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Chemometric optimization and validation of a novel dispersive liquid–liquid microextraction–HPLC method for gliclazide, glibenclamide and glimepiride quantitation in serum samples☆

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ABSTRACT

A dispersive liquid–liquid micro extraction (DLLME) liquid chromatographic method that allows extraction and separation of gliclazide, glibenclamide and glimepiride from serum was developed and optimized with the use of experimental design. The analyzed factors in optimization were type of extracting solvent, extracting solvent volume, dispersing solvent volume, pH and protein precipitation. The selected conditions for DLLME were dichloromethane 100 μL (extracting solvent), acetonitrile 1000 μL (dispersing solvent) and no protein precipitation. This procedure is simple, requires no sophisticated procedures and produces excellent analyte recoveries. Quantitation of glibenclamide, gliclazide and glimepiride in serum samples was carried out by HPLC. This analytical method has been developed and validated, allowing quantitation of the target analytes in presence of atenolol, enalapril and amlodipine besides other serum sample interferents. The chromatographic method is linear, accurate, precise and specific and has the ability to separate the antihyperglycemic drugs from antihypertensive drugs, which are usually found in serum of diabetic patients (LOQs ca. 0.12 μg L^{-1} for the three analytes).

1. Introduction

Type II diabetes (formerly noninsulin-dependent diabetes mellitus or adult-onset diabetes) is a metabolic disorder characterized by hyperglycemia due to cellular resistance to insulin, combined with insufficient pancreatic secretion of insulin. Over 300 million people suffer from diabetes worldwide [1]. In Argentina, in the year 2011, it was determined that diabetes affects over 9% of the population. Type II diabetes represents 90–95% of all reported cases in adults [2].

Sulfonylureas are oral antidiabetic drugs that increase insulin release from pancreatic beta cells. Gliclazide, glibenclamide and glimepiride are second-generation sulfonylureas used as initial treatment of type II diabetes in patients who cannot control hyperglycemia with diet and exercise [3].

Diabetic patients also have a high prevalence of hypertension. Pharmacological therapy frequently combines antihypertensive and antidiabetic drugs [4]. Atenolol belongs to the beta blocker drug group, enalapril is an angiotensin-converting enzyme inhibitor, and amlodipine is a calcium channel blocker, all of these with antihypertensive action. Usual serum concentration of the three antihypertensive and the three antidiabetic analyzed drugs are atenolol 0.30–0.70 μg mL^{-1}, amlodipine 0.004–0.017 μg mL^{-1}, enalapril 0.l5–0.30 μg mL^{-1}, gliclazide 2.00–8.00 μg mL^{-1}, gliblenclamide 0.14–0.35 μg mL^{-1} and glimepiride 0.20–0.31 μg mL^{-1} [5].

Several analytical methods have been described for the simultaneous determination of gliclazide, glibenclamide and glimepiride such as HPLC–UV [6], HPLC–fluorescence [7,8,9], CE–UV [10] and LC/API (MS) [11]. Due to the fact that serum is a highly complex matrix, and target compounds are at trace levels, an extraction step is mandatory in order to eliminate interfering compounds and pre-concentrate analytes. For the extraction of gliclazide, glibenclamide and glimepiride from serum, different methods have been reported, such as SPE [10,12] and liquid–liquid extraction [8,13,14,15,16,17].

In 2006, Assadi et al. [18] developed the dispersive liquid–liquid micro extraction (DLLME). This method is based on a ternary component solvent system in which both the extraction and disperser solvents are rapidly injected into the aqueous sample by syringe. The mixture is then gently shaken and a cloudy solution (water/disperser solvent/extraction solvent) is formed. Due to the large contact surface area of the two immiscible phases in DLLME, high extraction efficiency is achieved in a relatively short time [18].

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When attempting to discover the most relevant variables and then optimize a response by tuning these factors, experimental design gives a powerful suite of statistical methodology [19,20,21,22,23,24,25].

Response surface methodology (RSM) is a collection of statistical and mathematical techniques used to develop, improve and optimize processes. One of the strengths of RSM is that it may work well in cases where there is incomplete knowledge about the state and behavior of the system under study, as long as the system is stable and there is reasonable correspondence between set points and actual conditions [19]. In particular, factorial designs used in the screening phase and coupled with a central composite design (CCD) in the optimization one are an effective tool for optimizing a process involving several parameters at the same time [26].

