Bioanalytical method by column-switching with direct injection of human plasma for determination of sulphonylureas

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Sulphonylureas are widely used in the treatment of Diabetes mellitus, one of the main causes of death in human population. Their determination is essential in pharmacological research and in the development of new drugs. Generally, determination of sulphonylureas in biological matrices is performed using conventional sample preparation techniques, which frequently leads to an increase of analysis time and errors. In this context, a bioanalytical method for the simultaneous determination of sulphonylureas by direct injection of human plasma was developed and optimized. An automated column-switching high performance liquid chromatographic system with a restricted access media (RAM) column coupled to a fused-core column was employed. At the first dimension, a RAM column with mobile phase of ultrapure water pH 6.0 at a flow-rate of 1.0 mL min⁻¹ was used. The valve switching time was 3 minutes. At the second dimension, a C18 guard-column coupled to a C18 fused core column with mobile phase of acetonitrile and 10 mM phosphate buffer pH 3.0 (54:46 v/v) at a flow-rate of 0.8 mL min⁻¹ were employed. The column switching system was performed in backflush configuration with an analyte elution time of 1 minute. Flufenamic acid was used as the internal standard. The mean plasma protein exclusion percentage by the RAM-column was 104.5%. The developed and optimized method showed to be fast and simple, allowing the direct injection of biological sample into the chromatographic system and the simultaneous determination of three sulphonylureas in only 12 minutes, including the sample treatment, separation and detection.

Keywords: sulphonylureas, column-switching, restricted access media, fused core column, human plasma.

Introduction

The analysis of drugs and metabolites in biological samples, mainly in plasma, is a common procedure for clinical diagnoses, toxicological analysis and pharmacological research. Recurrent problems found in bioanalysis are analytes low concentration, interferences due to the presence of other compounds and plasma proteins, and analyte-protein binding (1,2).

When high-performance liquid chromatography (HPLC) is used for the analysis of biological matrices, a pre-treatment is necessary before the injection of the sample in the system. This procedure aims to concentrate the analyte and eliminate plasma proteins, since these macromolecules may suffer irreversible adsorption in the stationary phase of the analytical column, shortening its lifetime and increasing the system pressure. Therefore, the sample preparation is a critical and limiting step in the development of analytical methods for the determination of drugs in biological fluids by HPLC (1,3-5).

The sample preparation techniques may be classified in conventional and modern. Modern or miniaturized techniques are characterized by reduced analysis time and organic solvent consumption, as well as automation. Among the modern sample preparation techniques, online solid phase extraction (SPE) using restricted access media columns (RAM) deserve attention. This kind of support allows the direct injection of biological samples in the chromatographic system, because of its macromolecules exclusion mechanism, restraining their access to the support's adsorption sites (6,7).

The direct injection of biological samples in the chromatographic system, through online mode, allows the

reduction of the sample handling by the analyst, who will be less exposed to the sample and to toxic compounds. Also, the sample will be subject to less oxidation and evaporation, and the occurrence of error during the analysis will be lower. The automated mode employing RAM columns can be performed using a two-dimensional chromatographic system, controlled by a valve, with the extraction phase in the first dimension and the analytical step in the second dimension. First, the biological sample, usually plasma, is injected into the first dimension of the system and carried to the RAM column by the exclusion mobile phase (EMP), with the valve at position A. In the RAM column, the analytes are retained in the stationary phase and plasma protein excluded and discarded. After that, the automatic valve rotates to position B, and the analytical mobile phase (AMP) runs through the RAM column eluting the analytes into the analytical column, where separation occurs (Figure 1). In the backflush configuration, the AMP runs through the RAM column in an opposite flow direction of the EMP. Oppositely, in the foreflush configuration both AMP and EMP runs in the same direction through the RAM column (2,5,6,8-11).

