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Determination of benzophenone-3 and its main metabolites in human serum by dispersive liquid–liquid microextraction followed by liquid chromatography tandem mass spectrometry

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ABSTRACT

A new analytical method for the determination of benzophenone-3 (2-hydroxy-4-methoxybenzophenone), and its main metabolites (2,4-dihydroxybenzophenone and 2,2'-dihydroxy-4-methoxybenzophenone) in human serum is presented. The method is based on dispersive liquid–liquid microextraction (DLLME) as preconcentration and clean-up technique, followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Acidic hydrolysis and protein precipitation with HCl 6 M (1:1) (100 °C, 1 h) were carried out before extraction. The variables involved in the DLLME process were studied. Under the optimized conditions, 70 μL of acetone (disperser solvent) and 30 μL of chloroform (extraction solvent) were mixed and rapidly injected into 800 μL of hydrolyzed serum sample. Sample pH or ionic strength adjustment were not necessary. The method was validated by analyzing spiked human serum samples. No satisfactory recoveries were obtained when aqueous standards or standards prepared in synthetic serum were used, but excellent recoveries were achieved by using matrix-matched calibration standards. Moreover, limits of detection in the low μg L^{-1} level and good repeatability were obtained. In order to show the applicability of the proposed method in the study of percutaneous absorption processes, it was applied to the analysis of serum samples from two volunteers after topical application of a sunscreen cosmetic product containing 2-hydroxy-4-methoxybenzophenone.

1. Introduction

2-hydroxy-4-methoxybenzophenone (HMB), also known as benzophenone-3, has been used for many years as an UV filter in sunscreen cosmetic products, mainly due to its large molar absorptivity in both UVA and UVB ranges. Its use is allowed in the three main regulatory legislations on cosmetic products over the world [1], i.e. the European Union Cosmetics Directive (currently recast as the new Regulation on Cosmetic Products [2]), the United States Food and Drug Administration and the Japanese Pharmaceutical Affairs Law. However, the concentration of the UV filters in the final product is restricted by these legislations. Specifically, the use of HMB in the final product is allowed up to 10%, 6% and 5% (w/w), respectively [1].

UV filters are considered safe for topical application on human skin when employed in the established conditions. However, there are some studies that reveal that after dermal application of sunscreen cosmetic products, UV filters are absorbed through the skin, metabolized in the human body and finally excreted [3]. The percutaneous absorption of these compounds may result in different adverse health effects, such as photoallergy, endocrine disruption or carcinogenicity [4–8]. Specifically, HMB can be considered a potential allergen and contact photoallergen [4,9,10]. Moreover, different *in vitro* studies using human cell lines show that daily exposure to formulations containing HMB might have antiandrogenic and estrogenic activity in humans [5,7,11].

Regarding HMB metabolism, it was first proposed by Okereke et al. after oral [12] and dermal [13] administration to rats. Analysis of biological fluids and tissues revealed the formation of two phase I metabolites mainly, 2,4-dihydroxybenzophenone (DHB) and 2,2'-dihydroxy-4-methoxybenzophenone (DHMB), and a third one in a much lesser extent named 2,3,4-trihydroxybenzophenone (THB). These authors also found their corresponding phase II glucuronide conjugates.

It should be mentioned that metabolites remain in the human body longer than their parent compound, thus its adverse effects may be more important [14]. In fact, it has been shown that DHB has higher antiandrogenic activity than HMB [15] and both have similar estrogenic behavior than other well-established endocrine disruptors, such as bisphenol A, methoxychlor, endosulfan or dibutylphthalate. Moreover, they also shows synergistic effects when several UV filters are

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applied concurrently [11]. Furthermore, it has been shown that DHMB also displays estrogenic activity [16].

Studies about the pharmacokinetics of HMB showed its presence in blood stream, breast milk, semen and different tissues, being urine the major route of excretion. The literature concerning the analytical methods developed to determine this compound and its metabolites in biological fluids and tissues has been recently compiled in a review article written by some of the authors of the present work [3], to which more interested readers are referred to.

Regarding the determination of HMB, individually or together with its metabolites, in serum samples, different methods have been published, mostly based in liquid chromatography (LC), either with UV/Vis [12,13,17–25] or mass spectrometry (MS) detection [26]. Gas chromatography (GC) has been less employed due to the low volatility of these compounds. Derivatization by sylilation previous to the injection was required in this case [14].

It should be emphasized that, from a pharmacokinetic standpoint, in order to carry out a reliable determination of the target compounds, it is mandatory to hydrolyze the phase II glucuronide conjugates although only the free form of these compounds presents estrogenic activity. If not, only the free content would be determined and the conjugated fraction would be obviated. In any case, the difference between the total content (with hydrolysis step) and the free content (without hydrolysis step) results in the conjugated content. This step can be performed with HCl and heating (acidic hydrolysis) [12–14,19,20] or with β-glucuronidase/sulfatase (enzymatic hydrolysis) [26]. The acidic hydrolysis breaks the bound of the target compounds with glucuronic acid and also causes the protein denaturation, with its subsequent precipitation. In enzymatic hydrolysis, organic solvents, mainly pure acetonitrile or mixed with ethanol, need to be added to serum with the purpose of protein and enzyme precipitation [26].