In addition, when different objective functions (responses) have to be optimized simultaneously, the so-called "Derringer's desirability function" is a powerful strategy to be followed [27,28]. In a first step, a partial desirability function (di) must be created for each individual response using the fitted models and establishing the optimization criterion. The most desirable ranges for each design factor or response are selected, deciding if these factors or responses had to be maximized, minimized, maintained in the range or reach a target value. In addition, a weight (wi) or emphasis is given to each goal. After that, the Global Desirability function (D) is obtained using the following equation:

$$D = \left(d_1^{r_1} \times d_2^{r_2} \times \ldots\ldots \times d_n^{r_n}\right)^{\frac{1}{\sum ri}} = \left(\prod_{i=1}^{n} d_1^{ri}\right)^{\frac{1}{\sum ri}} \quad (1)$$

where n is the number of variables included in the optimization procedure and r_n is the importance of each factor or response relative to the others.

The measurement of drug concentrations in biological matrices and in pharmaceuticals is an important aspect of medicinal product development. The results of animal toxicokinetic studies and of clinical trials, including bioequivalence studies, are used to make critical decisions supporting the safety and efficacy of a medicinal drug substance or product [29].

It is therefore paramount that the applied bioanalytical methods used are well characterized and fully validated in order to yield reliable results. Acceptance criterion wider than those defined in Guideline on bioanalytical method validation of European Medicines Agency may be used in special situations [30].

In this paper, a novel DLLME procedure and an HPLC–UV method were developed, optimized and validated for the determination of gliclazide, glibenclamide and glimepiride in serum. As will be demonstrated, the advantages of this method are simplicity of operation, rapidity, low cost, high recovery, high enrichment factor and environmental benignity fitting the requirements of the green analytical chemistry [31].

2. Experimental

2.1. Apparatus and software

All experiments were performed using an Agilent 1100 Series liquid chromatograph equipped with a quaternary pump, degasser membrane, thermostated column compartment, auto sampler and DAD (Agilent Technologies, Waldbronn, Germany). Chromatograms were registered at 230 nm. The Chemstation version B 0103 was used for data acquisition and processing. The HPLC column was a Zorbax C18 (4.6 mm × 75 mm, 3.5 μm particle size) from Agilent. Experimental design, surface response modeling and desirability function calculations were performed using the StatEaseDesign–Expert 8.0.0 (Stat-Ease, Inc, Minneapolis, USA).

2.2. Chemicals and reagents

Analytical standards of atenolol, amlodipine, enalapril and glimepiride were provided by PLAMECOR (Medicinal Plant of Corrientes, Argentina). Glibenclamide and gliclazide were provided by Roemmers Argentina (Buenos Aires, Argentina). Acetonitrile and methanol HPLC-grade were obtained from Merck (Darmstadt, Germany). HPLC-grade water was obtained from a Milli-Q Biocel System (Millipore SAS, Molsheim, France). Sodium hydroxide, dichloromethane and monosodium phosphate of analytical grade were purchased from Cicarelli (Rosario, Argentina).

Solutions and solvents for mobile phase were always filtered through 0.45 μm nylon filters. Standards and sample solutions were also filtered through syringe 0.20 μm nylon membrane before injection in the chromatographic system.

2.3. Chromatographic conditions

The column temperature was controlled by setting the oven temperature at 25 °C. The mobile phase consisted of an acetonitrile:phosphate buffer 10 mmol L^{-1} (pH 2.6) (50:50). Samples were analyzed using gradient elution as follows: at 0 min 50% acetonitrile, at 5.5 min 59% acetonitrile and at 6.5 min 50% acetonitrile. The complete analysis was carried out in 8 min. The flow rate was maintained at 1.00 mL min^{-1}. An injection volume of 20 μL was used.

2.4. Standard solutions

Stock standard solutions of atenolol, amlodipine and enalapril 1.0 mg mL^{-1} were prepared in ultrapure water. Glibenclamide stock solution 1.0 mg mL^{-1} was prepared in acetonitrile. Gliclazide stock solution 1.0 mg mL^{-1} was prepared in methanol. Glimepiride stock solution 1.0 mg mL^{-1} was prepared in NaOH 0.1 mol L^{-1}. These solutions were conserved at 4°C in light-resistant containers and allowed to reach room temperature before use. Calibration standard solutions were prepared at the moment of use by diluting an appropriate volume of each stock standard solution in ultrapure water.

