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Determination of Non-Steroidal Anti-Inflammatory Drugs in Animal Urine Samples by Ultrasound Vortex-Assisted Dispersive Liquid–Liquid Microextraction and Gas Chromatography Coupled to Ion Trap-Mass Spectrometry

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Featured Application: The paper would like to show an easy, rapid, and affordable protocol to be used for determining four non-steroidal anti-inflammatory drugs (NSAIDs) (i.e., acetylsalicylic acid, ibuprofen, naproxen, and ketoprofen) in urine samples at trace levels. The method could be routinely used in several situations, from medicine and veterinary to doping issues.

Abstract: A low solvent consumption method for the determination of non-steroidal anti-inflammatory drugs (NSAIDs) in animal urine samples is studied. The NSAIDs were extracted with CH_2Cl_2 by the ultrasound vortex assisted dispersive liquid–liquid microextraction (USVA-DLLME) method from urine samples, previously treated with β-glucuronidase/acrylsulfatase. After centrifugation, the bottom phase of the chlorinated solvent was separated from the liquid matrix, dried with Na_2SO_4, and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) (99 + 1). After cooling at room temperature, the solution was concentrated under nitrogen flow, and 1 µL of solution was analyzed in gas chromatography/ion trap-mass spectrometry (GC-IT-MS). The enrichment factor was about 300–450 times and recoveries ranged from 94.1 to 101.2% with a relative standard deviation (RSD) of ≤4.1%. The USVA-DLLME process efficiency was not influenced by the characteristics of the real urine matrix; therefore, the analytical method characteristics were evaluated in the range 1–100 ng mL^{-1} ($R^2 \geq 0.9950$). The limits of detection (LODs) and limits of quantification (LOQs) were between 0.1 and 0.2 ng mL^{-1} with RSD ≤4.5% and between 4.1 and 4.7 ng mL^{-1} with RSD ≤3.5%, respectively, whereas inter- and intra-day precision was 3.8% and 4.5%, respectively. The proposed analytical method is reproducible, sensitive, and simple.

Keywords: non-steroidal anti-inflammatory drug (NSAID); urine; doping analysis; dispersive liquid–liquid microextraction (DLLME); gas chromatography mass spectrometry (GC-MS)

1. Introduction

Anti-inflammatory drugs, used for reducing inflammation, are of two types, i.e., cortisone-based and non-steroidal anti-inflammatory drugs (NSAIDs). The latter are, in all likelihood, the best known and most used category of anti-inflammatory drugs in therapy [1]. NSAIDs are a wide class of drugs showing anti-inflammatory, analgesic, and antipyretic action and include some of the best-known molecules used to fight pain [2]: ibuprofen, nimesulide, ketoprofen, naproxen, and diclofenac. They are

able to stop the inflammation process by their mechanism of action, i.e., interfering with the synthesis of prostanoids; molecules that play a fundamental role in these processes [3]. To do this, the NSAIDs block one or more passages of the metabolism of arachidonic acid, which is the precursor of prostaglandins [4]. Further, NSAIDs can also be used as pain relievers and antipyretics [5,6].

NSAIDs are associated with a small increase in the risk of a heart attack, stroke, or heart failure [7]. However, even in this case, the real danger depends on the type of molecule taken, the duration of the treatment, and the doses taken. Short-term use can instead trigger less serious but sometimes serious adverse effects, such as ulcers, gastric bleeding, and kidney damage [8–10]. In addition, NSAIDs can trigger allergic reactions and interfere with the activity of antihypertensive drugs [11].

Furthermore, NSAIDs are commonly used in animal medicine in different inflammatory situations (e.g., for curing musculoskeletal problems in equines) [12–14]. On the other hand, these drugs are improperly used for masking inflammation and pain of an animal, especially before horse racing. NSAIDs are substances prohibited in horse competitions and are considered one of the main doping agents [15–18]. For instance, salicylic acid, a NSAID used for the treatment of pain and fever, has an allowed threshold of 750 μg mL^{-1} in urine, or 6.5 μg mL^{-1} in plasma, for equines [19].

