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Determination of non-steroidal anti-inflammatory drugs residues in animal muscles by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A confirmatory method for the determination of residues of nine non-steroidal anti-inflammatory drugs and one metabolite in animal muscles has been developed. After enzymatic hydrolysis samples were extracted with acetonitrile and cleaned up using alumina and C_{18} SPE cartridges. Liquid chromatography–tandem mass spectrometry was used for the separation and determination of analytes. The method was validated in bovine muscles, according to the Commission Decision 2002/657/EC criteria. Applicability of the method in the analysis of swine, horse and chicken muscles was checked by precision and recovery experiment. The influence of matrix effect on the quantification of non-steroidal anti-inflammatory drugs residues was investigated. The method was used for the confirmation of phenylbutazone and oxyphenbutazone in horse muscle sample.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used both in human and in veterinary medicine because of their ability to reduce inflammatory process. In veterinary practice NSAIDs are used in the treatment of musculoskeletal disorders, coliform mastitis, pulmonary diseases and enteritis in several animal species. NSAIDs, however, because of their toxicity, can affect the gastro-intestinal, hematopoietic and renal systems. Gastro-intestinal ulcerations are the most common and serious side effect of NSAIDs, especially in cases of overdose or chronic abuse.

NSAIDs are also authorized for food producing animals. The widespread use of these drugs presents the potential risk for the consumers if food containing residues enters the food chain. For this reason there is a need for the control of residues and development of methods to monitor their compliance with legislation [1].

Multitude of analytes and low limits of NSAIDs residues in the tissues (Table 1) make LC–MS/MS the preferable technique for both screening and confirmatory purposes. Despite using sensitive analytical instrument, the proper pre-treatment of the sample is necessary to obtain reliable results. Therefore, the development of multi-residue procedure for drugs differing in chemical properties is the main challenge in determination of NSAIDs residues in tissues.

Many articles describing the methods for the determination of NSAIDs in biological matrices (mainly plasma) [2,3] exist, but only few procedures have been published concerning residues of these drugs in food of animal origin. Single-residue methods were published [4,5] but do not fulfil criteria of practicability. Recently, multi-residue methods for NSAIDs residues in milk [6] and kidneys [7] were published. Van Hoff et al. published LC–MS^2 method for the determination of acetylsalicylic acid, flunixin, meloxicam, phenylbutazone, tolfenamic acid and ketoprofen in bovine muscle tissues [8]. Igualada published the procedure devoted to analysis of muscle and liver samples, allowing to determine flunixin, meloxicam, carprofen and tolfenamic acid [9]. The latest trend is involving NSAIDs in the multi-class methods [10,11]. The main disadvantage of these procedures is a narrow range of the analytes and/or sometimes impropriate (high) levels of analytes in the spiked samples in method validation. Stoyke et al. published comprehensive method for determination of 14 of NSAIDs in bovine muscle, liver and kidney [12]. Our experience with this method, especially problems with matrix effects and degradation of phenylbutazone induced us to modify this method.

In this paper, we present a multi-residue confirmatory method which allows to determine residues of NSAIDs with established MRL value (flunixin, diclofenac, meloxicam, carprofen and tolfenamic acid) and non-MRL substances (mefenamic acid, phenylbutazone, oxyphenbutazone, naproxen and ketoprofen) in animal muscles.

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Table 1
Maximum residue limits [19] and recommended concentrations (RC, proposed by Community Reference Laboratory [16] for NSAIDs in animal muscles.

Pharmacologically active substance	MRL/recommended concentration (RC) ($\mu g\,kg^{-1}$)			
	Bovine	Porcine	Equine	Chicken
Carprofen [CPF][a]	500	Not established	500	Not established
Diclofenac [DC]	5	5	Not established	Not established
Flunixin [FLU]	20	50	10	Not established
Ketoprofen [KET]	No MRL required			
Mefenamic acid [MEF]	10 (RC)	10 (RC)	10 (RC)	10 (RC)
Meloxicam [MEL]	20	20	20	Not established
Naproxen [NAP]	10 (RC)	10 (RC)	10 (RC)	10 (RC)
Oxyphenbutazone [OPB]	5 (RC)	5 (RC)	5 (RC)	5 (RC)
Phenylbutazone [PBZ]	5 (RC)	5 (RC)	5 (RC)	5 (RC)
Tolfenamic acid [TOL]	50	50	Not established	Not established

[a] Marker residue expressed as sum of carprofen and carprofen glucuronide conjugate.

2. Experimental

2.1. Chemicals and standards

Acetonitrile, acetic acid, ethyl acetate, n-hexane, methanol, all HPLC grade and SPE columns C_{18} (500 mg, 6 mL) were obtained from J.T. Baker, Germany. Sodium acetate, puriss p.a. was purchased from POCh, Poland. β-Glucuronidase from Helix pomatia, formic acid, LC–MS grade and ascorbic acid were from Sigma–Aldrich, USA. SPE cartridges SepPak Alumina N ("Plus long" format, 1710 mg, 1.2 mL) were from Waters, USA. Syringe filters (Puradisc – PTFE 0.45 μm) were from Whatman, UK. Ultrapure water (resistance >18 mΩ) was obtained from Milli-Q system (Millipore, France).