2.5. Sample preparation

Aliquots of 250 μL of serum sample or standard solutions were transferred into 1.5 mL centrifuge tubes, and 100 μL of dichloromethane (extractive solvent) and 1000 μL of acetonitrile (dispersing solvent) were added (see optimization step below). Finally, the samples were vortexed for 1 min, centrifuged at 1000g for 3 min and the organic phase was transferred to glass tubes. The organic phase was evaporated to dryness under a gentle stream of nitrogen gas. The residue was dissolved in 50 μL acetonitrile:buffer phosphate 10 mmol L^{-1} pH 2.6 mixture (50:50) and injected into the HPLC system. Consequently, an enrichment factor of 5 was reached with the pre-treatment.

Table 1
Spiked concentration of the analytes and interferents in the recovery test set samples.

Sample	Gliclazide	Glibenclamide	Glimepiride	Atenolol	Amlodipine	Enalapril
(μg mL^{-1})						
S1	0.53	0.08	2.62	0.07	0.50	2.65
S2	2.78	0.53	1.07	0.54	2.72	1.12
S3	0.12	2.00	0.51	2.05	0.10	0.59

Table 2
Placket–Burman design used for method robustness study

Std	Run	F1[a]	F2[b]	F3[c]	F4[d]	F5[e]	F6[f]	F7[g]	F8[h]	F9[i]	F10[j]	F11[k]	R1[l]
3	1	110	990	2.7	11.0	0.95	26	51	1	−1	−1	−1	3.08
1	2	110	1010	2.5	11.0	1.05	26	49	−1	−1	1	−1	3.40
7	3	110	990	2.5	9.0	1.05	24	51	1	−1	1	1	3.47
12	4	90	990	2.5	9.0	0.95	24	49	−1	−1	−1	−1	3.08
6	5	90	990	2.5	11.0	0.95	26	51	−1	1	1	1	2.97
11	6	110	990	2.7	11.0	1.05	24	49	−1	1	−1	1	2.17
10	7	90	1010	2.7	11.0	0.95	24	49	1	−1	1	1	2.75
4	8	90	1010	2.5	11.0	1.05	24	51	1	1	−1	−1	1.78
5	9	90	990	2.7	9.0	1.05	26	49	1	1	1	−1	2.43
2	10	90	1010	2.7	9.0	1.05	26	51	−1	−1	−1	1	4.46
9	11	110	1010	2.7	9.0	0.95	24	51	−1	1	1	−1	2.63
8	12	110	1010	2.5	9.0	0.95	26	49	1	1	−1	1	2.49

[a]Extracting volume solvent (μL). [b]Dispersing volume solvent (μL). [c]pH. [d]Phosphate buffer concentration (mmol L^{-1}). [e]Chromatographic flow (mL min^{-1}). [f]Column temperature (°C). [g]Mobile phase composition (% acetonitrile). [h,i,j,k]Dummy variables. [l]Resolution between glibenclamide and glimepiride peaks.

2.6. DLLME: experimental design and optimization

In order to evaluate the main factors affecting the DLLME procedure, a reduced factorial design (resolution IV) of 12 experiments was built. The evaluation consisted in analyzing aliquots of 250 μL sample serum spiked with the six drugs (at 1.0 μg mL^{-1} of each analyte). The analyzed factors were type of extracting solvent, extracting solvent volume, dispersing solvent volume, pH and protein precipitation, each of them evaluated at two levels. These factors were chosen, given their relevance in DLLME and the selected levels, according to prior experience. Three responses were studied: %recovery of glibenclamide, gliclazide and glimepiride.

The factors showing significant effects were then taken into account to build a central composite design in order to find optimum factor levels for all the response signals by optimizing an objective function. The central composite design consisted of 11 experiments, which corresponded to combinations of the selected independent variables in the following ranges: dispersing solvent volume, 500–1000 μL, and extracting solvent volume, 50–150 μL. These levels were selected based on preliminary studies. A single block rotatable design ($\alpha = 1.414$) with 3 central points was built. In each case, %recovery of glibenclamide, gliclazide and glimepiride were evaluated.

Finally, the multiple response criterion using the desirability function was successfully implemented to optimize the recovery percentages of glibenclamide, gliclazide and glimepiride.

