% for both substances.

This developed and validated HPLC method was applied for the simultaneous determination of GB and LA from new pharmaceutical systems, type chitosan-GB-LA microparticles, obtained through ionic gelation method. Using this method the encapsulation efficiency and drug release from the polymeric matrix were determined.

Table VI

The mean recovery for each substance and statistical analysis

Level of conc.	GB				LA			
	Theoretical conc. (mg/mL)	Peak area (mUA*s)	Calculated conc. (mg/mL)	Recovery (%)	Theoretical conc. (mg/mL)	Peak area (mUA*s)	Calculated conc. (mg/mL)	Recovery (%)
80%	0.2	1045.4	0.212	106.10	1	508.4	0.972	97.25
		1051.8	0.213	106.87		509.1	0.970	97.39
		1042.3	0.211	105.86		509.5	0.974	97.4
100%	0.25	1235.3	0.252	100.80	1.25	604.1	1.162	92.98
		1239.8	0.253	101.43		607.7	1.169	93.55
		1234.5	0.252	100.80		605.3	1.164	93.17
120%	0.3	1453.5	0.298	99.63	1.5	715.2	1.382	92.17
		1474.2	0.303	101.09		712.4	1.377	91.80
		1434.9	0.294	98.31		719.9	1.391	92.79
Statistical data		Mean recovery = 102.32% SD = 3.11 RSD% = 3.04%				Mean recovery = 94.27% SD = 2.35 RSD% = 2.50%		

Conclusions

The present study proposed a new simple, selective, accurate and precise HPLC method for the simultaneous quantitative determination of glibenclamide (GB) and lipoic acid (LP). This method could be recommended for efficient routine assays that allow the determination of these substances associated in multi-component dosage formulations such as microparticles based on chitosan.

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Conflict of interest

The authors declare no conflict of interest.

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