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Simultaneous determination of ibuprofen and its metabolites in complex equine urine matrices by GC-EI-MS in excretion study in view of doping control

Emilia Waraksa[a,b], Marzena Wójtowicz-Zawadka[c,*], Dorota Kwiatkowska[c], Anna Jarek[c], Anna Małkowska[d], Robert Wrzesień[e], Jacek Namieśnik[a]

[a] Gdańsk University of Technology, Faculty of Chemistry, Department of Analytical Chemistry, G. Narutowicz 11/12 Street, 80-233 Gdansk, Poland
[b] Institute of Sport – National Research Institute, Trylogii 2/16 Street, 01-982 Warsaw, Poland
[c] Institute of Sport – National Research Institute, Department of Anti-Doping Research, Trylogii 2/16 Street, 01-982 Warsaw, Poland
[d] Medical University of Warsaw, Department of Toxicology, Banacha 1 Street, 02-097 Warsaw, Poland
[e] Medical University of Warsaw, Central Laboratory of Experimental Animal, Banacha 1B Street, 02-097 Warsaw, Poland

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ABSTRACT

A novel assay for the simultaneous determination of ibuprofen (IBU) and its four probable metabolites, 1-hydroxyibuprofen (1-OH IBU), 2-hydroxyibuprofen (2-OH IBU), 3-hydroxyibuprofen (3-OH IBU) and carboxyibuprofen (CBX IBU) in equine urine samples with the application of Gas Chromatography-Electron Ionization-Mass Spectrometry (GC-EI-MS) has been developed and elaborated. The new approach for sample preparation including minimizing matrix effects by the application of weak cation exchange solid-phase extraction together with strong cation exchange solid-phase extraction has been applied. The GC-EI-MS method was validated to demonstrate specificity, matrix effect, linearity, limit of detection (LOD) and quantification (LOQ), precision, trueness, carry-over and stability by using the matrix-matched quality control samples. Additionally, extraction yield was evaluated. The assay achieved the LOQ of 1.75 $\mu g\,mL^{-1}$, 0.62 $\mu g\,mL^{-1}$, 4.15 $\mu g\,mL^{-1}$, 0.58 $\mu g\,mL^{-1}$ and 4.04 $\mu g\,mL^{-1}$ for IBU, 1-OH IBU, 2-OH IBU, 3-OH IBU and CBX IBU, respectively. The developed method has been successfully applied to the excretion study in horses, in which a single oral IBU dose was administered to twelve horses (mares and geldings) and equine urine samples were collected for 5 or 6 days after the drug administration. Data on the detection and determination of three IBU metabolites, 2-OH IBU, 3-OH IBU and CBX IBU in equine urine samples has been presented for the first time. The obtained results indicated the rapid excretion of IBU and its metabolites that were detectable only in the first day after the drug administration. IBU was mainly the most abundant compound detected in equine urine samples (with two exceptions in the case of samples collected from two horses, for which the highest instrumental responses were obtained for CBX IBU). The received results have indicated that two major IBU metabolites, CBX IBU and 2-OH IBU can be important markers for the IBU abuse in view of doping control in equestrian sports.

1. Introduction

Metabolic studies are of great importance in doping control field [1]. They are the basic tool in the selection of the most appropriate marker for the detection of the misuse of prohibited substances. Metabolites of prohibited substances detected in biological samples may provide additional information and evidence of drug abuse, even when prohibited substances are not detected. Therefore, a number of articles concern metabolic studies *in vivo* in humans [2–4] and in horses [5–7] in view of doping control.

The system of regulations that govern equine doping and medication control is the Equine Anti-Doping and Controlled Medication Regulations, which were implemented in conformity with the undertakings of the Fédération Equestre Internationale (FEI) governing bodies in the spirit of the World Anti-Doping Code [8]. According to the regulations, the use of substances that may influence on equine performance and health with a high potential for misuse, is contrary to the integrity of equestrian sports and the welfare of the horses. Therefore, the FEI established the Prohib-

* Corresponding author at: Institute of Sport – National Research Institute, Department of Anti-Doping Research, Trylogii 2/16 Street, 01-982 Warsaw, Poland.
E-mail address: marzena.wojtowicz@insp.waw.pl (M. Wójtowicz-Zawadka).

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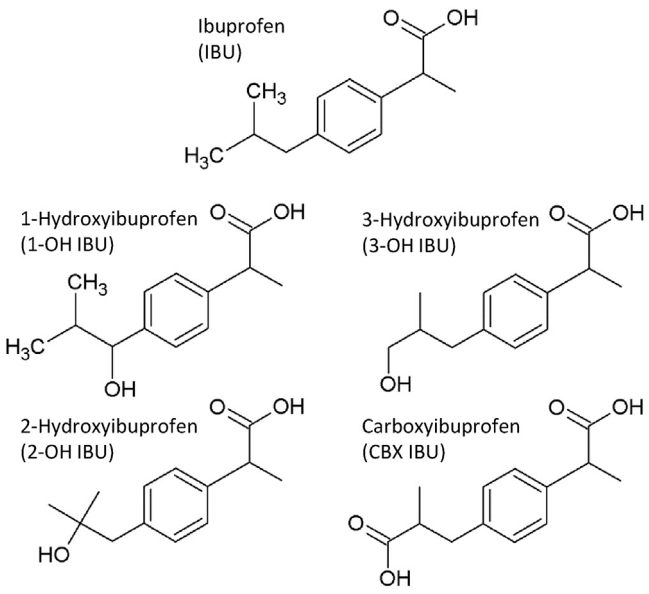

Fig. 1. Chemical structure of IBU and its probable metabolites in equine urine.

ited Substances List (the FEI List) updated each year concerning banned and controlled substances belonging to different groups of compounds, e.g. non-steroidal anti-inflammatory drugs, anabolic-androgenic steroids, stimulants and diuretics. Additionally, the FEI List is not limited to compounds that it contains, but also extends to compounds with similar chemical structure or biological effect(s) [9].