2.7. Method validation: limit of detection (LOD), limit of quantitation (LOQ), matrix effect, linearity, precision, accuracy and robustness

LOD was calculated as the concentration of analyte giving a signal three times the noise level (S/N = 3) using standard solutions in ultrapure water processed as described in Section 2.5. Additionally, the LOD was computed implementing Eq. (2), which is recommended by IUPAC [32]:

$$\mathrm{LOD}_u = t(\alpha,\nu)\sigma_{c,0} + t(\beta,\nu)\sigma_{c,\mathrm{LOD}} = \frac{3.3 s_{y/x}}{A}\sqrt{1 + h_0 + \frac{1}{I}} \quad (2)$$

where $t(\alpha,\upsilon)\sigma_{c,0}$ and $t(\beta,\upsilon)\sigma_{c,LOD}$ are Student coefficients with υ degrees of freedom and α and β probabilities, respectively; $\sigma_{c,0}$ and $\sigma_{c,\mathrm{LOD}}$ are the concentration standard errors for the blank and LOD_u levels; A is the slope of the univariate calibration graph; I is the number of calibration samples; and $S_{y/x}$ is the residual standard deviation. Assuming $\sigma_{c,0} = \sigma_{c,\mathrm{LOD}}$, 95% confidence level ($\alpha = \beta = 0.05$) and a large number of degrees of freedom, the right-hand side of Eq. (2) is obtained, where h_0 is the leverage for the blank sample:

$$h_0 = \frac{\bar{c}_{\mathrm{cal}}^2}{\sum_{i=1}^{I}(c_i - \bar{c}_{\mathrm{cal}})^2} \quad (3)$$

where $\bar{c}_{\mathrm{cal}}$ is the mean calibration concentration and c_i is each of the calibration concentration values.

LOQ was calculated as the concentration of analyte giving a signal ten times the noise level (S/N = 10), using standard solutions processed as described in Section 2.5. Additionally, the LOQ was computed implementing Eq. (4), which is recommended by IUPAC [32]:

$$\mathrm{LOQ}_u = \frac{10 s_{y/x}}{A}\sqrt{1 + h_0 + \frac{1}{I}} \quad (4)$$

where the factor 10 ensures a maximum relative prediction uncertainty of 10%.

Calibration curves were built preparing seven standards in triplicate of each analyte at the following concentration levels: 0.04, 0.10, 0.20, 0.52, 1.00, 1.52 and 2.80 μg mL^{-1}. These solutions were processed (see Section 2.5) and then introduced into the instrument in a randomized way and calibration plots were built.

Matrix effect was evaluated by comparing the calibration graphs obtained by spiking basal human serum with convenient volumes of standard solutions of glimepiride, gliclazide and glibenclamide, and the calibration graphs obtained from the standard solutions prepared and processed (see Section 2.5).

For recovery studies (accuracy), aliquots (250 μL) of serum sample were enriched with the six drugs in order to reach the concentration levels indicated in Table 1 and processed (see Section 2.5). Final solutions were injected into HPLC. Each sample was prepared by quintuplicate.

Repeatability was assessed by replicate analysis ($n = 5$) of standard solutions at three different concentration levels S1, S2 and S3 (see Table 1), prepared by spiking blank human serum with appropriately prepared standard solutions. Intermediate precision was evaluated by

Table 3
DLLME. Reduced factorial design experiments and responses

Std	Run	F1[a]	F2[b]	F3[c]	F4[d]	F5[e]	R1[f]	R2[g]	R3[h]
1	1	Tetrachloride	100	1500	With	With	0	0	0
12	2	Tetrachloride	100	300	With	Without	0	3	4.8
3	3	Tetrachloride	25	300	With	With	3.7	1.3	7.9
4	4	Tetrachloride	25	1500	With	Without	115.3	97.9	197.9
7	5	Dichloromethane	25	300	Without	Without	52.5	50	55.7
9	6	Dichloromethane	100	1500	Without	Without	78.1	87.6	84.3
2	7	Dichloromethane	100	300	With	Without	41.1	30.2	54.4
6	8	Dichloromethane	25	1500	Without	With	0	0	0
8	9	Tetrachloride	25	1500	Without	With	75.5	54	68.1
10	10	Dichloromethane	100	300	Without	With	5.6	7.2	6.1
11	11	Dichloromethane	25	1500	With	With	3.5	9.5	6.9
5	12	Tetrachloride	100	300	Without	Without	2.3	5.5	1.2

[a]Type of extracting solvent. [b]Extracting solvent volume (μL). [c]Dispersing solvent volume(μL). [d]pH (with or without buffer). [e]Protein precipitation (with or without precipitation). [f]Glibenclamide recovery percentage (%). [g]Gliclazide recovery percentage (%). [h]Glimepiride recovery percentage (%).