NSAIDs are considered safe drugs, but acute overdose or chronic abuse can give serious toxic effects [20,21]. They are weak in acid (pK_a 3–5) and some of them show short half-lives (e.g., ibuprofen 2–3 h [22]), whereas others show long half-lives (e.g., phenylbutazone residual can also be detected after 24 h [23]). A screening procedure is necessary for detecting such drugs in urine samples. Different analytical methods are present in literature, mainly based on liquid–liquid extraction (LLE) or solid-phase extraction (SPE), followed by chromatographic methods (i.e., HPLC with fluorescence detector HPLC-FLD, HPLC-diode array detection (DAD), gas chromatography mass spectrometry (GC-MS), GC-MS/MS, UHPLC-MS/MS, capillary electrophoresis CE-DAD, and CE-MS) [20,24–33]. Further, a derivatization step is necessary before the GC-MS analysis [30,31,34].

Recently, Rezaee et al. introduced the dispersive liquid–liquid microextraction (DLLME) [35]. The extraction is based on the addition of both an immiscible solvent with higher density to the aqueous sample and a dispersant solvent for increasing the contact between the two immiscible solvents. For many years, researchers have deepened this method by applying it to different matrices [36–38], especially for avoiding (at least, for reducing) the use of highly toxic chloro-solvents [39]. In this way, several protocols based on ultrasound vortex assisted DLLME (USVA-DLLME) for determining toxic compounds in foodstuffs have been investigated and set up [40–44].

The aim of this study was to develop a simple method for the simultaneous screening and confirmation of four NSAIDs, i.e., acetylsalicylic acid (ASA), ibuprofen (IBP), naproxen (NAP), and ketoprofen (KPF), in animal urine samples. The entire procedure, not previously reported in literature, starts with the extraction procedure, i.e., the USVA-DLLME method, followed by the NSAID derivatization step with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)-trimethylchlorosilane (TMCS) to form the relative trimethylsilyl (TMS) derivates: gas chromatography coupled with an ion trap-mass spectrometry detector (GC-IT-MS) has allowed us to detect the NSAID residues in real samples.

2. Materials and Methods

2.1. Materials

Ethanol, C_2Cl_2, $CHCl_3$, $C_2H_4Cl_2$, $C_2H_2Cl_4$, and acetone were of pesticide grade (Carlo Erba, Milan, Italy), whereas NaCl, acetic acid, NaOH, HCl, and Anhydrous Na_2SO_4 were of analytical grade (Carlo Erba). Standards of acetylsalicylic acid, ibuprofen, naproxen, and ketoprofen were purchased as powder from Sigma–Aldrich (Milan, Italy), whereas anthracene, used as the internal standard (IS), was provided by LabService Analytical (Anzola Emilia, Bologna, Italy). Beta-glucuronidase/arylsulfatase and BSTFA-TMCS (99 + 1) solutions were given by Sigma–Aldrich.

The solutions (1 mg mL^{-1}) of each analyte, i.e., acetylsalicylic acid, ibuprofen, naproxen, and ketoprofen (Table 1), were prepared in acetone. These solutions were further diluted for preparing final working standard solutions for spiking both the blank solutions (simulated urine samples) and real samples.

Table 1. The non-steroidal anti-inflammatory drugs (NSAIDs) investigated in this paper, with their corresponding abbreviations, Chemical Abstracts Service (CAS) number, chemical structure, molecular weight (MW), target, and qualifier ions (selected ion monitoring (SIM), abundance 100%).

Compound [a]	# CAS	Formula	MW	Target Ion [b]	Qualifier Ion [b]	II Ion [b]
ASA	50-78-2	$C_9H_8O_4$	180.16	252 $[C_{12}H_{16}O_4Si]^+$	209 $[C_{10}H_{13}O_3Si]^+$	149 $[C_8H_5O_3]^+$
IBP	15687-27-1	$C_{13}H_{18}O_2$	206.29	278 $[C_{15}H_{26}O_2Si]^+$	160 $[C_{12}H_{16}]^+$	263 $[C_{15}H_{18}O_2Si]^+$
NAP	22204-53-1	$C_{14}H_{14}O_3$	230.26	302 $[C_{17}H_{22}O_3Si]^+$	185 $[C_{13}H_{13}O]^+$	243 $[C_{14}H_{12}O_3]^+$
KPF	22071-15-4	$C_{16}H_{14}O_3$	254.28	325 $[C_{19}H_{22}O_3Si]^+$	282 $[C_{15}H_{14}O_3Si]^+$	295 $[C_{17}H_{15}O_3Si]^+$

[a] Abbreviations: acetylsalicylic acid (ASA), ibuprofen (IBP), naproxen (NAP), ketoprofen (KPF); [b] target and qualifier ions of the trimethylsilyl derivates.