Non-steroidal anti-inflammatory drugs (NSAIDs) are widespread used in human and veterinary medicine in the treatment of variety of pain-related conditions, including arthritic and other related diseases [10,11]. Unfortunately, NSAIDs are sometimes misused in competing horses in order to hide signs of pain or inflammation [1]. NSAIDs due to their wide range of structures, therapeutic use and the need to control their use in equine sports are extensively studied group of compounds in equine samples with various composition of the matrix (e.g. plasma, serum, urine, feces, hair) [12–15]. However, equine urine is the best testing specimen for doping control because it contains the highest proportion of metabolites of doping substances and its collection is relatively non-invasive [1]. Unfortunately, equine urine samples have a highly complex matrix composition comprising salts, acids, bases, proteins, and other organic compounds, that can hinder the analytes detection [16].

Ibuprofen (IBU) is one of the most commonly used NSAID in human and veterinary medicine for the treatment of acute and chronic pain and many rheumatic and musculoskeletal disorders [10]. Unfortunately, it is sometimes misused in competing horses in order to hide injury or illness. Therefore, IBU is listed in the FEI List as a controlled substance. In the literature, several analytical methods are known for determination of IBU in human urine samples. These methods include GC–MS, HPLC-DAD, HPLC-FLD, UHPLC–MS/MS, CE-DAD, CE-ESI–MS and HPCE-UV [17–23]. Reported methods are used with various techniques as a sample preparation step, i.e. liquid–liquid extraction, solid phase extraction (SPE), continuous SPE, fiber-based liquid phase microextraction and ultrasound-assisted emulsification-microextraction. Additionally, the use of GC–MS technique required the derivatization step in the sample preparation procedure [17,18]. Although IBU has been extensively metabolically studied in humans [18–23] (taking also into account the stereoselectivity of IBU metabolism [23–26]), only few articles concern the determination of IBU profile and its pharmacokinetics in the case of horses [27,28]. In the seventies, IBU has been determined in urine and plasma samples collected from one gelding after single and fourth oral administration with the application of GC-FID after acidic extraction and silanization [27]. In the study, samples have been collected for about 24 h with the use of the catheter. In the nineties, the pharmacokinetics of IBU were determined in equine serum and urine samples after intravenous and oral administration to seven foals [28]. However, in both papers metabolites of IBU have not been investigated. In recent years, there have been no reports in the scientific literature addressing the gap in knowledge on this subject.

The development of a novel assay for the simultaneous determination of IBU and its metabolites in equine urine samples with the application of Gas Chromatography-Electron Ionization-Mass Spectrometry (GC-EI-MS) has been described in the present study. The new approach for sample preparation including minimizing matrix effects by the application of weak cation exchange (WCX) SPE together with strong cation exchange (SCX) SPE has been presented. The developed method has been successfully applied to the excretion study in horses, in which the metabolites of IBU have been detected and determined for the first time in equine urine samples. The excretion study has included the administration of IBU to twelve horses (mares and geldings) and collecting urine samples from natural urination process post administration for 5 or 6 days.

2. Experimental

2.1. Chemicals and materials

Racemic-ibuprofen (IBU), *racemic*-3-hydroxyibuprofen (3-OH IBU) and fenoprofen reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Racemic*-1-hydroxyibuprofen (1-OH IBU), *racemic*-2-hydroxyibuprofen (2-OH IBU) and *racemic*-carboxyibuprofen (CBX IBU) reference standards were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). The reference standards were of a minimum purity of 97%. The chemical structure of IBU, 1-OH IBU, 2-OH IBU, 3-OH IBU and CBX IBU is shown in Fig. 1.

Acetonitrile, diethyl ether, ethyl acetate and acetic acid (HPLC grades) were received from J.T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid (35–38%; pure p.a.), sodium hydrogen carbonate (pure p.a.), disodium hydrogen phosphate anhydrous (pure p.a.), sodium dihydrogen phosphate dihydrate (pure p.a.), sodium hydroxide (pure p.a.), sodium acetate anhydrous (pure p.a.), potassium carbonate anhydrous (pure p.a.) were purchased from POCH (Gliwice, Poland). L-cysteine, acetonitrile and methanol (hypergrade for LC–MS) were obtained from Merck KGaA (Darmstadt, Germany). Methyl iodide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium sulphate anhydrous was received from Honeywell International Inc. (Muskegon, MI, USA).

WCX SPE Supelco cartridges (3 mL, 500 mg) and SCX SPE Supelco cartridges (3 mL, 500 mg) were obtained from Sigma-Aldrich (St. Louis, MO, USA). AMBERLITE MB150 resin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Stock and working solutions

Stock solutions of IBU (1 mg mL^{-1}), 1-OH IBU (1 mg mL^{-1}), 2-OH IBU (1 mg mL^{-1}), 3-OH IBU (1 mg mL^{-1}) and CBX IBU (1 mg mL^{-1}) were prepared by dissolving 5 mg of each of six mentioned compounds in 5 mL of methanol. The working standard solutions of IBU, 1-OH IBU, 2-OH IBU, 3-OH IBU and CBX IBU were prepared by serial dilution with methanol at the concentration of 100, 10 and 1 μg mL^{-1}. A stock solution of fenoprofen (1 mg mL^{-1}) was prepared by dissolving 5 mg of fenoprofen in 5 mL of methanol and