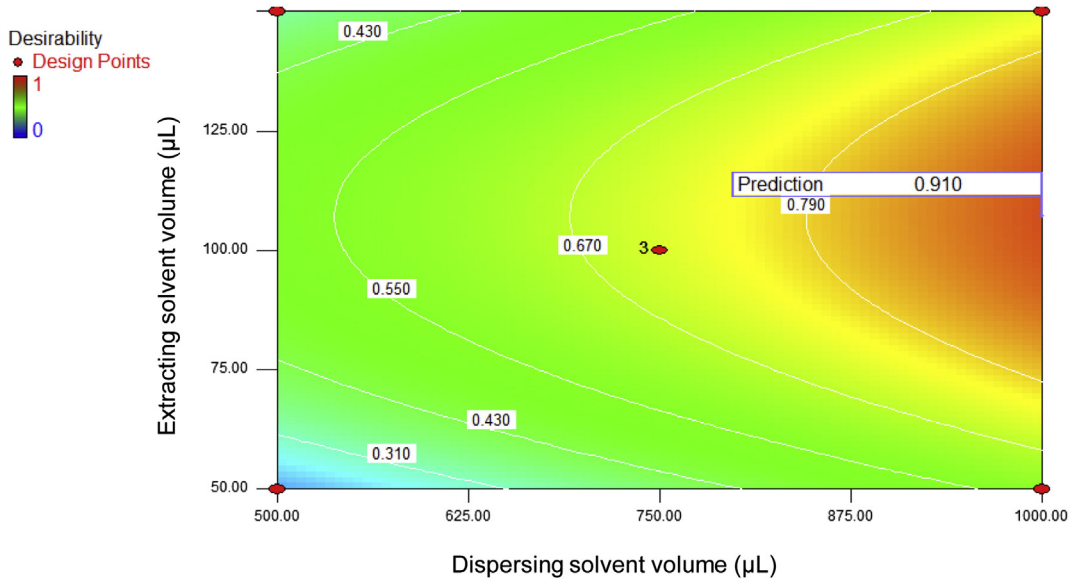

Fig 1. Contour plot corresponding to the desirability function when optimizing simultaneously the three recoveries for the target analytes.

replicate analysis of the same standard samples during two consecutive weeks. Relative standard deviation (RSD %) was calculated in both precision studies.

In order to assess the method robustness, different extraction and chromatographic parameters were varied within a realistic range and the influence of these variables on resolution was studied. A twelve experiment Plackett–Burman design was built considering small variations in extracting volume solvent, dispersing volume solvent, pH, buffer phosphate molarity, chromatographic flow, column temperature and mobile phase composition (% acetonitrile) (Table 2).

3. Results and discussion

3.1. Extraction procedure: preliminary studies

Solid-phase extraction (SPE) was considered, and two classes of cartridges were studied (C18 and HLB) [10,12]. Very low recoveries were obtained in both cases, discarding SPE as a possible extraction method. Liquid–liquid extraction was then studied. Three extraction systems were tested: System A, 1.0 mL dichloromethane:hexane (50:50); System B, three successive extractions (B1, B2, B3) with 300 µL dichloromethane:hexane (50:50); and System C (DLLME), 100 µL dichloromethane:hexane and 500 µL acetonitrile [8,13–17]. The highest recovery percentages were obtained with system C (DLLME); therefore, an experimental design was built for DLLME optimization.

3.2. DLLME optimization

3.2.1. Screening phase

A reduced factorial design was performed in order to find the best conditions for DLLME procedure (see Section 2.6). Table 3 shows the studied factors and the %recovery obtained in each case. An ANOVA test was applied to the experimental data. As a conclusion of this analysis, two factors (B, extracting solvent volume and C, dispersing solvent volume) were shown to be significant ($p < 0.05$) for all the three responses. This information was used to build the central composite design used in optimization.

3.2.2. Response surface method

The model coefficients of the central composite design were computed by backward multiple regression and validated by ANOVA. All the three responses were adjusted by quadratic models.

3.2.3. Multiresponse optimization

Multiresponse optimization was carried out with the central composite design described in Section 2.6. The three responses were