The anthracene solution (1 mg mL^{-1}) was prepared in ethanol, and by further dilution the working solution was obtained. NaOH 1 M, HCl 1 M, and CH_3COOH 1 M was used to adjust the pH of the blank, and real samples were prepared with ultrapure water (resistivity 18.2 MΩ cm^{-1}) and obtained by means of a Milli-Q purification system (Millipore, Bradford, MA, USA).

2.2. Sample Preparation

2.2.1. Preparation of Simulated Urine Samples

For simulating a urine sample, an aqueous solution containing the most present components was prepared as follows: urea 14 g L^{-1}, creatinine 0.4 g L^{-1}, uric acid 0.05 g L^{-1}, glucose 0.06 g L^{-1}, mono potassium phosphate 0.2 g L^{-1}, and sodium chloride 13 g L^{-1}.

2.2.2. Preparation of Animal Urine Samples

Animal urine samples were provided by small farm owners near Campobasso (Molise, Italy). Each sample was filtered through a 0.45 μm pore size cellulose acetate filter and buffered at pH 5 with few drops of acetic acid, with the addition of a few μL of NaOH 1 M. Before performing the extraction and derivatization procedures, the animal urine samples were subjected to enzymatic hydrolysis. With total of 9 mL of sample and 100 μL of β-glucuronidase/arylsulfatase [45], the IS (5 μL of anthracene, 60 ng μL^{-1}) were incubated overnight at 37 °C.

2.2.3. USVA-DLLME and Derivatization Procedure

The extraction procedure was performed as follows: the mixture of dispersive (1 mL of acetone) and extraction (250 μL of CH_2Cl_2) solvent was injected above the sample level of the solution previously kept at room temperature at pH 3 with a few μL of HCl [46]. The solution was subjected to vortex for 1 min and ultrasounds for 2 min: This occurrence was repeated three times, followed by centrifugation for 10 min at 4000 rpm at room temperature. The organic phase was withdrawn with a micro-syringe and placed in a vial with the addition of a few grains of anhydrous sodium sulphate. A total of 50 μL of BSTFA + TMCS (99 + 1, v + v) were added [47] and the vial was closed and heated up to 50 °C for 30 min. Afterwards, the vial was cooled at room temperature and the organic phase was concentrated to a final volume of 20–50 μL under a slight nitrogen flow and 1 μL were analyzed in GC-IT-MS.

2.3. GC-IT-MS Apparatus

Analysis and data acquisition were performed using a gas chromatograph Finnigan Trace GC Ultra, equipped with an ion trap mass-spectrometry detector Polaris Q (Thermo Fisher Scientific, Waltham, MA, USA), a programmed temperature vaporizer (PTV) injector, and a PC with a chromatography station Xcalibur 1.2.4 (Thermo Fisher Scientific).

A fused-silica capillary column with a chemically bonded phase (SE-54, 5% phenyl-95% dimethylpolysiloxane) was prepared in our laboratory [48–50] with the following characteristics: 30 m × 250 μm i.d.; N (theoretical plate number) 132,000 for *n*-dodecane at 90 °C; K′, capacity factor, 7.0; d_f, (film thickness) 0.246 μm; u_{opt} (optimum linear velocity of carrier gas, hydrogen) 39.5 cm s^{-1}; utilization of theoretical efficiency (UTE) 95%. A 1 μL sample was injected into the PTV injector in the splitless mode. A total of 10 s after, the injection the vaporizer was heated from 110 °C to 290 °C at 800 °C min^{-1}; the splitter valve was opened after 120 s (split ratio 1:50). The transfer line and ion source were held at 270 °C and 250 °C, respectively. Helium (IP 5.5) was used as a carrier gas at a flow rate of 10 mL min^{-1}. The oven temperature program was as follows: 100 °C for 60 s, 10 °C min^{-1} up to 290 °C, and held for 120 s. The IT/MS was operated in the electron ionization mode (70 eV), and the analytes were qualitatively identified in the full-scan mode (*m*/*z* 100–500) and quantified in the selected ion monitoring (SIM) mode (Table 1). The quantitative analysis was performed by calibration graphs of ratio $Area_{(NSAID)}/Area_{(IS)}$ plotted versus each NSAID concentration (ng mL^{-1}). All the samples were determined in triplicate.