Diabetes mellitus (DM) is one of the main causes of death in human population. It is estimated that DM affects the lives of about 382 millions of people in the world. It can be classified in type 1 – autoimmune disease in which a catabolic disturb leads to the absence of insulin, and type 2 – heterogenic pathology encompassing many forms of diabetes, which have in common the insulin insufficiency or tissue insensitivity. Its treatment involves lifestyle change, maintenance of ideal weight and use of oral antidiabetic drugs, such as sulphonylureas. Figure 2 shows the chemical structure and some physical-chemical properties of the sulphonylureas analyzed in this study. These drugs are

completely and rapidly absorbed by the gastrointestinal tract and are bound to plasma proteins, mainly albumin, by about 90-99%. They are metabolized in the liver and their metabolites are excreted in urine (12-15).

Figure 1. Backflush and foreflush configurations for the two-dimensional chromatographic system.

Figure 2. Chemical structures of the analyzed sulfonylureas (GB, GM and GZ) and the internal standard (AF).

Sulphonylurea determination in biological fluids is commonly performed employing conventional sample preparation techniques. When modern techniques are used they are not automated. In a review of the recent literature on the quantification of sulphonylureas in biological samples using HPLC, the majority of the studies refers to the use of protein precipitation (PP) (16-18) or liquid-liquid extraction (LLE) (19-21). Lately, modern approaches have been described, such as dispersive SPE with magnetic material (22) and microextraction by packed sorbent (23). Despite of

presenting some advantages over conventional techniques, their main limitation is the lack of automation. One study was found describing the determination of glimepiride (GM) in plasma using column-switching with RAM column. The total time spent to determine only one sulfonylurea was 40 minutes (24).

In this context, the present study describes the optimization of a rapid and simple method, using a column-switching HPLC with RAM column (sample preparation) and fused core column (analytical separation), for the simultaneous determination of three sulfonylureas: glibenclamide (GB), GM and gliclazide (GZ), in human plasma.

Experimental

Chemicals and reagents

GB active pharmaceutical ingredient (API), 99.50% of purity, from Cadila Pharmaceuticals (Ahmedabad, Gujarat, India) was donated by Fundação Ezequiel Dias – FUNED (Belo Horizonte, Brazil). GZ API, 99.10% of purity, from Shandong Keyuas Pharmaceutical (Jinan, Shandong, China) and GM API, 99.96% of purity, from Mantena Laboratories (Naldonga, Dist, Andhra Pradesh, India) were donated by Cifarma (Belo Horizonte, Brazil). Flufenamic acid (FA), used as internal standard, was from Sigma-Aldrich (Steinheim, Germany) (Figure 2). Acetonitrile Panreac (Barcelona, Spain) and methanol Sigma-Aldrich (Steinheim, Germany) were of HPLC grade. Other analytical solvents and reagents were: acetic acid and monobasic potassium phosphate Sigma-Aldrich (Steinheim, Germany), ortho-phosphoric acid Merck (Darmstadt, Germany) and sodium hydroxide Quimex (São Paulo, Brazil).

Equipment and materials

Chromatography was performed in a Shimadzu (Kyoto, Japan) system composed by a SCL-10A VP controller, two LC-10AD VP pumps, a CTO-10A VP oven, a SIL-10AD VP automated injector, a SPD-M10A VP diode array detector (DAD), and a Cheminert six port valve (VICI - Brockville, Canada). A Poroshell 120 EC-C18 (100 x 4.6 mm, 2.7 μ m) fused core column from Agilent Technologies (Santa Clara, USA) and a C18 pre-column (4.0 mm x 3.0 mm) from Phenomenex (Torrance, USA) were used for separation. Extraction was performed in a RAM Merck Lichrospher C18 ADS (25 x 4.0 mm, 25 μ m) column (Darmstadt, Germany).

Biological sample

The human plasma samples, obtained by donation from healthy volunteers, were collected in the Laboratório de Hematologia Clínica from Faculty of Pharmacy - Federal University of Minas Gerais. The study was approved by the ethical committee (CAAE number 01892012.0.0000.5149). The blood collection was performed in the morning, with the volunteers fasted or having a light breakfast, characterized by low calorie food and without coffee and chocolate.

Heparin tubes were used for collection. The blood samples were centrifuged at 480 x g for 10 minutes at 4 °C and the obtained supernatant (plasma) was separated and stored at -70 °C until its use.