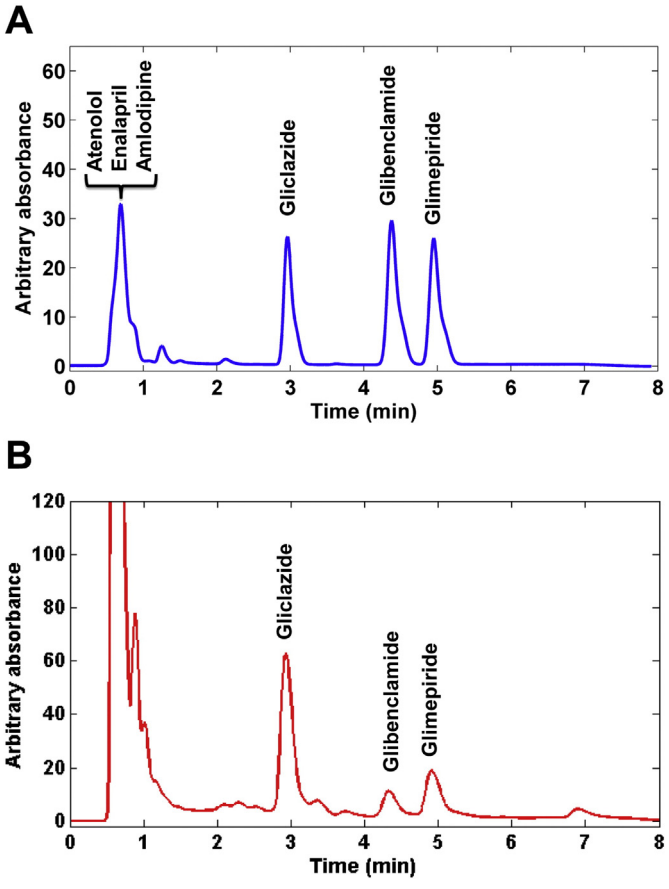

Fig. 2. Chromatograms corresponding to (A) standard mixture solution in water containing gliclazide 0.97 µg mL^{-1}, glibenclamide 1.06 µg mL^{-1} and glimepiride 1.07 µg mL^{-1}, atenolol 0.99 µg mL^{-1}, amlodipine 1.01 µg mL^{-1} and enalapril 1.00 µg mL^{-1}. (B) Standard mixture solution in serum (S2 of the recovery test set), containing gliclazide 2.78 µg mL^{-1}, glibenclamide 0.53 µg mL^{-1} and glimepiride 1.07 µg mL^{-1}, atenolol 0.54 µg mL^{-1}, amlodipine 2.72 µg mL^{-1} and enalapril 1.12 µg mL^{-1}.

Table 4
LOD, LOQ and linearity ranges.

	Analyte		
	Gliclazide	Glibenclamide	Glimepiride
LOD (μg mL^{-1})			
IUPAC	0.045	0.047	0.077
S/R	0.032	0.031	0.029
LOQ (μg mL^{-1})			
IUPAC	0.12	0.12	0.15
S/R	0.11	0.10	0.14
Linearity range (μg mL^{-1})	0.12–2.80	0.12–2.40	0.15–2.70
Intercept[a]	−14 (9)	−8 (6)	−0.8 (0.9)
Slope[a]	250 (9)	260 (20)	240 (20)
F_{exp}[b]c	2.30	0.85	1.01
r^2	0.991	0.990	0.997
Lack of fit	0.99	0.24	0.67

[a] Values between parenthesis indicate standard deviation.
[b] $F_{tab} = 3.185$. *F*-test for linearity determination
[c] Since the *p*-value for the lack of adjustment is greater than or equal to 0.10, the model seems to be adequate for the observed data.

simultaneously optimized by using the desirability function, as defined in Eq. (1). The criterion followed for the optimization of the individual responses was their maximization, given that the three responses correspond to the recoveries of each hypoglycemic drug. Lower and upper limits were as follows: dispersing solvent volume: 390–1100 μL, and extracting solvent volume 30–170 μL, based on previous experience with this system. The importance assigned to each response was the same, given that each response corresponds to the recovery of each studied drug, and they are equally important. Under the above-mentioned optimization criterion, the experimental conditions corresponding to a maximum in the desirability function ($D = 0.910$) (Fig. 1) are as follows: dispersing solvent volume: 1000 μL and extracting solvent volume 107.2 μL. In order to simplify experimental measurements, 100 μLwas chosen as extracting solvent volume. Interestingly, a visual inspection of Fig. 1 reveals that this change in the optimal volume of 107.2 μL to 100.0 μL does not produce any significant variation in the *D* value ($D = 0.904$). Finally, the predicted recoveries for the three responses, setting the latter experimental conditions, were the following: R1 = 98.0%, R2 = 96.4% and R3 = 95.7%.

3.3. Chromatography conditions

Yao et al. [33] developed an HPLC method for various sulfonylureas. Based on the latter report, changes were introduced in the present work to fit with the chemical characteristics of the studied analytes. Consequently, considering the pK_a of the analytes, pH was adjusted to 2.60 with phosphoric acid. The mobile phase was delivered at 1.0 mL/min. UV chromatograms were registered at 230 nm, with the wavelength corresponding to the maximum absorbance value of gliclazide, glibenclamide and glimepiride.