3. Results and Discussion

For USVA-DLLME extraction of the four investigated NSAIDs from animal urine samples, several parameters that control the optimal extraction performance were investigated and optimized using the one variable at a time method. It should be highlighted that the entire analytical methodology has been studied by means of simulated urine samples, prepared according to what reported in Section 2.2.1 and after applied to real urine samples. Simultaneously, the use of β-glucuronidase was welcome because it increased the IBP detection [33].

3.1. Parameter Optimization

The parameter optimization was addressed to find out the best analytical conditions for achieving high recoveries and accurate and precise determinations of the NSAIDs in animal urine samples. In this way, extraction solvent and volume, sample pH, and NaCl effect were deeply investigated.

First, the study dedicated its attention on the choice of organic extraction solvent. This issue plays a key role in the extraction efficiency. Chlorinated solvents are generally used because they show characteristics (higher density than water, low solubility in water) appropriate to obtaining high extraction efficiency and worthy gas chromatographic performance. Following these considerations, our attention was focused to five solvents: dichloromethane (CH_2Cl_2; d = 1.3255 g mL^{-1}), chloroform ($CHCl_3$; d = 1.4788 g mL^{-1}), carbon tetrachloride (CCl_4; d = 1.5940 g mL^{-1}), 1,2-dichloroethane ($C_2H_4Cl_2$; d = 1.2454 g mL^{-1}), and 1,1,2,2-tetrachloroethane ($C_2H_2Cl_4$; 1.5953 g mL^{-1}). Table 2 reports the results of the performance of a 300 μL volume of each solvent on simulated urine samples spiked with 20 ng mL^{-1} of each NSAID: Dichloromethane shows the best recoveries, ranging between 94.6% and 98.5% for IBP, NAP, and KPF, respectively, and 82.5% for ASA with a relative standard deviation (RSD, %) below 3.0. The recoveries are calculated as the accuracy (IS added before the extraction) [51].

The extraction recovery, defined as the percentage of the total analyte (n_0), that was extracted to the sediment phase (n_{sed}) has been determined according to the formula reported in a previous paper [36]. Over the extraction solvent choice, another quite important parameter is its volume, used to achieve the highest recoveries. The strength of the DLLME regards an extraction solvent volume as low as possible for obtaining good performance. Leong and Huang [39] highlighted that an extraction

solvent volume leads to a change in the sediment phase volume and therefore in the enrichment factors (EFs). For these reasons, the effect of different dichloromethane volumes (200, 250, 300 µL) were investigated (Table 3): a volume of 250 µL is sufficient to obtain good recoveries for all the NSAIDs, i.e., 94.2% for ASA, 100.1 for IBP, 99.8 for NAP, and 101.2 for KPF with RSDs ≤3.1.

Table 2. Effect of different extraction solvents on the NSAID recovery accuracy (%). The conditions were as follows: 9 mL of simulated urine samples spiked with NSAIDs (20 ng mL^{-1} of each), 1 mL of acetone, 300 µL of extraction solvent, and 5 µL of anthracene (I.S.; 60 ng μL^{-1}). In brackets are reported the relative standard deviations (RSDs, %); each analysis was in triplicate.

Compound	Accuracy (%)				
	CH_2Cl_2	$CHCl_3$	CCl_4	CH_2ClCH_2Cl	$CHCl_2CHCl_2$
ASA	82.5 (2.5)	79.2 (3.2)	74.1 (2.7)	76.1 (2.1)	74.2 (3.0)
IBP	94.6 (3.0)	81.7 (2.6)	82.3 (3.0)	83.2 (2.7)	85.2 (2.9)
NAP	96.8 (2.9)	83.4 (3.1)	84.5 (3.1)	85.4 (3.0)	86.3 (3.1)
KPF	98.5 (2.8)	85.2 (3.0)	86.5 (2.9)	89.1 (2.9)	86.2 (2.9)

Table 3. Effect of different volumes of CH_2Cl_2 on the NSAID recoveries (%). The conditions were as follows: 9 mL of simulated urine samples spiked with NSAIDs (20 ng mL^{-1} of each), 1 mL of acetone, different volumes of CH_2Cl_2 as extraction solvent, and 5 µL of IS (60 ng μL^{-1}). In brackets are reported the RSDs (%); each analysis was in triplicate.

