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Matrix effect marker for multianalyte analysis by LC–MS/MS in biological samples

Eva Tudela, Gloria Muñoz, Jesús A. Muñoz-Guerra*

Doping Control Laboratory Madrid, State Anti-Doping Agency, El Greco s/n, ES-28040, Madrid, Spain

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ABSTRACT

Matrix effects (ion suppression/enhancement) are a well-observed phenomenon in analyses of biological matrices by high-performance liquid chromatography–mass spectrometry (LC–MS). However, few simple solutions for detecting and minimizing these adverse effects have been described so far in multianalyte analysis, especially in the field of doping control.

This study describes an exhaustive characterization of matrix effects in one hundred urine samples fortified with 41 analytes (glucocorticoids and diuretics). It introduces a novel marker to identify samples in which the reliability of the results is compromised because of acute ion suppression. This new strategy strengthens the rigor of the analysis for screening purposes. Once the matrix effect is identified, a selective sample preparation is introduced to minimize the adverse ion suppression effect. That selective extraction together with the use of a deuterated internal standard permits enhancing the ruggedness of the estimation of glucocorticoid concentration in urine.

1. Introduction

Doping control analysis permits the detection in biological matrices, like urine or blood, of the presence of the parent compounds and/or metabolites of any of the substances included in the World Anti-Doping Agency (WADA) List of prohibited substances and methods in sport. Diuretics and glucocorticoids are two of the fifteen classes of illicit compounds or methods present on this list. Diuretics increase the urinary flow and their consumption is banned in sport for two main reasons: they can be used to dilute the urine thus masking the administration of other prohibited substances; or in sports where weight categories are involved, they can help to achieve acute weight loss (Fig. 1). Glucocorticoids (Fig. 1) are included in the prohibited list due to their anti-inflammatory properties (category S9) [1]. They are prohibited only in-competition when they are administered orally, or by intravenous, intramuscular or rectal routes. To discriminate between permitted and forbidden routes of administration, WADA recommends accredited laboratories not to report any sample with an estimated concentration of glucocorticoids or their metabolites under 30 ng mL^{-1} as an adverse analytical finding [2]. It is mandatory to implement methods that permit accurate estimation of the concentration of glucocorticoids in order to report reliable results that are consistent among WADA accredited laboratories.

WADA accredited anti-doping laboratories analyze a high number of urine samples every year, for this reason, it is necessary to use generic methods of sample preparation to extract and analyze together a wide number of compounds from different therapeutic classes in order to improve time economy, laboratory productivity and reduce the volume of urine required. Frequently, laboratories use the same screening method to analyze diuretics and glucocorticoids by LC–MS/MS, due to difficulties in analyzing them by gas chromatography mass spectrometry (GC–MS).

High-performance liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) is one of the current analytical methods of choice for doping control analysis due to the ability to analyze a wide range of doping substances in biological matrices simultaneously and with a very high level of selectivity. However, in contradiction to the common perception about LC–MS/MS based methods, the selectivity obtained with selected reaction monitoring (SRM) acquisition modes has been questioned [3]. The presence of unknown and undetectable (by SRM) components in biological matrices could induce an alteration of the analyte response that could limit and compromise the reliability of the results. This alteration of the analyte response is known as matrix effects and may be reflected in an increased (ion enhancement) or a decreased (ion suppression) signal. This complex phenomenon was first reported by Kebarle and Tang in 1993 [4] and since then, the origin, the possible mechanism, and methods to eliminate or reduce the effects have been widely discussed in relation to electrospray ionization (ESI) mass spectrometry [5–9]. These effects have also been described with the use of atmospheric pressure chemical ionization (APCI), which calls into question the mechanism of this phenomenon [10–12].

There are two main techniques to assess matrix effects: post-column infusion [10] and post-extraction addition [13]. The

* Corresponding author. Tel.: +34 678312222; fax: +34 915437290.
E-mail address: jmunoz@aea.gob.es (J.A. Muñoz-Guerra).

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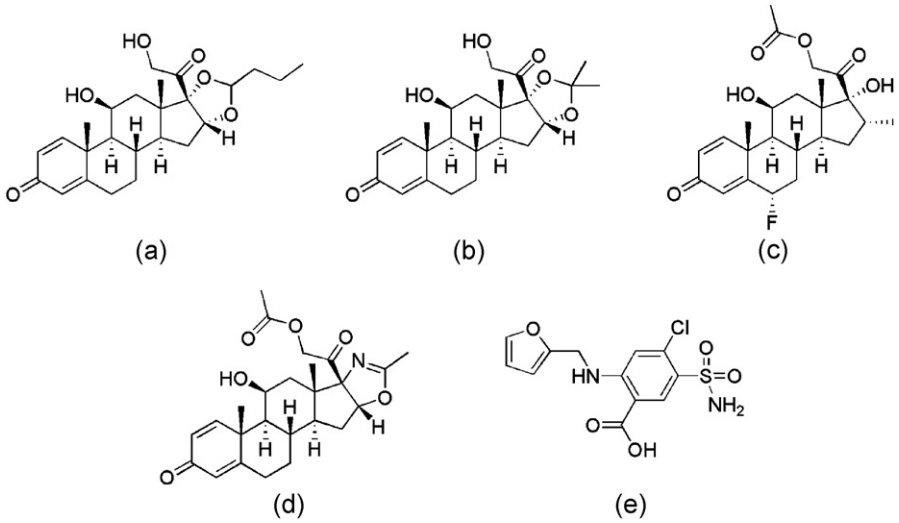