Preparation of chemicals solutions

Stock solutions of GB, GM and GZ at 0.5 mg mL⁻¹ in a mixture of acetonitrile and water 4:1 (v/v) were individually prepared. Work solutions were prepared diluting 1 mL of each stock solution in acetonitrile and water 50:50 (v/v). The final concentration was 100 µg mL⁻¹. Internal standard work solution was prepared at 0.1 mg mL⁻¹ in methanol.

Spiked plasma samples

After thawing, the plasma samples were centrifuged at 10410 x g for five minutes, spiked with the work solutions and, then, vortexed for 30 seconds in 2200 rpm. The final concentration was 5 µg mL⁻¹.

Evaluation of protein exclusion by RAM-ADS column

First, different volumes of blank human plasma (20, 50, 100, 200 and 500 µL) were injected in a chromatographic system without the analytical and RAM columns. Water as mobile phase at 1 mL min⁻¹ and UV detection at 280 nm were used. Afterwards, the RAM-ADS column was added to the chromatographic system, still without the analytical column, and the same volumes of blank human plasma were injected. By comparing the peak's areas from the two configurations described above, the protein exclusion percentage of the RAM column for the tested volumes was established.

Optimization of instrumental parameters and chromatographic conditions

First, the UV-visible spectra (from 190 to 800 nm) of the sulfonylureas and of the internal standard at 0.1 mg mL⁻¹ were obtained. Next, the time needed to EMP exclude the proteins from plasma samples was established by analyzing the chromatograms obtained during the evaluation of the protein exclusion by the RAM-ADS column. At this moment, the valve was at position A. After complete exclusion of plasma proteins, the valve was switched to position B, when the AMP ran through the RAM-ADS column eluting the retained analytes. The influence of the valve position (maintaining in the position B or returning to position A) in the peak area was evaluated. In the case of returning to position A, different switching times (30, 60, 90 and 120 seconds) were tested.

Ultrapure water in different pH values (4.0, 5.0, 6.0 and 7.0) was tested as EMP. Different compositions of the AMP were tested, using acetonitrile as organic phase and acetic acid 1% or 10 mM potassium phosphate buffer as aqueous phase, both with pH 3.0). Also, different proportions and flow-rates were evaluated, as long as the elution mode: isocratic or gradient. After that, different sample volumes (20, 50, 100, 200 and 500 µL) were injected, in order to evaluate the effect on the method detectability.

In order to establish the more appropriate column-switching configuration for the simultaneous analysis of the three sulfonylureas, backflush and foreflush were assessed (Figure 1).

Protein precipitation

The effect of a previous PP on the analyte's peak area was tested. The analyte's peak area obtained with PP, RAM-ADS, both alone, and with the combination of RAM and PP were compared. For PP, acetonitrile was added to the spiked plasma in the proportion of 1:1 (v/v) (25) followed by vortex for 1 minute and centrifugation at 10410 x g during 5 minutes. Then, the supernatant was collected and filtered in regenerated cellulose membrane filter (0.45 µm).

Results and discussion

Evaluation of protein exclusion by RAM-ADS column

After the analysis of blank plasma samples using the chromatographic system without (representing the concentration of 100% of plasma proteins), and with (representing plasma proteins excluded) the RAM-ADS column, plasma protein exclusion percentage by RAM-ADS column, for each volume injected, was calculated (Table 1). The values obtained were considered satisfactory, since it was higher than 100% for all tested volumes. The mean was 104.5%, with a relative standard deviation of only 2.15%, indicating total exclusion of plasma proteins by the RAM-ADS column in less than 2 minutes (Figure 3).

Table 1 – Plasma protein exclusion percentages by the RAM-ADS column.

Injection volume (µL)	Peak area		Exclusion (%)
	Without RAM	With RAM	
20	20509651	20674455	100.8
50	32340063	34123606	105.5
100	45236230	48327986	106.8
200	49928515	52410799	104.9
500	50935121	53198585	104.4
Mean			104.5%
RSD			2.15

Figure 3. Chromatograms obtained before and after plasma protein exclusion by the RAM-ADS column. Sample volume: 200 μ L. Mobile phase: ultrapure water, flow-rate: 1.0 mL min⁻¹. Wavelength detection: 280 nm.