Compound	Recovery (%)		
	200 µL	250 µL	300 µL
ASA	82.3 (3.1)	94.2 (2.8)	82.5 (2.5)
IBP	95.2 (2.7)	100.1 (3.1)	94.6 (3.0)
NAP	96.8 (2.9)	99.8 (2.5)	96.8 (2.9)
KPF	95.1 (3.2)	101.2 (3.0)	98.5 (2.8)

Another parameter influencing the extraction is the pH of the solution. In fact, it should be remembered that NSAIDs are weak acids. Particularly, ASA shows a pK_a of 3.5 [52], IBP of 5.3 [53], NAP of 4.14, and KPF of 4.45 [54]. Solutions of simulated urine samples at different pH were tested for studying the best acidic conditions. Table 4 evidences that the best recoveries and RSDs are obtained at pH 3: in fact, they range between 93.5 and 100.1% and between 3.4 and 4%, respectively.

Table 4. The effect of pH on the NSAID recoveries (%). The conditions were as follows: 9 mL of simulated urine samples spiked with NSAIDs (20 ng mL^{-1} of each), 1 mL of acetone, 250 µL of CH_2Cl_2, and 5 µL of IS (60 ng μL^{-1}). In brackets are reported the RSDs (%); each analysis was in triplicate.

Compound	Recovery (%)				
	pH 2	pH 3	pH 4	pH 5	pH 6
ASA	94.1 (5.6)	93.5 (3.5)	86.1 (4.0)	82.0 (4.2)	70.0 (4.3)
IBP	100.2 (3.3)	99.7 (3.4)	97.2 (3.9)	92.5 (4.1)	82.2 (4.1)
NAP	99.8 (3.8)	100.1 (3.9)	96.8 (4.1)	91.0 (4.0)	81.5 (4.0)
KPF	101.0 (3.7)	99.7(4.0)	97.2 (4.2)	90.2 (3.9)	84.7 (4.3)

Finally, the effect of different NaCl quantities on the NSAID recoveries was evaluated. Table 5 shows that the salt decreased the NSAID solubility (salting out) below and above 13 g L^{-1} concentration. Further, the decision to perform the whole study at NaCl concentration of 13 g L^{-1} was essentially due to two considerations: (1) this concentration was the average of those reported in the real urine samples, which was between 10 and 16 g L^{-1} of NaCl [55]; (2) the percentage NSAID recoveries obtained and reported in Table 5 were very similar to each other for NaCl concentrations between 10 and 15 g L^{-1}.

Finally, it should be highlighted that two other interesting parameters, such as vortex time and ultrasonication time, were extensively studied in previous papers by this group [41–44].

Table 5. Effect of different NaCl amounts on the NSAID recoveries (%). The conditions were as follows: 9 mL of simulated urine samples spiked with NSAIDs (20 ng mL^{-1} of each), pH 3, 1 mL of acetone, 250 µL of CH_2Cl_2, and 5 µL of IS (60 ng μL^{-1}). In brackets are reported the RSDs (%); each analysis was in triplicate.

Compound	Recovery (%)					
	5 g L^{-1}	**10 g L^{-1}**	**13 g L^{-1}**	**15 g L^{-1}**	**20 g L^{-1}**	**25 g L^{-1}**
ASA	80.1 (4.0)	89.5 (3.9)	93.2 (3.6)	93.8 (3.5)	85.2 (4.1)	83.5(3.9)
IBP	92.5 (3.7)	97.2 (3.5)	99.4 (3.5)	99.5 (3.7)	92.5 (3.8)	90.1 (4.0)
NAP	94.2 (4.1)	97.1 (4.2)	100.6 (3.9)	101.0 (4.0)	91.6 (4.2)	89.2 (4.1)
KPF	95.2 (3.9)	97.5 (4.0)	99.8 (3.9)	100.1 (7.8)	93.2 (4.0)	90.2 (3.8)

