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Study of Dried Blood Spot Reliability for Quantitative Drug Analysis by UHPLC-PDA-FLUO

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Abstract: In this work, the reliability of Dried Blood Spot (DBS) as a sampling technique for drug analysis was studied by Ultra High Performance Liquid Chromatography coupled to Photodiode-Array and Fluorescence Detection (UHPLC-PDA-FLUO). DBS microsampling, a technique based on placing a drop of blood in a cotton support that is allowed to air dry, has lately noticed an increase in use in bioanalysis. Even thought it offers several advantages compared to common blood sampling methods, it also shows some limitations for quantitative analysis due to the dependence on different factors. In this study, the influence of some of them (hematocrit, blood volume and sampling position) has been investigated, using amiloride, propranolol and valsartan drugs as model compounds. According to the results, it has been concluded that the sampling position and the hematocrit have influence in the accuracy and precision of the quantitative results, therefore limiting the use of this technique. On the other hand, dispersion of the analytes in the blood drop depends on their physicochemical properties which implies that the distribution of each analyte must be carefully studied during method development.

Keywords: DBS; UHPLC-PDA-FLUO; Bioanalysis

1. Introduction

Dried blood spot (DBS) sampling method was first used with human blood in 1963 by Roberth Gurthrie to detect metabolomic diseases (phenylketonuria) in newborn infants. Since then, it has been used in many different areas such as toxicokinetics and pharmacokinetic studies, diagnostic screening, and therapeutic drug monitoring [1,2]. Due to the simplicity of this technique and thanks to the improvement in terms of sensitivity of the analytical instrumentation, its use has been notably increased in the last years.

DBS microsampling is a simple technique in which a drop of blood is placed in a support that

is left to air dry prior to analysis. The support is usually cellulose paper chemically treated to prevent the growth of bacteria and other microorganisms [3] and sampling is done by putting a drop of blood from a heel, ear or finger prick in the support [2,4].

Once the blood is placed and dried, the analytes are extracted from the support usually by a liquid extraction of a punch done in the drop, and then analyzed [3]. DBS technique is used as a sampling method for a wide range of analytical techniques such as DNA-based assays, enzyme activity assays, immunoassays, direct mass spectrometry, and liquid chromatography coupled to different detectors [1].

DBS technique shows many advantages compared with conventional blood, plasma or serum collection procedures [5]. Among them, the stability of the cellulose-fixed analytes, the little blood volume required, the possibility of automation of the sample processing [6], the easy storage and transportation [4], or the lower biological risk in comparison with liquid blood samples. Despite these advantages there are also some disadvantages to consider. For example, when they are used in quantitative analysis, some factors can affect the reliability of the results[4,7,8], such as the type of blood (venous or capillary) [9,10], the type of support [11,12], the hematocrit [3,9,10,13-15] or the blood volume placed [16,17].

The aim of this work is the study of the influence of the blood volume, the hematocrit and the punching position on the quantitative results of a DBS based method in order to increase the reliability of the analysis and better understand the dispersion of the analytes in the support. For this purpose three drugs with different physico-chemical properties (amiloride, propranolol and valsartan) were selected as model compounds (Figure 1). For the analysis of these compounds a robust quantitative method was optimized using Ultra High Performance Liquid Chromatography coupled to Photodiode-Array and Fluorescence Detection (UHPLC-PDA-FLUO).

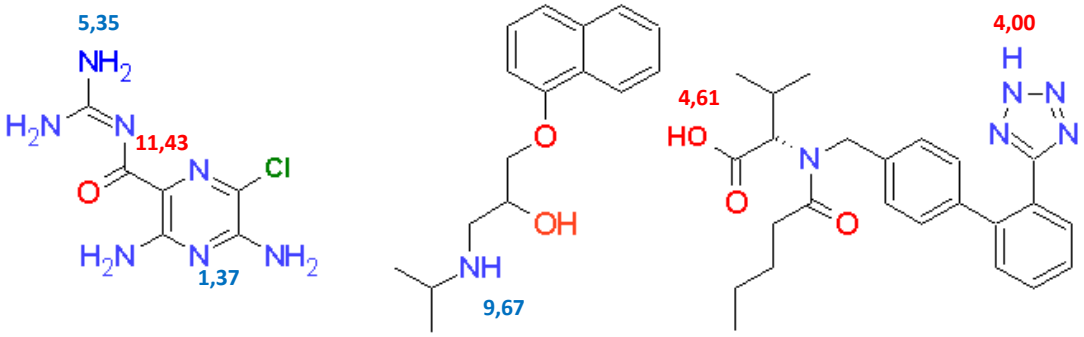

Figure 1. Amiloride, propranolol and valsartan. Chemical structure, molecular formula and weight, and fluorescence excitation and emission wavelengths. In blue, the pKa for basic protons and in red for acidic protons.