Optimization of instrumental parameters and chromatographic conditions

In order to choose the internal standard for the bioanalytical method, different compounds available in our study group, with similar physical-chemical properties, retention time and maximum absorption wavelengths as the sulfonylureas, were evaluated. Considering these requirements, FA (logP of 5.25 and pKa of 3.88) was chosen as the internal standard. A maximum absorption at 230 nm was observed for the sulfonylureas and FA, when solutions at 0.1 mg mL⁻¹ in methanol were tested from 190 to 800 nm. Therefore, 230 nm was chosen for the chromatographic method.

As can be observed in Figure 3, after 3 minutes 100% of plasma proteins were excluded from the RAM-ADS column for all volumes tested. Therefore, at 3 minutes after injection the valve was switched from position A to position B.

The chromatograms of blank and spiked plasma, without returning the valve to the initial position (position A) are presented in Figure 4. The interference of the blank plasma in the peaks of the analytes makes their integration inappropriate. Thus, the returning of the valve from position B to position A was necessary. Different time intervals (30, 60, 90 and 120 seconds) were tested. At 90 and 120 seconds, the determination of GZ peak area was not possible due to a co-elution with a plasma interference. Therefore, 60 seconds was selected, since there was an increase in the peak area for all the analytes and the internal standard when compared to 30 seconds (Figure 5).

Ultrapure water, a weak solvent in reversed phase, was used as EMP because of its low affinity for the analytes and IS, assuring a high interaction of these analytes with the RAM-ADS column. Besides that, water has the ability to ionize the sample proteins favoring their exclusion by electrostatic repulsion inside the RAM-ADS column. Aiming to evaluate the elimination of the proteins, four values of pH were evaluated: 4.0, 5.0, 6.0 and 7.0. When neutral pH was used, a lack of resolution between the chromatographic peaks was observed. At pH 4.0, a residual effect related to the IS was observed in the chromatogram of blank plasma obtained after the analysis of spiked plasma. Since this phenomenon

was not observed in pH 5.0 and 6.0, this range was employed.

Figure 4. Chromatograms obtained for the analysis of spiked and blank plasma, without returning the valve from position B to A. Sample volume: 200 μ L. EMP: ultrapure water, flow-rate: 1.0 mL min⁻¹. AMP: acetonitrile and 10 mM phosphate buffer pH 3.0 (54:46 v/v), flow-rate: 0.8 mL min⁻¹. Wavelength detection: 230 nm.

Figure 5. Peak areas of GB, GM, GZ and IS in different time intervals, after which the valve was switched from position B to position A.

Different AMP compositions were tested using acetonitrile (organic phase) and acetic acid 1% or 10 mM phosphate buffer, both at pH 3.0 (aqueous phase), varying the proportion of the phases, flow-rate and elution mode (gradient or isocratic). In view of the obtained results in terms of resolution and baseline noise, AMP was composed by acetonitrile and 10 mM phosphate buffer pH 3.0, in isocratic mode (54:46 v/v), in a flow-rate of 0.8 mL min⁻¹.

Different sample volumes of human plasma were evaluated. As can be observed in Table 2, there was an improvement in the chromatographic peak areas and heights when the injection volumes were increased. An exception was a decreasing in peak areas and heights when the volume changed from 200 to 500 μ L of plasma. A possible reason is the saturation of the RAM column, reducing its capacity to adsorb the analytes. Due to the decrease in peak area and height when 500 μ L of the sample was injected in the

chromatographic system, 200 μL was defined as the injection volume, since there was an increase in the peaks when compared to the injection of 100 μL of plasma, improving the method detectability. It is important to note

that during this step of the method development, blank plasma samples were injected between spiked samples analysis and no carry-over effect was observed.

Table 2 – Variation of peaks areas and heights as a function of the volume of injected plasma(μL).