3.2. GC-IT-MS Method Validation

Using optimized parameters, all the analytical data were investigated. Table 6 shows the correlation coefficients (R^2) in the range 1–100 µg L^{-1}, along with the limits of detection (LODs) and limits of quantification (LOQs), repeatability (as intra-day precision) and reproducibility (as inter-day precision), and EFs of each NSAID considered. LODs and LOQs were determined according to Knoll's definition [56,57], i.e., an analyte concentration that produces a chromatographic peak equal to three times (LOD) and seven times (LOQ) the standard deviation of the baseline noise. All the compounds show a good linearity in the investigated range (≥0.995) and LODs and LOQs between 0.1–0.2 µg L^{-1} and 4.1–4.7 µg L^{-1}, respectively, with high intra- and inter-day precision (≤3.8 and ≤4.5, respectively). The EFs, defined as the ratio between the analyte concentration in the sediment phase (C_{sed}) and the initial analyte concentration (C_0) in the sample ($EF = C_{sed}/C_0$) [35], were also studied, ranging between 350–450.

Table 6. Correlation coefficients (R^2) calculated in the range 1–100 µg L^{-1}, limit of detection (LOD; µg L^{-1}) and limit of quantification (LOQ; µg L^{-1}) and inter- and intra-day precision (expressed as RSD, %) of each NSAID determined by GC-IT-MS.

Compound	R^2	LOD	LOQ	Intra-day	Inter-day	EF
ASA	0.9950	0.2	4.1	3.8	4.5	350
IBP	0.9972	0.1	4.7	3.2	4.0	450
NAP	0.9987	0.1	4.7	3.5	4.3	385
KPF	0.9981	0.1	4.5	3.3	4.2	412

Finally, for a complete analytical methodology evaluation, the recoveries have been studied in the investigated matrices, i.e., animal urine, at two different spiked NSAID concentrations (20 ng mL^{-1} and 50 ng mL^{-1}). Table 7 shows these data: recoveries in animal urine samples between 93.8 and 102 with RSDs ≤3.2.

Table 7. Average NSAID recoveries (%) obtained at different spiking concentrations on real urine samples. In brackets are reported the RSDs (%); each analysis was in triplicate.

Compound	Recovery (%)	
	Animal Urine [a]	
	20 ng mL^{-1}	50 ng mL^{-1}
ASA	93.8 (3.2)	94.3 (2.9)
IBP	99.8 (3.0)	100.2 (3.0)
NAP	101.0 (3.2)	102.0 (3.1)
KPF	100.2 (2.9)	99.8 (3.2)

[a] Goat urine sample.

Finally, Table 8 shows a comparison among different methods present in literature [58–63] for analyzing NSAIDs. The extraction methods were different: three papers were based on hollow-fiber liquid microextraction [59,60,62], whereas two papers were on rotating disk sorptive [63] and liquid–liquid extraction [61]. According to the analytical techniques, three studies used HPLC with ultraviolet (UV) [58,61,63] and one the diode array detection (DAD) [59], one used the ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) [62], and one used GC equipped with a flame ionization detector (FID) [60]. Looking at the comparison among the different studies with parameters developed in this study, the main advantages regard LODs and LOQs, recoveries, and RSDs, whereas EFs are good except those reported by Payan et al. [59]. On the other hand, the whole procedure can be routinely applied and does not require particular technology, such as the use of rotating disks or hollow fiber.

Table 8. Linear range (LR, μg mL^{-1}), volume of extraction solvent (Ex. Solv., μL), extraction time (Ex. Tm., min), enrichment factor (EF), limit of detection (LOD, μg L^{-1}), limit of quantification (LOQ, μg L^{-1}), recovery (%), and relative standard deviation (RSD, %) of each NSAID investigated in this study and relative comparison with similar studies present in literature.

