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Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood

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ABSTRACT

In this study, a fast and simple blood sampling and sample pre-treatment method based on the use of the dried blood spot (DBS) cards and ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) for the quantification of olive oil phenolic metabolites in human blood was developed and validated. After validation, the method was applied to determine hydroxytyrosol metabolites in human blood samples after the acute intake of an olive oil phenolic extract. Using the FTA DMPK-A DBS card under optimum conditions, with 20 μL as the blood solution volume, 100 μL of methanol/Milli-Q water (50/50, v/v) as the extraction solvent and 7 disks punched out from the card, the main hydroxytyrosol metabolites (hydroxytyrosol-3-*O*-sulphate and hydroxytyrosol acetate sulphate) were identified and quantified. The developed methodology allowed detecting and quantifying the generated metabolites at low μM levels. The proposed method is a significant improvement over existing methods to determine phenolic metabolites circulating in blood and plasma samples, thus making blood sampling possible with the volunteer pricking their own finger, and the subsequent storage of the blood in the DBS cards prior to chromatographic analysis.

1. Introduction

In recent years, the biological effects of food bioactive compounds (FBCs) have been related to their bioavailability and their temporal and spatial distribution in the body. The concept of bioavailability is complex and includes: (i) availability for absorption or "bioaccessibility"; (ii) absorption; (iii) tissue distribution and (iv) bioactivity [1]. Based on the intense biological metabolism of some FBCs, mainly phenolic compounds, it is very important to determine the metabolites circulating for a better understanding of the fate of the parent compounds in the food. Only when the circulating forms and the pharmacokinetics of the FBCs are known, a more complete picture can be obtained about their bioavailability and possible correlation with bioefficacy [1]. That is why absorption studies of FBCs in general, and polyphenols in particular, are of great importance for establishing the dose-exposure relationship, the impact of the composition of the food matrix, metabolism after being consumed, and the kinetic of absorption, among other aspects. The knowledge of these factors that determine the polyphenol absorption is essential to all those involved in food production and nutritional assessment. Although human clinical trials are mandatory for testing a functional ingredient, these involve an extremely complex organization of volunteers, and are a huge technical and economical investment [1]. Ideally, as a first step, feasibility studies should be performed in a small group of humans as a *proof-of-concept* to explore the effect of the dose and food matrix composition on absorption based on the analysis of the FBCs or their metabolites circulating in blood or plasma samples. This first step is achieved through a so-called *acute intake* or *post-prandial* study. In these studies, the volunteers are cited on the day of the experiment after fasting overnight. After the acute ingestion of the test food, blood samples are obtained by venipuncture from volunteers and are collected under fasting conditions and at different times, usually between 6 and 8 h after the intake of the test food. After blood has been collected, plasma samples are obtained by centrifuging the Vacutainer™ tubes containing the EDTA-K_2 anticoagulant. After that, the extraction of the FBC metabolites from the plasma samples is usually carried out by liquid-solid extraction prior to the chromatographic analysis.

Nevertheless, for these *post-prandial* studies, the collection of blood samples has serious limitations because, in general, is necessary to insert a cannula into the vein for multiple blood sampling, resulting in intense discomfort for the volunteers.

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Additionally it requires staff qualified in blood extraction and special infrastructure where the volunteers must spend a long time. A strategy to overcome these limitations and simplify the blood sampling and sample-pretreatment procedure could be the use of dried blood spot (DBS) cards. Filter paper has been used for the collection and analysis of human blood sampling to screen newborn babies for congenital metabolic diseases for over 50 years [2]. Over the last few years, DBS sampling has been used for clinical and pre-clinical pharmacokinetic studies, taking advantage of smaller sampling needs and simplified sample collection and handling [3]. Briefly, DBS sampling involves collecting and storing a small volume of blood obtained from a human or study animal, via a simple prick (on the heel, finger, toe, or tail) or other means, on a card made of cellulose or polymer materials. Due to the ease of collection, DBS cards can even be used at home by the volunteers themselves in the human clinical or epidemiological studies [4,5]. Besides the simple sample collection, this technique allows the storage of blood samples on the card and recovery of the target compounds with an extraction solvent (sample pre-treatment for *clean-up*) prior to their chromatographic analysis. The future of this simple idea for collecting and transporting a valid biological sample seems to be unlimited.

In recent years, DBS with liquid chromatography coupled to mass spectrometry (LC-MS) has gained significant interest as a potentially powerful tool for analysing small molecules in different areas, such as therapeutic drug monitoring, toxicology and pharmaceuticals [6–8]. Recently, we developed a method using DBS cards to determine hydroxytyrosol metabolites in urine samples after a sustained intake (21 days) of phenol-enriched olive oil [9]. Due to the satisfactory results obtained, and to expand the applicability of DBS cards to bioavailability studies, the aim of this study was to develop and validate a method for detecting and quantifying hydroxytyrosol metabolites in blood samples using DBS cards combined with ultra-performance liquid chromatography coupled to tandem MS (UPLC-MS/MS). The method developed was applied to the analysis of hydroxytyrosol metabolites in human blood samples collected from three volunteers after an acute intake of an olive oil phenolic extract (OOE) at different post-intake times, from 0 to 120 min. To our knowledge, this is the first study in which DBS cards have been applied for the extraction of hydroxytyrosol metabolites in blood samples.

