Multi-Residue Determination of Non-Steroidal Anti-Inflammatory Drug Residues in Animal Serum and Plasma by HPLC and Photo-Diode Array Detection

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Abstract

The European Union regulated the use of non-steroidal anti-inflammatory drugs (NSAIDs) in animal production and set the official analytical controls to detect their residues in plasma, serum, and milk within the frame of national monitoring programs in each member state. In this work, a multi-residue reversed-phase high-performance liquid chromatography with diode array detector (DAD) method is described for the simultaneous determination of 13 NSAIDs in serum and plasma of farm animals. Chromatographic separation by a C12 stationary phase column with a linear gradient is able to resolve all the compounds considered: salicylic acid, ketoprofen, flurbiprofen, phenylbutazone and its metabolite (oxyphenbutazone), carprofen, ibuprofen, naproxen, niflumic acid, suxibutazone, diclofenac, mefenamic acid, and tolfenamic acid. These compounds are chosen as the most representative of the different NSAID chemical sub-classes. The DAD analysis allows the confirmation of all drugs on the basis of their own UV–vis spectrum, according to the requirements of the European Council Decision 2002/657/EC. Moreover, the method is in-house validated, evaluating mean recoveries, specificity, repeatability, and within-laboratory reproducibility as the performance parameters required by the Decision. The results of this study indicate the method is specific and repeatable, with the mean percentage recoveries of the drugs ranging between 72.5% and 104.5%. Only salicylic acid has poor recovery, with results ranging between 36.3% and 54.9%.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for therapeutic aims in veterinary medicine to treat inflammatory processes and the related clinical effects (1), but they are also used to treat some bovine respiratory diseases in conjunction with antibiotics (2). Their routine or prolonged use can cause several toxic side effects, such as gastrointestinal bleeding, intestinal ulceration, aplastic anaemia, inhibition of platelet aggregation, and changes in renal function (3). Besides for therapeutic purposes, NSAIDs are used in animal productions for their secondary pharmacological effects and to improve some quality characteristics of meats. As a matter of fact, when high doses of NSAIDs are orally supplied to farm animals close to slaughtering, platelet aggregation is inhibited and a rapid bleeding after slaughter occurs. This causes the pale look of pig and calf meats, which relevant in some cases as quality parameter consumers find appealing. Furthermore, they can reduce lypogenesis and, consequently, edible fat (4). Moreover, it is well known that NSAIDs are used as a doping agent in racing horses to improve performance and disguise pathologies of the muscle-skeletal apparatus (5). However, they have been prohibited by many equine governing bodies (6). In the USA, a survey in 1992 proved that phenylbutazone, licensed for treating inflammatory diseases in dogs and horses (7,8), was misused by veterinarians for the treatment of dairy and beef cattle (9). But long-term exposure to phenylbutazone has proven to induce kidney tumors in rats and liver tumors in mice (10). Because of their good oral availability, the involuntary intake of NSAIDs as residues in foods could pose a health risk to consumers, the possible side effects are already described for the correspondent pharmaceutical preparations. Both side effects of NSAIDs and their misuse in food-producing animals induced the European Commission to monitor the presence of their residues in the frame of the surveillance programs carried out by the member states. For many NSAIDs, a maximum residue level (MRL) tolerable in foods has been established by the European Commission through the Council Regulation 2377/90/EEC (11) and its amendment (12). Most of the NSAIDS (carprofen, diclofenac, tolfenamic acid, vedaprofen, and flunixin) are in Annex I of the Regulation, which sets provisional MRLs for different animal species and target matrices such as liver, muscle, kidney, and fat. Ketoprofen, salicylic acid, and salicylates, as well as acetylsalicylic acid and acetylsalicylates, are in Annex II of the Regulation and are allowed substances only for some species and applications,