2. Results and Discussion

2.1. Study of the influence of hematocrit and sample volume in DBS analysis

The influence of hematocrit in the quantitative application of DBS has been already reported by some authors [3,5,9,18]. In this work seven

hematocrit values (25%, 30%, 35%, 40%, 45%, 50% and 55%) were studied in order to cover a wide interval of the hematocrit values of the population. Three sample volumes (15µL, 25µL and 35µL) were studied for each hematocrit level.

Using ANOVA, at 95% confidence level, it was observed for amiloride and propranolol that the results are influenced by the hematocrit value but not by the sample volume. In the case of valsartan, both variables have influence, being the effect of the hematocrit much more significant. In Figure 2 the increase of the chromatographic response of propranolol with the hematocrit value can be noticed. The extreme values (25% and 55%) were compared using a t-test (95% confidence level) and a significant difference was confirmed. The reason of this difference is probably the change in the blood drop area. As it decreases when the hematocrit value increases (due to the density/viscosity difference), the blood volume present in the sample punch is higher for the high hematocrit value samples, and also the amount of analyte.

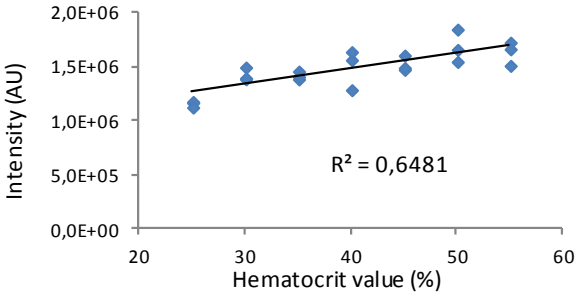

Figure 2. Chromatographic responses obtained for propranolol samples with different hematocrit values, using a 15 µL blood drop.

2.2. Study of the influence of the sampling position

Due to a heterogeneous dispersion of the blood or to chromatographic processes involving the analytes, the distribution of the drugs in the blood spot may not be homogeneous. In those cases the position where the punch that will be analysed is done becomes a critical factor.

To study the influence of the dispersion of the different analytes, the blood drop area has been divided in three zones: central, upper and lower peripheral. In this way a radial distribution of the analytes could be studied as well as the effect of the paper position during the drying step. In order to obtain a sample above the quantitation limit of the method 15 mini punches (1.2 mm diameter) were collected in each zone.

The results of the analysis can be observed in Figure 3. The statistical analysis did not show significant differences in terms of precision but a clear difference in analytes distribution behavior was noticed. On the one hand, amiloride concentration is higher in the central zone, being this behavior more obvious/perceptible at lower hematocrit values. On the other hand, propranolol and valsartan behave differently, with higher concentration values at the peripheral zone (phenomena known as volcano effect and already observed by other authors [11,12]).

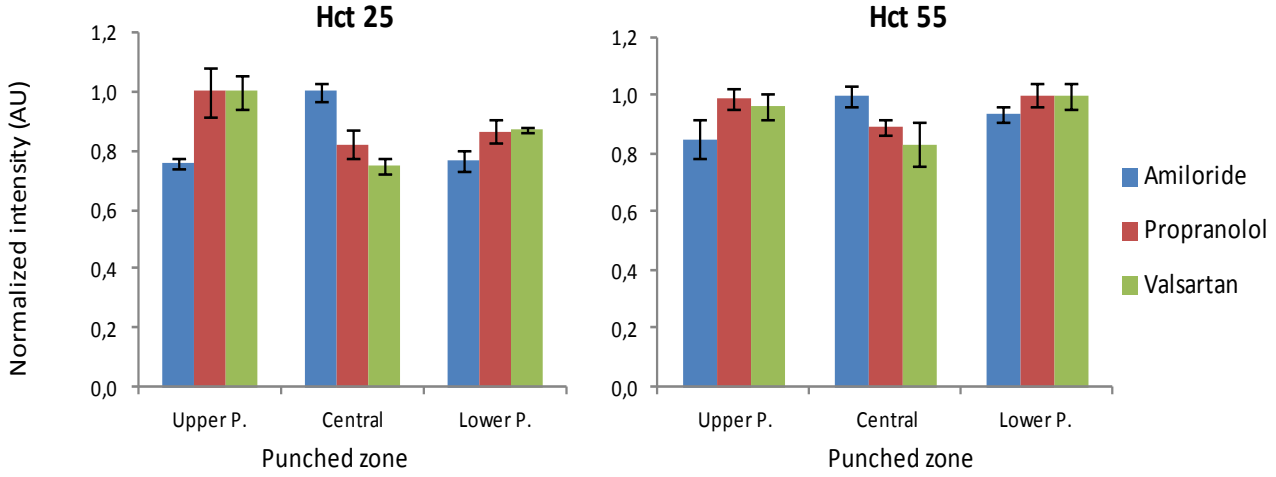

Figure 3. Normalized results for the chromatographic responses of the analytes belonging to central, upper and lower peripheral zones of the blood spot.