The analytical standards were supplied by the following manufacturers: carprofen (CPF), diclofenac (DC), flunixin meglumine (FLU), flunixin-d3 (FLU-d3), ketoprofen (KET), mefenamic acid (MEF), meloxicam sodium (MEL), meloxicam-d3 (MEL-d3), naproxen (NAP), tolfenamic acid (TOL) – Sigma, USA; phenylbutazone (PBZ) and oxyphenbutazone monohydrate (OPB) – LGC Promochem, Germany; diclofenac-d4 (DC-d4) and carprofen-d3 (CPF-d3) – Toronto Research Chemicals Inc., Canada; phenylbutazone-d10 (PBZ-d10) – Cambridge Isotopes Laboratories, USA; tolfenamic acid-d4 (TOL-d4) – QChem, Germany. TOL, TOL-d4 and MEF were gifted by the Community Reference Laboratory for Drug Residues in Berlin.

2.2. Preparation of standard solutions

Stock standard solutions (1000 $\mu g\,mL^{-1}$) were prepared by weighing of 10.0 mg of each analyte and dissolving in 10.0 mL of methanol (stable for 12 months). Working standard solutions (100 and 10 $\mu g\,mL^{-1}$) were prepared by the dilution of the proper solutions with methanol and were stable for 6 months.

Two mixed standard solutions were prepared by the dilution of suitable aliquots of working standard solutions (100 or 10 $\mu g\,mL^{-1}$) and used for bovine muscle samples spiked on three levels (Table 2). The first one contained 0.5 $\mu g\,mL^{-1}$ PBZ and OPB and was stable for 1 month. The second one contained 25 $\mu g\,mL^{-1}$ CPF, 2.5 $\mu g\,mL^{-1}$ TOL, 1.0 $\mu g\,mL^{-1}$ FLU and MEL, 0.5 $\mu g\,mL^{-1}$ KET, MEF, NAP and 0.25 $\mu g\,mL^{-1}$ DC and was stable up to 6 months. The mixed solution of internal standards (IS) contained 2.0 $\mu g\,mL^{-1}$ of each IS (CPF-d3, DC-d4, FLU-d3, MEL-d3, PBZ-d10, TOL-d4) and was stable for 6 months. Mixed standards solutions used for spiking swine, horse and chicken muscle samples were prepared to obtain spike levels described in Table 3. All standards solutions were stored at 2–10 °C. Stability of the standards solutions was based on the results of the stability tests performed by the Community Reference Laboratory [13].

2.3. Equipment

In sample preparation vortex mixer (IKA, Germany), rotary evaporator (Heidolph, Germany), nitrogen evaporator (VLM, Germany), SPE system (J.T. Baker, Germany) and laboratory centrifuge (Sigma, Germany) were used. The analysis was performed using 1100 liquid chromatograph (Agilent Technologies, Germany) connected to triple-quadrupole API 3000 mass spectrometer (Applied Biosystems Sciex, Canada), controlled by Analyst 1.5 software.

2.4. Sample preparation

2 ± 0.01 g of previously minced muscles were weighed into 50 mL polypropylene centrifuge tube. 20 μL of IS solution was added, the sample was vortex-mixed and allowed to rest for 10 min. Then, 4 mL of acetate buffer (100 mL of buffer contained 2.7 g of sodium acetate and 0.17 g of ascorbic acid, pH 4.5 was adjusted with acetic acid) and 50 μL of β-glucuronidase were added. The sample was mixed and incubated for 1 h at the temperature of 37 °C. After cooling the sample, 10 mL of acetonitrile was added, the sample was vigorously mixed and centrifuged (4120 × *g*, 10 min). The supernatant was transferred into clean 50 mL polypropylene tube. The extraction was repeated with a 5 mL portion of acetonitrile and the sample was centrifuged (4120 × *g*, 10 min). The combined extracts (ca. 19 mL) were passed through Sep Pak Alumina N cartridge and collected entirely. The eluate was transferred to round-bottom flask and evaporated to ca. 4 mL in the rotary evaporator (50 °C). The extract was loaded on the C_{18} cartridge preconditioned with 2 mL of methanol and 2 mL of 0.02 M ascorbic acid. The round-bottom flask was washed with two 5 mL portions of 0.02 M ascorbic acid and both portions were loaded on the column. The cartridge was then washed with 2 mL of 0.02 M ascorbic acid and 2 mL of water and dried under vacuum for 45 min. The cartridge was washed with 1 mL of n-hexane and vacuum dried for 2 min. The analytes were eluted from the column with 4 mL of the mixture of n-hexane and ethyl acetate (1:1, v/v) and evaporated to dryness (N_2, 50 °C). The dry residue was reconstituted in 0.2 mL of acetonitrile: 0.1% formic acid (30:70, v/v), filtered through syringe filters and transferred into the vial.

2.5. Optimisation of mass spectrometry parameters

Influence of different mobile phases (MeOH/ACN: 0.1% formic acid, MeOH/ACN: 0.1% acetic acid, MeOH/ACN: 0.05 M ammonium acetate solution pH 4.0, MeOH/ACN: 0.05 M ammonium formate solution pH 4.0) on analytes' signal intensity in the electrospray ionisation was tested. 5 μL of standard solution (2.2) was injected on the LC system, which pumped a mobile phase and was connected directly (without the HPLC column) to mass spectrometer.