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excluding animals producing milk and eggs for human consumption. It should be noted that no MRLs are established for phenylbutazone, flurbiprofen, ibuprofen, suxibutazone, meclofenamic acid, and mefenamic acid, which are considered to be prohibited substances. Besides, the use of carprofen and diclofenac is prohibited for milk-producing animals. As a consequence, the European Commission recommended stringent control of these substances in food-producing animals (4) because a considerable amount of NSAIDs can be presumed to be used, and teratogenic and carcinogenic potential activities by some of them cannot be excluded. To this aim, plasma, urine, and milk have been indicated as the target matrices. It is noteworthy that NSAIDs are a heterogeneous group of acidic drugs that can be divided into the following main four sub-classes: (i) salicylic acid derivatives; (ii) propionic acid derivatives; (iii) pyrazole derivatives; and (iv) aniline derivatives, including both anthranilic acid and nicotinic acid derivatives. These compounds share common pharmacological activity, but they are not structurally related, making it difficult to develop a multi-residue method for their determination. Several methods for NSAID determination in tissue, milk, urine, and plasma, both by high-performance liquid chromatography (HPLC) (13–15) and liquid chromatography–mass spectrometry (16–21), regarding just one or a few molecules have been published. This work describes a multi-residue method to determine 13 NSAIDs belonging to different sub-classes. The NSAIDs determined are salicylic acid (usually considered as the drug from which all NSAIDs can be formally derived), ketoprofen, carprofen, flurbiprofen, ibuprofen, naproxen (as arylpropionic acid derivatives), suxibutazone, phenylbutazone and its metabolite (oxyphenbutazone) (as pyrazolidinedione derivatives), diclofenac, mefenamic acid and tolfenamic acid (as anthranilic derivatives), and niflumic acid (as nicotinic acid derivative). The method was developed by introducing slight changes in the clean up procedure by Gowik et al. (14), and it was based on a liquid extraction from samples and subsequent C18 solid-phase extraction (SPE) purification, followed by the HPLC separation of the 13 analytes on a C12 reversed-phase column, with identification and quantitation by a diode array detector (DAD). The method was in-house validated above and below the action limit of 0.100 µg/mL, as indicated in the Italian National Residue Monitoring Programme by evaluating specificity and calculating mean recoveries, repeatability, and within laboratory reproducibility; then, the method was applied to the analysis of bovine, pig, and equine plasma and serum samples.

Experimental

Reference materials

Salicylic acid was supplied by Carlo Erba (Milan, Italy); carprofen was from Pfizer Limited (Walton Oaks, Surrey, UK); ketoprofen, phenylbutazone, oxyphenbutazone, flurbiprofen, ibuprofen, naproxen, niflumic acid, diclofenac, and tolfenamic acid were supplied by Sigma-Aldrich, (Milan, Italy); suxibutazone and mefenamic acid were from the European Reference Laboratory for Residues of Veterinary Drugs in Berlin. All the reference materials were analytical grade purity.

Chemicals

Phosphoric acid (85%, w/v), hydrochloric acid (37%, w/v), diethyl ether (Carlo Erba, Milan, Italy), and ascorbic acid (Merck, Darmstadt, Germany) were all analytical-grade reagents. HPLC-grade acetonitrile, methanol, and n-hexane were supplied by J.T. Baker (Mallinckrodt Baker B.V., Deventer, Holland). HPLC-grade water was produced by a laboratory MilliQ system (Millipore, Bedford, MA).

SPE columns

End-capped C18 SPE cartridges with 1 g sorbent content and 6 mL reservoir volume were from Isolute (Step-Bio, Bologna, Italy).

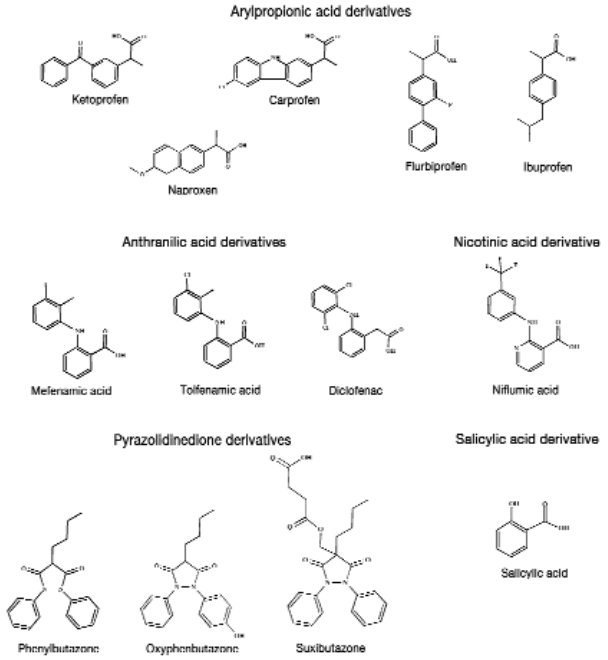

Figure 1. The molecular structures of the 13 NSAIDs studied.

Table I. The Retention Times and Absorbance Relative Maximum Wavelengths of the NSAIDs Separated by Reversed-Phase HPLC

NSAID	Retention time (min)	Absorbance relative maximum wavelength (nm)
Salicylic acid	8.79	304
Ketoprofen	15.95	256
Naproxen	16.24	232
Oxyphenbutazone	16.46	239
Carprofen	20.28	239
Flurbiprofen	20.75	246
Suxibutazone	21.09	237
Niflumic acid	21.85	285
Diclofenac	22.39	275
Ibuprofen	23.09	230
Phenylbutazone	24.63	240
Mefenamic acid	25.89	279
Tolfenamic acid	27.31	285

Apparatus

An SPE vacuum manifold system (Waters, Milford, MA) was used.