Fig. 1. Chemical structure of the glucocorticoids budesonide (a), desonide (b), paramethasone acetate (c), deflazacort (d) and the diuretic furosemide (e).

post-column infusion method involves continuous infusion of the analyte using a syringe pump connected via a "tee" at a point between the chromatographic column and the mass spectrometer ion source. Simultaneously, a blank extract sample is injected under the desired chromatographic conditions and the response of the analyte is monitored. This technique allows identifying the chromatographic area where the analyte will be influenced by matrix effects in a qualitative way. Every compound must be infused separately to evaluate matrix effects. This is a disadvantage if several analytes are determined in one method. Otherwise, post-extraction addition provides a quantitative assessment of matrix effects by comparison of the response of some analytes added to a post-extraction sample and the direct injection of the same amount of analytes in mobile phase. In order to quantify the matrix effect of several compounds analyzed in the same run, the post-extraction addition was chosen as the best technique.

In general, two approaches to counter matrix effects can be applied: to improve chromatographic separation or to modify the sample preparation. It is possible to adjust the chromatographic conditions to prevent the elution of the analytes in the region where ion suppression is observed. However, this generally involves increasing the chromatography times, and this can be very difficult when several compounds are analyzed simultaneously in the same run. For this reason, alternative sample extraction protocols based on different interaction mechanisms were studied.

In doping control, prevention of matrix effects is complicated due to the wide variety of endogenous and exogenous compounds that could be present in the urine. Athletes usually take medicines and dietary and nutritional supplements leading to high concentrations of the main components in the urinary sample. Many of these components present in the matrix may co-elute with doping substances present in the sample so that the response of these analytes could be affected.

To the best of our knowledge, very few papers have evaluated matrix effects on doping control in depth, although these phenomena have a serious impact on the sensitivity, accuracy and ruggedness of LC–MS/MS based methods and may lead to the non-detection of an existing analyte or underestimation of its concentration, with immediate consequences in terms of false negative reporting. In fact, in other analytical areas, these factors are taken into account. The Food and Drug Administration (FDA) recommend the identification of matrix effects during the validation process [14].

Herein we describe a simple method to characterize matrix effects in a multi-residue analysis. A novel marker to detect the presence of an acute ion suppression sample is introduced, avoiding a false negative. Additional experiments to overcome ion suppression and correctly estimate glucocorticoid concentration are proposed.

2. Experimental

2.1. Chemicals

Desoximethasone, fluorometholone, flunisolide, triamcinolone acetonide, triamcinolone, 6α-methylprednisolone, beclomethasone, betamethasone, budesonide, dichlorisone acetate, fludrocortisone acetate, flumethasone, fluocinolone, prednisolone, prednisone, altiazide and clopamide were purchased from Sigma–Aldrich (Madrid, Spain). Desonide and 1-dehydrocortexolone were purchased from Steraloids (Naxxar, Malta). Fluocortolone pivalate and 4-amino-6-(trifluoromethyl)benzene-1,3-disulphonamide (bendroflumethiazide impurity) were purchased from European Pharmacopeia (Strasbourg, France). Fluticasone propionate was purchased from British Pharmacopeia (London, United Kingdom). Paramethasone acetate, ethacrynic acid, bendroflumethizide, benzthiazide, bumetanide, chlorothiazide, hydrochlorothiazide, cyclothiazide, dichlorphenamide, hydroflumethiazide, indapamide, methyclothiazide, metolazone, polithiazide, quinethazone, trichlormethiazide and torasemide were purchased from United States Pharmacopeia (Basel, Switzerland). D_8-budesonide and deflazacort were purchased from Toronto Research Chemicals (Toronto, Canada). Chlortalidone, furosemide and probenecid were purchased from the World Health Organization Center for Chemical Reference Substances (Stockholm, Sweden).

Stock solutions of all compounds were individually prepared in methanol (LC grade) purchased from Scharlau (Barcelona, Spain).

The enzyme β-glucuronidase (*Escherichia coli*) was supplied by Roche Diagnostics Mannheim, Germany).

Deionized water, obtained with a Milli-Q plus apparatus Millipore (Molsheim, France) was used to prepare the mobile phase. Ammonium acetate (reagent grade) and acetonitrile (LC–MS grade) were purchased from Scharlau (Barcelona, Spain).

For the extraction procedure, formic acid (98–100%), ammonia solution 32% and *tert*-butyl methyl ether were purchased from Scharlau (Barcelona, Spain).