Then, in order to improve the HPLC separation, methanol was substituted by acetonitrile, implementing an elution gradient. This modification resulted in higher resolution, besides of better peak shapes. Atenolol, enalapril and amlodipine could not be quantitated but do not interfere in the separation and quantification of gliclazide, glimepiride and glibenclamide. Serum components do not interfere either. Chromatographic runs corresponding to standard prepared in both water and basal serum are shown in Fig. 2 (A–B).

3.4. Method validation

3.4.1. Limit of detection (LOD) and limit of quantitation (LOQ)

According to the results presented in Table 4, it can be concluded that the three analytes can be accurately quantitated in serum samples of patients under treatment of the studied antidiabetic drugs, whose therapeutic ranges are 2.00–8.00, 0.14–0.35 and 0.20–0.31 μg mL^{-1} for glicazide, glibemclamide and glimepiride, respectively [5].

3.4.2. Matrix effect

Matrix effect was evaluated as indicated in Section 2.7. Comparisons between pure standard solutions and basal human serum samples spiked with standards at the same concentration levels, provided $p > 0.1$ for the three cases, which means that matrix effect is absent.

3.4.3. Linearity

Calibration curves were obtained with seven standards of glibenclamide, gliclazide and glimepiride, covering the whole linear range and each point in triplicate. They showed a good linear relationship ($r^2 > 0.990$); calibration parameters are listed in Table 4. However, for assessment of the linearity of an analytical method, the goodness of fit was tested by comparing the variance of the lack of fit against the pure error variance. The adequacy of the model was estimated by an *F*-test as described by Olivieri in his Tutorial [32]. The calibration model is considered suitable if F_{exp} is less than the one-tailed tabulated value $F_{(\alpha,I-2,I-L)}$ ($I = 7$ is the number of calibration samples and $L = 3$, the number of concentration levels) (see Table 4).

3.4.4. Accuracy

Solutions prepared as described above were injected into the HPLC system, and the recoveries of the known amounts of added analytes were computed for each sample. Recoveries were calculated by interpolation of these signals on the calibration graph. The results obtained are indicative of the good accuracy reached with the proposed methodology since the mean recovery was in the range of 93–109% of the target concentration (in the worst case). In addition, a hypothesis to evaluate if the average recovery for each analyte at each level is significantly different from 100% or not was applied (see details in Ref. [32]). In all the cases, the critical value of $t_{(0.05,\,4)} = 2.776$ was higher than the experimental *t* value, fact that allowed us to conclude about the accuracy of the method. Detailed results are shown in Table 5.

3.4.5. Precision

The precision was calculated as the RSD (%); the values obtained are listed in Table 5. From this study, it can be concluded that the precision

Table 5
Results obtained in the recovery and precision study[a]

Variable	Experimental value for S1, S2 and S3 [a]								
	Gliclazide			Glibenclamide			Glimepiride		
	S1	S2	S3	S1	S2	S3	S1	S2	S3
Recovery (%)	102(9)	100(4)	95(8)	99(9)	95(4)	108(7)	95(9)	98(3)	93(9)
Intra-assay precision RSD (%)[b]	8.6	4.5	8.1	9.3	3.9	6.8	10.3	3.5	10.1
Inter-assay precision RSD (%)[b]	10.8	3.1	6.1	10.3	5.5	7.8	7.8	5.6	10.9
p-value[c]	0.73	0.25	0.10	0.27	0.18	0.08	0.15	0.07	0.35

[a] S1, S2 and S3, see Table 1. Values between parentheses indicate standard deviation ($n = 5$).
[b] Acceptable criterion: RSD ± 15% (Ref. [30]).
[c] Because the *p*-value is greater than or equal to 0.05, there is no statistically significant difference between the mean values.

of the method could be considered acceptable (acceptable RSD values are ≤15%) [30].

For further evaluation of inter-assay precision, an analysis of variance was applied for the recoveries obtained for each concentration level during the 2 weeks, in such a way that both within-condition and between-condition variances were taken into account (Table 5). The *p*-values obtained, which were >0.05, enable us to conclude there were no significant differences between the mean recoveries for each level during the 2 weeks studied with a confidence level of 0.05 for each analyte.