HPLC–DAD analysis

HPLC analysis was performed by a Waters system, equipped with a 600E quaternary pump, a 717 Plus autosampler, and a 2996 photodiode array detector (Waters, Milford, MA). Chromatographic separation was performed injecting 50 µL sample volume on a 4-µm particle 250- × 4.6-mm Max-RP 80 Å Synergi stainless steel column (Phenomenex, Torrance, CA), at a 1.2 mL/min flow rate, using 0.010 mol/L o-phosphoric acid as mobile phase A and acetonitrile as mobile phase B. A linear gradient was carried out for elution, according to the following program: from 35% B at time 0 to 75% B in 25 min, then to 100% B in 5 min, held for 5 min, and finally to 35% B in 3 min. The UV–vis spectra were acquired in the range 220–350 nm, setting 230 and 254 nm as the monitoring wavelengths. Quantitative analysis was carried out by interpolation of the external standard calibration curves, calculated daily by linear regression of peak area versus standard solution concentrations, in the linear range from 0.25 to 2.0 µg/mL of each NSAID tested. During each analytical session, a blank reagent and blank and spiked samples of bovine, pig, or equine plasma or serum were analyzed. The spiked concentrations were 0.050, 0.100, and 0.150 µg/mL, corresponding, respectively, to 0.5, 1.0, and 1.5 fold the action limit, apart for salicylic acid, which was added at 0.100, 0.200, and 0.300 µg/mL of serum or plasma (or both).

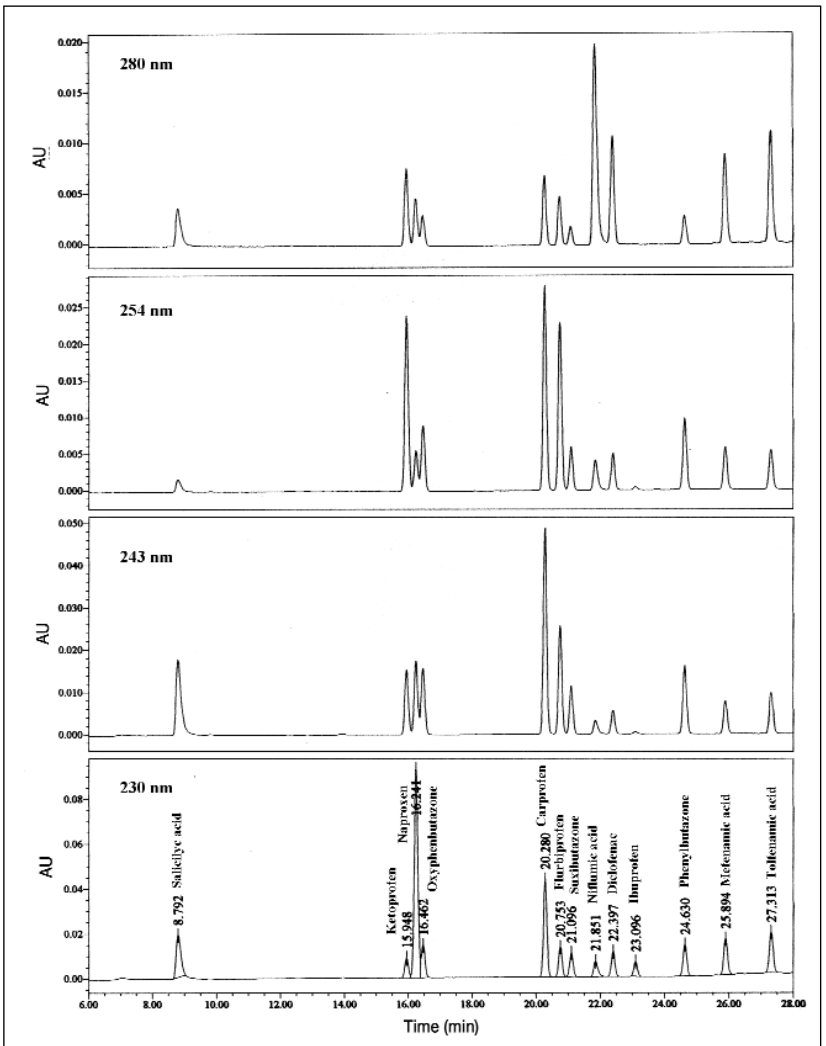

Figure 2. The stacked chromatograms at 230, 243, 254, and 280 nm of a standard mixture of NSAIDs at 1 µg/mL.