2. Experimentation

2.1. Chemicals and reagents

Catechol as the internal standard (IS) and hydroxytyrosol were from Sigma-Aldrich (St. Louis, MO, USA) and Seprox Biotech (Madrid, Spain), respectively. Hydroxytyrosol-3-*O*-sulphate and hydroxytyrosol acetate sulphate were kindly supplied by Dr. de la Torre (Human Pharmacology and Clinical Neurosciences Research Group, IMIM-Institut de Recerca, Hospital del Mar, Barcelona, Spain), and synthesized according to the method reported by Khymenets et al. [10]. Stock solutions of individual phenolic standard compounds were prepared by dissolving each compound in methanol at a concentration of 2000 mg/L, and storing these in dark flasks at 4 °C. Methanol (HPLC grade), acetonitrile (HPLC grade), and acetic acid were purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Ortho-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). The water was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

2.2. Blood sample collection

The olive oil phenolic extract (OOE) was prepared in our laboratory and the contents of hydroxytyrosol and the oleuropein derivatives (secoiridoids) were quantified by UPLC-MS/MS [11]. The OOE extract (2,4 g) was dissolved in 50 mL of water equivalent to a dose of 120 mg of hydroxytyrosol and oleuropein derivatives.

The protocol of the study was approved by the Ethical Committee of Human Clinical Research at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEIC-164 1326). The volunteers (three healthy adults aged between 25 and 30) gave written informed consent before starting the experiment protocol. The volunteers were asked to follow a diet free of virgin olive oil for a week before the study day. On the day of the study, each volunteer ingested the OOE extract solution (50 mL) after fasting overnight. The whole blood was taken by pricking the volunteers' fingers with disposable lancets (Unistik®, Owen

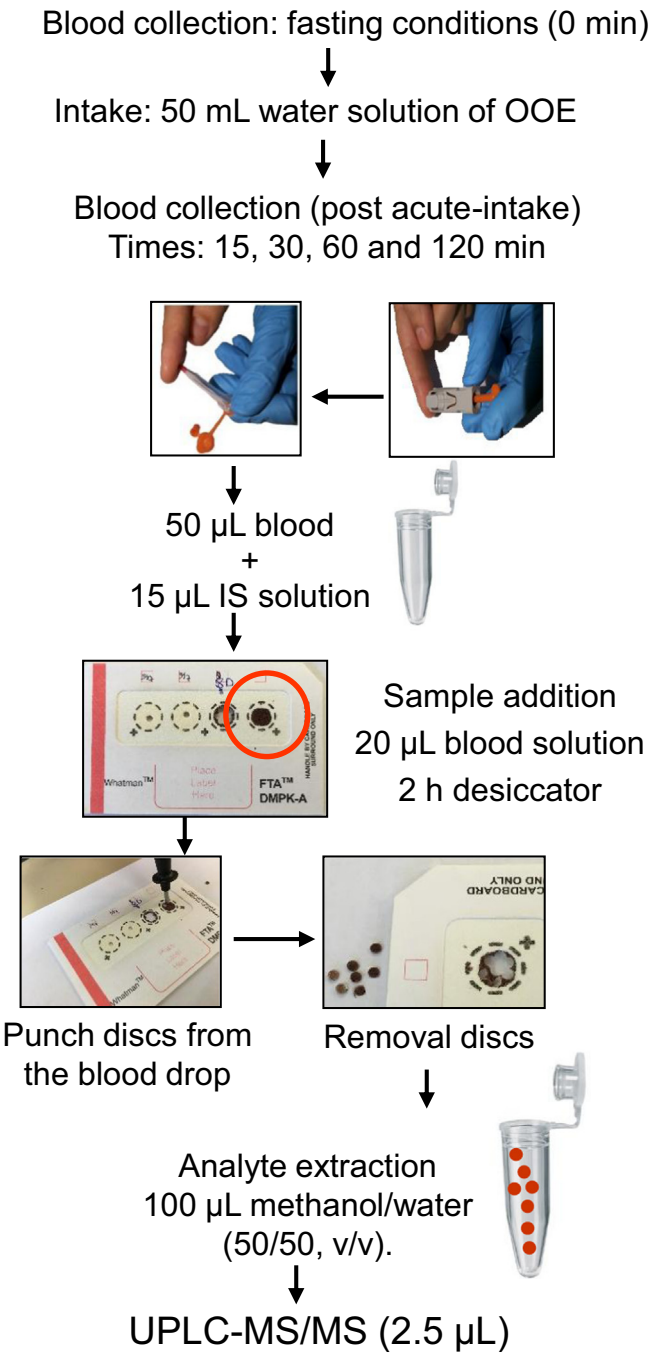

Fig. 1. Schematic procedure of the blood sampling and blood sample pre-treatment using DBS cards.