3. Materials and Methods

3.1. Chemicals

Amiloride hydrochloride was purchased from Sigma-Aldrich (St. Louis, USA), propranolol hydrochloride from Fluka (Burch, Switzerland) and valsartan from Novartis Pharma AG (Basel, Switzerland). Methanol used for the preparation of stock and working solutions was provided by Romil (Cambridge, England). For the preparation of the different pH solutions dipotassium phosphate (>99%), acetic acid (LC-MS grade), sodium dihydrate citrate anhydrous (>99%) and disodium hydrogen citrate sesquihydrate (>99%) were purchased from Fluka. Potassium dihydrogen phosphate, sodium acetate, trisodium dihydrate citrate, ammonium (25%) and ammonium chloride all of Pro-Analysi grade, and sodium hydroxide, EMSURE (Millipore's premium grade), were obtained from Merck (Darmstad, Germany). Phosphoric acid (85%) and citric acid (PA-ACS-ISO) were supplied by Panreac (Barcelona, Spain). Acetonitrile used for the preparation of the mobile phases was obtained from Romil. LC-MS quality grade formic acid was purchased from Fluka. Ultrapure analytical water was obtained from a Milli-Q Element A10 sysrem (Millipore, Milford, USA).

3.2. Instrumentation

The DBS cards used were Protein saver 903 of Whatman (NJ, USA). The puncher used was a regular office puncher with a diameter of 5.9 mm and the mini puncher was purchased from Harris (CA, USA) with a diameter of 1.2 mm. Analyses were performed on an Acquity UPLC system (Waters, Mildford, USA), coupled to a PDA detector and FLUO detector with a scheduled excitation and emission wavelength method. System control, data collection and data processing were accomplished using Empower 2 software. The chromatographic column used was Acquity BEH C18 (2.1x50, 1.7 µm) of Waters with a filter pre-column. The pH was measured with a Crison GPL22 pH-meter (Barcelona, Spain). To draw the molecules of the analytes ChemSketch 10.02 has been used and for the statistical treatment of the results Microsoft Excel 2010 and Unscrambler softwares have been used.

3.3. Standard solutions and blood samples

Standard stock solutions of 1000 mg/L were prepared in methanol for each analyte separately. With those solutions 50 mg/L working solutions were prepared. 15 blood samples covering the range of 22%-55% hematocrit value were generously provided by the University Hospital

of Basurto (Bilbao, Spain), Samples were stored at -80ºC until analysis. The blood samples were spiked from working solutions to concentration of 1 mg/L.

3.4. Chromatographic conditions

The mobile phase consisted of solvent A (0.01% formic acid) and solvent B (acetonitrile). The gradient applied was the following: 0-0.5 min, 1% B; 0-3 min, 1 to 99% B; 3-3.5 min, 95% B; 3.5-3.6 min, 95 to 1% B; 3.6-4.5 min 1% B. Flow was kept at 0.55mL/min. During the chromatographic analysis the column was thermostated at 35 °C and samples were kept at 10±1°C in the autosampler. The PDA wavelength range was set from 190 to 400 nm and the excitation and emission wavelengths of the FLUO detector were as follows: from 0.00-1.39 min, 363/415 nm; 1.40-1.59 min, 287/340 nm; 2.00-4.50 min, 237/371nm).

3.5. Extraction conditions

The extraction conditions were carefully optimized. Extraction solution was methanol: pH 2 phosphate buffer (75:25). 200 µL of this solution was added to a 5.9 mm punch and after sonication and centrifugation the supernatant was transferred to a chromatographic vial.

4. Conclusions

In this work, some limitations of DBS as a sampling technique for small molecule quantitative analysis have been observed. On the one hand, it has been demonstrated that the chromatographic response of samples with the same analyte concentration but different hematocrit values are significantly different. This effect is attached to the difference in blood drop area, which decreases when hematocrit value increases. On the other hand, due the heterogeneous dispersion of the analytes in the blood drop the punching position is a critical parameter that affects the quantitative results. Furthermore, it has been observed that this dispersion is different depending on the analyte which means that during method development the behavior of each analyte must be carefully studied since, currently, anticipating the distribution of the analyte in the blood drop is not possible.

To prevent these problems the analysis of the whole blood drop can be an alternative approach. Nevertheless, this would require a more time- and solvent-consuming method and would make the automation of the sample treatment more complicated. In addition, from the point of view of a reliable quantitation all the blood drops (calibration and study samples) should have the same volume. Although that is not a problem for samples prepared in a laboratory it would make sampling more complicated under other conditions (hospitals, third-world countries, etc.) which is one of the main advantage of the DBS technique. Therefore, analytical methods based on DBS and punching should be studied more in depth in order to guarantee a reliable quantitation.

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Author Contributions

Conceived and designed the experiments: BU, OG, RA. Performed the experiments: BU, OA. Analyzed the data: BU, OG. Wrote the paper: BU, OG

Conflicts of Interest

The authors declare no conflict of interest

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