		20 $\rightarrow$ 50 (%)	50 $\rightarrow$ 100 (%)	100 $\rightarrow$ 200 (%)	200 $\rightarrow$ 500 (%)
Area	GZ	133.1	95.9	18.1	- 3.8
	GB	89.4	226.0	7.3	- 5.5
	GM	200.1	313.1	4.4	- 8.4
	IS	88.0	173.3	2.8	- 2.6
	Mean	127.6	202.1	8.2	- 5.1
Height	GZ	133.5	96.2	7.1	- 2.9
	GB	116.6	232.2	3.6	- 4.9
	GM	228.2	315.5	2.9	- 8.3
	IS	137.6	197.3	2.8	- 2.2
	Mean	154.0	210.3	4.1	- 4.6

After the optimization of the chromatographic conditions, backflush and foreflush modes were compared for the simultaneous determination of GB, GM and GZ. In foreflush configuration a baseline noise was observed, which compromised the appropriate peak integration. Moreover, the resolution between the sulfonylureas was poor. A possible reason is the flow direction of the AMP, which is the same of the EMP. Therefore, the analytes eluted through the whole length of the RAM column before achieve the analytical column. This might lead to the adsorption of the analytes along the RAM column, resulting in peak broadening.

On the other hand, in backflush mode, the flow direction of the AMP is opposite to the EMP. Therefore, the analytes adsorbed in the head of the RAM column were eluted directly into the analytical column, going through a small pathway resulting in less extra column volume and avoiding peak broadening. In view of the obtained results and the small amount of time spent in the analysis of the studied sulfonylureas, only 12 minutes, backflush was the selected column-switching configuration.

Protein precipitation

When PP was used as a single technique, the peak area for all analytes increased with higher injected plasma volumes. This gain was much more pronounced when the volume was 100 μL (Figure 6). Possibly, the increase would be higher with 200 and 500 μL ; however, it was not possible to identify the compound's peaks due to baseline noise and poor resolution between the analytes. As can be observed, the peak areas were higher for PP when compared with RAM-ADS and RAM-ADS + PP. A possible reason is the disruption of drug-protein binding by the organic solvent, improving analytes recovery.

Despite the higher peak areas observed with PP, it was not considered for the analysis of the studied sulfonylureas, in

view of the increased analysis time, chance of errors, and analyst exposure, since it is a non-automated technique.

The peak areas obtained with RAM-ADS column were similar to those obtained with the combination of both techniques (PP + RAM), another indication of a possible saturation of the RAM column. Therefore, the RAM column was maintained as the only sample treatment technique, since its combination with PP did not add any improvement. Figure 7 shows the chromatogram of blank and spiked plasma samples obtained with the optimized method.

Figure 6. Peak areas of GB, GM, GZ and IS as a function of the sample treatment technique and of the volume of injected plasma (μL).

Figure 7. Chromatograms obtained after the injection of spiked plasma followed by blank plasma using the optimized method. Sample volume: 200 μL . EMP: ultrapure water, pH: 6.0; flow: 1.0 mL min^{-1} . AMP: acetonitrile and phosphate buffer 10 mM pH 3.0 (54:46), flow: 0.8 mL min^{-1} . Wavelength: 230 nm.

Conclusion

The column-switching system using the backflush configuration, which employed a six-port valve, allowed the use of two dimensions in the chromatographic system, one for sample treatment and the other for separation. This system allowed the direct injection of human plasma samples in the chromatographic system.

The developed method showed several advantages over those already published in the peer-review literature dealing with sulphonylureas determination: less exposure of the analyst to the biological sample and to the solvents used in the analysis, less chances of errors due to the handling of the samples and reduction of the time spent in the analysis, increasing the productivity and reducing the expense of organic solvents.

The developed method demonstrated to be simple and fast, in addition to allowing simultaneous determination of three sulphonylureas, glibenclamide, glimepiride and gliclazide, in human plasma. The total analysis time, encompassing the chromatographic step (8 minutes) and the sample treatment step (3 minutes for the protein exclusion and 1 minute for the analytes elution) was only 12 minutes.

Conflict of interest

The authors declare there is no conflicts of interest.

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