Method	Matrix	Analyte	LR	Ex. Solv.	Ex. Tm.	EF	LOD	LOQ	Recovery	RSD	Ref.
LLE-HPLC-UV [1]	human plasma	KPF, NAP, FPC [7], IBP, DIC [7]	100–100,000	600	n.r. [8]	n.r. [8]	11.5–75	n.r. [8]	97.1–146.6	<10.1	[58]
HF-LPME-HPLC-DAD (or FLD) [2]	human urine	DIC [7], ASA, IBP	41–10,000	50	15	70–1010	12.3–52.9	n.r. [8]	82.3–99	<1.8	[59]
HFLM-SPME-GC-FID [3]	human urine	IBP, NAP, DIC [7]	0.08–400	6	80	46.5–60.5	0.03–0.07	0.08–0.1	80.2–98.5	<12.1	[60]
SPE-SUPRASF-HPLC-UV [4]	human urine, water	DIC [7], MFA [7]	10–300	1500	25	431–489	0.4–7.0	n.r. [8]	90.4–103.8	<6.2	[61]
HF-LPME-UPLC-(MS/MS) [5]	water, juice, soda, energy drink	ASA, IBP, NAP, DIC [7]	1–5000	15	30	195–350	0.5–1.25	2.0–5.0	87.9–115.2	<12.0	[62]
RDSE-HPLC-UV [6]	human urine	DIC [7], IBP, KPF, NAP	200–2000	200	20	15–18	21.7–44.0	72.4–146.6	100–110	<12.0	[63]
USVA-DLLME-GC-IT-MS	animal urine	ASA, IBP, NAP, KPF	1–100	250	19	300–450	0.1–0.2	4.1–4.7	94.1–101	<4.1	This study

[1] Liquid–liquid extraction-high performance liquid chromatography-ultraviolet (LLE-HPLC-UV) detector; [2] hollow-fiber liquid-phase microextraction-high performance liquid chromatography-diode-array detection; [3] hollow-fiber liquid membrane-protected solid-phase microextraction-gas chromatography flame ionization detector; [4] solid phase extraction combined with supramolecular solvent-high performance liquid chromatography-UV; [5] hollow-fiber liquid-phase microextraction-ultra performance liquid chromatography-tandem mass spectrometry; [6] rotating disk sorptive extraction HPLC-UV; [7] diclofenac (DIC), fenoprofen (FPC), mefenamic acid (MFA); [8] not reported.

3.3. Application to Real Animal Urine Samples

Using the entire analytical USVA-DLLME-GC-IT-MS protocol previously developed (briefly resuming: 9 mL of simulated urine sample solution at pH 3 containing 5 µL of I.S., 60 ng mL^{-1}, addition of 1 mL of acetone and of 250 µL dichloromethane as extraction solvent, three times of 1 min vortex and 2 min ultrasounds, centrifugation for 10 min at 4000 rpm, 1 µL injection into GC-IT-MS), some animal urine samples have been analyzed, particularly three animal urine samples, i.e., two from goats and one from a sheep. All the subjects were healthy. No residues (i.e., levels below the LODs) were found in all the samples. The analysis allows us to investigate the presence of such compounds at trace levels in these matrices, but it does not furnish evidence as to whether there was a previous assumption of such molecules. As an example are shown in Figure 1, the gas chromatograms in SIM mode of a simulated sample of urine (a) and one of goat urine sample (b) both additions with 30 ng mL−1 of each NSAID. The peaks are well-solved and the determinations are precise and accurate.