Taking into account that serum is a complex matrix where the target compounds are at trace levels, the hydrolyzed serum without proteins is usually subjected to an extraction step to eliminate potentially interfering compounds and to preconcentrate the target compounds. In this sense, liquid–liquid extraction (LLE) [12–14,18] and solid phase extraction (SPE) [24–26] have been employed in some cases. However, no evidences of the use of the highly-potential microextraction techniques have been found. Among them, it should be emphasized the so-called dispersive liquid–liquid microextraction (DLLME), developed by Assadi and coworkers in 2006 [27]. The fundament of the DLLME has been explained elsewhere, as well as the advantages over the traditional extraction techniques and other microextraction techniques [27,28].

Finally, it is important to note that the *in vivo* experiments performed to determine phase I metabolites of HMB in plasma or serum has been carried out only by means of experimental animals (rats or piglets) [12–14,18,21,22]. *In vivo* studies in humans have only been carried out for the determination of the parent compound [20,23,26,29]. Taking into account that the European legislation forbids the use of experimental animals to evaluate the effectiveness or safety of cosmetic products, it is necessary to advance in the development of new analytical methods to study the processes of absorption and/or excretion of the UV filters without the use of experimental animals.

In order to contribute with the study of the percutaneous absorption of HMB, the aim of this work is to develop an analytical method to determine the total (i.e., free plus conjugated) content of HMB and its main metabolites (DHB and DHMB) in human serum, based on DLLME as microextraction technique before LC-MS/MS determination. The minor metabolite, i.e., THB, was finally excluded from this study because it was not efficiently nor repeatably extracted from serum by DLLME, probably due to its higher polarity. In any case, it has been shown that it is formed in negligible amounts in animal plasma compared to the other two main metabolites [13,14,21,22].

2. Experimental

2.1. Reagents and samples

2-Hydroxy-4-methoxybenzophenone (HMB) 98%, 2,4-dihydroxybenzophenone (DHB) 99% and 2,2'-dihydroxy-4-methoxybenzophenone (DHMB) 98% from Sigma-Aldrich (Steinheim, Germany) were used as standards. dihydroxy-4,4'-dimethoxybenzophenone (DHDMB) 98% also from Sigma-Aldrich was used as surrogate.

LC-grade absolute ethanol from Scharlab (Barcelona, Spain) was used as solvent to prepare the standard stock and working solutions. De-ionized water, obtained by means of a NANOpure II water purification system from Barnstead (Boston, USA), LC-grade methanol (MeOH) also from Scharlab and formic acid from Fluka (Steinheim, Germany) were used for the mobile phase.

Analytical reagent-grade chloroform and analytical reagent-grade acetone, both from Scharlab, were used as extraction and disperser solvent, respectively.

Analytical reagent-grade sodium chloride (NaCl) 99.5% from Scharlab was used in the study of the ionic strength.

Formic acid from Fluka and acetic acid, trichloroacetic acid, sulfuric acid, hydrochloric acid and acetonitrile, all from Scharlab, were tested to protein precipitation.

Sodium chloride, sodium di-hydrogen phosphate monohydrate from Panreac (Barcelona, Spain) and di-sodium hydrogen phosphate dodecahydrate from Scharlab were used to prepare synthetic serum.

Typical cosmetic-grade ingredients from Guinama S.L. (Valencia, Spain) such as emollients, hydrating agents, preservatives, etc. were used to prepare a laboratory-made cream, according to an adapted procedure [30].

Nitrogen (99.9%), used as nebulizing and desolvation gas in the ESI source, was provided by a high-purity generator (CLAN Tecnológica, Sevilla, Spain). Argon (99.9992%) (Carburos Metálicos, Paterna, Spain) was used as collision gas in the MS system.

Blank serum samples used for the method development and validation were obtained from different healthy volunteers who were known not to use cosmetic products containing HMB. Moreover, serum samples from other healthy volunteers who topically applied a laboratory-made sunscreen cream containing 5% of HMB were employed for method application. Each volunteer gave written informed consent to participate in this study, which was conformed to the ethical guidelines of the Declaration of Helsinki, and was approved by the Ethical Committee of the University of Valencia (Spain).

2.2. Preparation of synthetic serum

Synthetic serum was prepared by diluting NaCl (2.4 g), $NaH_2PO_4 \cdot H_2O$ (1.2 g) and Na_2HPO_4 (4.3 g) in 1 L of deionized water.

2.3. Serum samples obtention

Blood extractions were carried out into non heparinized tubes. After extraction, the tubes were centrifuged at 5000 rpm during 15 min to obtain serum. All the serum samples were stored at -20 °C in the freezer until the analysis.