3.4.6. Robustness

By combining changes in conditions and performing a set of experiments, one can determine which factors have a significant or even critical influence on the analytical results (see Table 2). An ANOVA test was applied to the experimental data employing the effects of dummy variables to obtain estimates of standard errors. The ANOVA allowed us to conclude that small variations in extracting volume solvent, dispersing volume solvent, pH, buffer phosphate molarity, chromatographic flow, column temperature and mobile phase composition (% acetonitrile) have no significant effect in resolution between glibenclamide and glimepiride peaks. In addition, all three analytes can be quantitated with acceptable accuracy. Nevertheless, these variables are an important issue to be considered when quantifying antihyperglycemic drugs in serum and should be maintained as fixed as possible.

3.5. Analysis of real samples

Sixteen serum samples corresponding to patients under treatment with one of the analyzed substances plus other drugs as the mentioned antihypertensives (atenolol, enalapril and amlodipine) were analyzed according to the procedure described in Section 2.5. The predictions are presented in Table 6. In these samples, atenolol, enalapril and amlodipine were considered as potential interferents together with the serum components.

The concentration of the three analytes measured in this work ranged between 0.16 and 1.42 μg mL^{-1}. These levels agree well with those reported in the bibliography [5]. This confirms the suitability of this environmentally benign method, which is simpler than others reported previously for monitoring patients being treated with these drugs.

In addition, to demonstrate that the method developed is valid for real samples, the results were compared between spiked and non-spiked samples. Three samples were chosen: M4, M6 and M15 (see Table 6), and each one was spiked with 0.5 mg mL^{-1} of drug standard solution. Recovery % was calculated in each case. Results are shown in Table 7. These high recoveries (95–102%) are indicative that the method can be applied in real samples.

Table 6
Determination of glicazide, glibenclamide and glimepiride in real serum samples corresponding to patients under treatment.

Sample	Gliclazide	Glibenclamide	Glimepiride
	Concentration (μg mL^{-1})		
M1	–	–	0.31 (0.02)
M2	–	–	0.18 (0.01)
M3	–	–	0.16 (0.01)
M4	–	–	0.17 (0.01)
M5	1.06 (0.07)	–	–
M6	1.42 (0.06)	–	–
M7	1.23 (0.09)	–	–
M8	–	0.61 (0.04)	–
M9	–	–	0.27 (0.02)
M10	–	–	0.18 (0.01)
M11	–	–	0.23 (0.02)
M12	–	–	0.18 (0.01)
M13	0.77 (0.05)	–	–
M14	–	–	0.26 (0.02)
M15	–	0.26 (0.02)	–
M16	–	0.18 (0.01)	–

– means not detectable. Values in parentheses indicate standard deviation.

Table 7
Determination of glicazide, glibenclamide and glimepiride in real serum samples spiked with drug standard solution 0.5 mg mL^{-1}.

Sample	Gliclazide	Glibenclamide	Glimepiride
	Recovery %		
M4	–	–	95 (3)
M6	99 (4)	–	–
M15	–	102 (4)	–

– means not detectable. Values in parentheses indicate standard deviation.

Finally, a comparison with other published methods in the literature was conducted with the aim to show the advantages of the method reported in this paper. It is apparent that one important achievement is the possibility to quantitate the three analytes together [6,11,16,34,35]. Other important achievement is the substantial reduction of analysis time: results can be obtained in 5–25 min lesser [6,11]. Furthermore, it is important to remark the diminution in the use of solvents during the extraction phase (100–300% less) [6,8,16] and in the volume of sample (100–400% less) [6,8,16], a fact that is of paramount importance when working with human serum samples. It should be remarked that the LOQs achieved by use of this method are low enough for monitoring the drugs in patients as was demonstrated in the present report [11,20].Very recently, Oliveira Vianaet al. developed a microextraction by packed sorbent (MEPS) method [36]; the latter requires the use of internal standards and has many aspiration/ejection cycles, which implicates the use of a greater volume of solvents and long times before analysis. The DLLME method proposed in this paper does not require internal standards in the quantification stage, has only one extraction step and has much better LODs and LOQs.

4. Conclusions

A validated HPLC method for the determination of glibenclamide, gliclazide and glimepiride in serum samples has been developed. This method allows quantification in presence of atenolol, enalapril and amlodipine. A DLLME method that allows extraction of these drugs from serum was developed and optimized. This method is simple, requires no sophisticated procedures and produces excellent analyte recoveries. The chromatographic method is linear, accurate, precise and specific and has the ability to separate the antihyperglycemic drugs from antihypertensive drugs which are usually found in serum of diabetic patients.

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