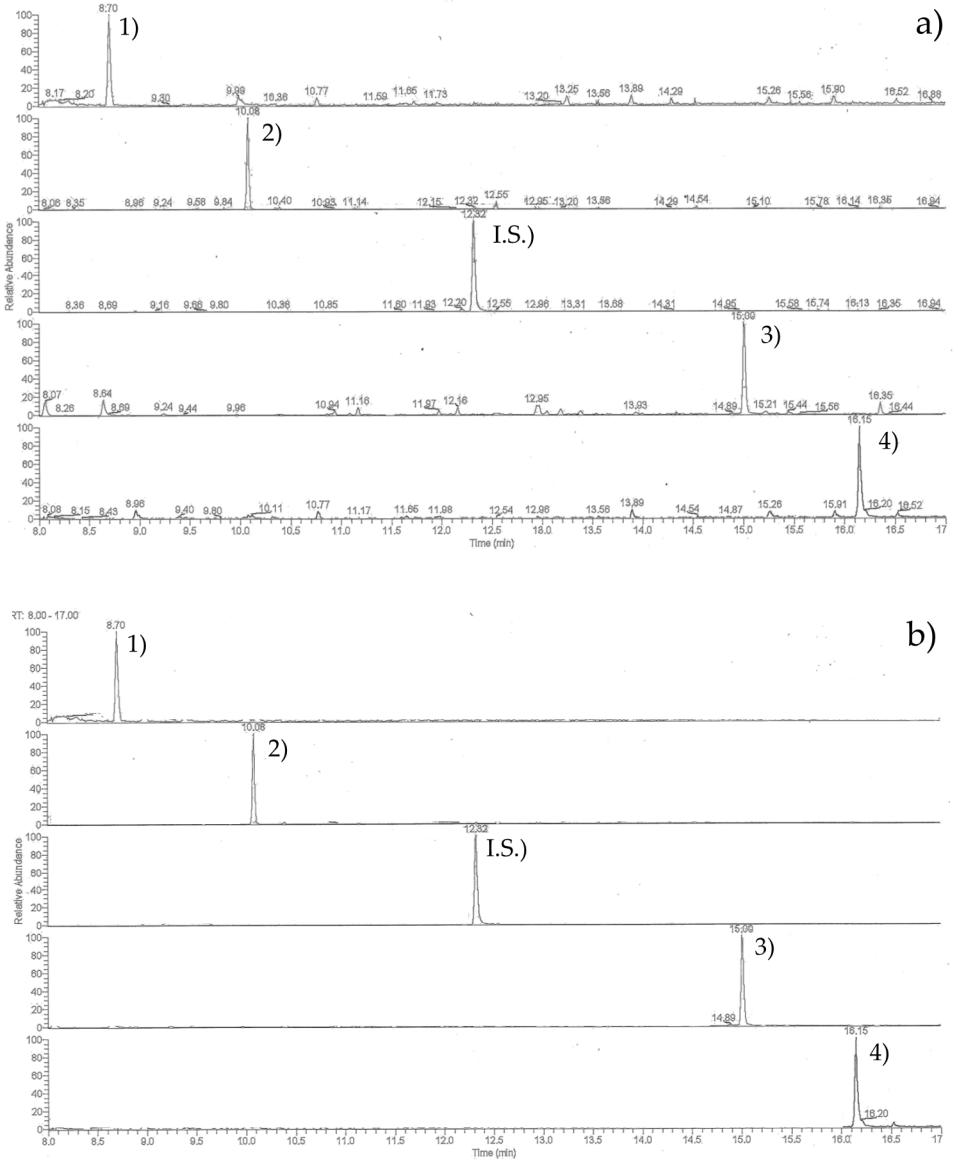

Figure 1. Gas chromatography/ion trap-mass spectrometry (GC-IT-MS) chromatograms in SIM mode of (**a**) simulated urine and (**b**) goat urine samples, both spiked with 30 ng mL^{-1} of each NSAID. For experimental conditions, see text. Peak list: 1. acetylsalicylic acid; 2. ibuprofen; internal standard (IS); 3. naproxen; 4. ketoprofen.

4. Conclusions

This paper highlights an affordable method for analyzing NSAIDs in animal urine samples. The method used for the animal urine samples in this study can also be applied to human urine samples, as a lead on to the discussion about athletes. In fact, athletes often make excessive use of anti-inflammatories in order to compete, even in less than optimal physical conditions. Many athletes take NSAIDs to compete or even simply train, even in the presence of pain, joint inflammation, trauma etc. Incorrect use of these drugs can lead to serious damage to health. Further, with regards to "premedication" in the sports field, it should be highlighted that the NSAIDs are not among the substances prohibited by the anti-doping measures and are therefore only drugs at risk of easy inappropriate abuse. Equine doping can also be defined as "the use of any exogenous agent (pharmacological, endocrinological, hematological, etc.) or clinical manipulation, which, in the absence of suitable and necessary therapeutic indications, is aimed to improve performance, outside the adjustments induced by training. In this view, this paper shows a simple, rapid, and sensitive method for determining four NSAIDs in animal urine samples. The very low LODs and LOQs and the high precision reached by means of a modified DLLME method coupled with GC-IT-MS allow us to apply the entire procedure to routine screening and monitoring of such compounds in doping cases or other similar situations.

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