Standard solutions

Standard stock solutions at 1000 µg/mL were prepared by dissolving 10.0 ± 0.1 mg of each drug in 10 mL of acetonitrile–methanol (90:10, v/v). A standard solution mixture of NSAIDs at 10 µg/mL, containing all 13 drugs, was prepared by diluting 0.100 mL of each standard stock solution up to 10 mL with acetonitrile–methanol (90:10, v/v). All standard solutions were stable if stored at –20°C for at least 6 months. NSAIDs mixed working standard solutions at 0.25, 0.50, 1.0, and 2.0 µg/mL were prepared daily by diluting the standard solution at 10 µg/mL with methanol.

Plasma and serum clean-up

Bovine, pig, and equine plasma and serum samples were obtained from farm animals. Five millilitres of sample were centrifuged at 1086 × g for 5 min, then the supernatant was separated and added with 500 µL HCl 1 mol/L, to make the pH approximately 3.0. This step allowed both denaturation of plasmatic proteins and the hydrolysis of bound drug residues. After 10 min at room temperature (~ 20°C), the sample was added with 25 mL of ascorbic acid buffer (0.010 mol/L, pH 3.0). Then the sample was loaded at an approximately 1 mL/min flow rate onto a C18 (EC) 1-g SPE cartridge, previously rinsed and equilibrated with 3 mL n-hexane–diethyl ether (1:1, v/v), 3 mL methanol, and 5 mL ascorbic acid buffer (0.010 mol/L, pH 3.0). The SPE cartridge was washed with 3 mL ascorbic acid buffer (0.010 mol/L, pH 3.0) and 3 mL MilliQ water, then dried under vacuum for 10 min. Finally, elution of analytes was carried out by 3 mL n-hexane–diethyl ether (1:1, v/v). The eluate was evaporated to dryness under a nitrogen stream without heating. The residue, dissolved in 500 µL of methanol, was ready for HPLC–DAD analysis.

Recovery and precision data

During the in-house validation study, several uncontaminated plasma or serum samples from all species, at 0.050, 0.100, and 0.150 µg/mL of each drug studied (apart salicylic acid, which was added at 0.100, 0.200, and 0.300 µg/mL), were analyzed over a period of 4 months. Method repeatability was calculated by analyzing six different spiked samples at each contamination level. Within laboratory reproducibility was calculated by testing, by a different analysts, two plasma or serum samples at each spiking level, repeated during three different working sessions. The overall data were considered for calculating mean recoveries at all spiking levels.

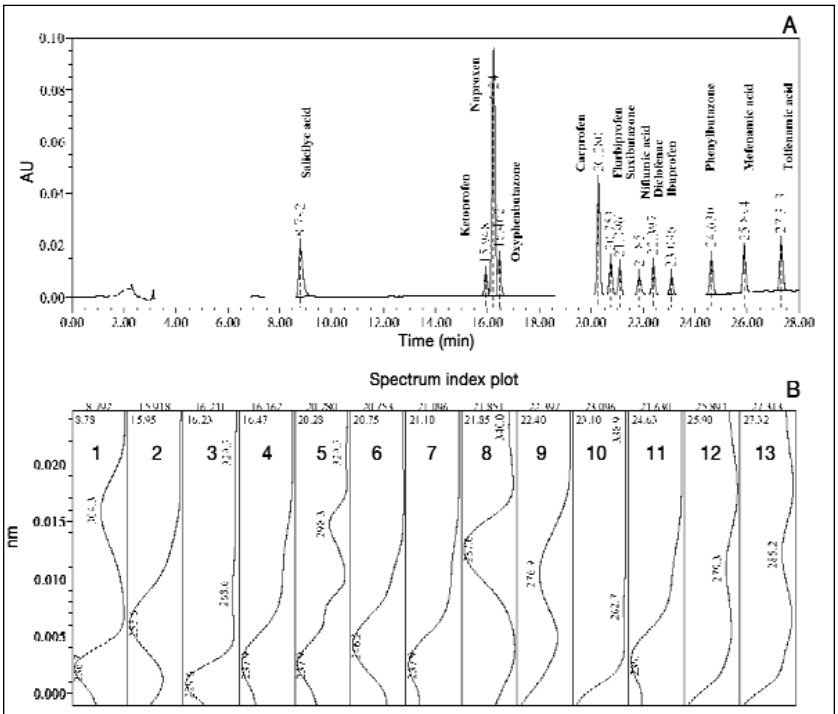

Figure 3. The chromatograms at 230 nm of a standard mixture of NSAIDs at 1 µg/mL (A) and the UV–vis spectrum between 220 and 350 nm of each analyte (B). Analyte numbers for the spectrum are: Salicylic acid, 1; ketoprofen, 2; naproxen, 3; oxyphenbutazone, 4; carprofen, 5; flurbiprofen, 6; suxibutazone, 7; niflumic acid, 8; diclofenac, 9; ibuprofen, 10; phenylbutazone, 11; mefenamic acid, 12; and tolfenamic acid, 13.

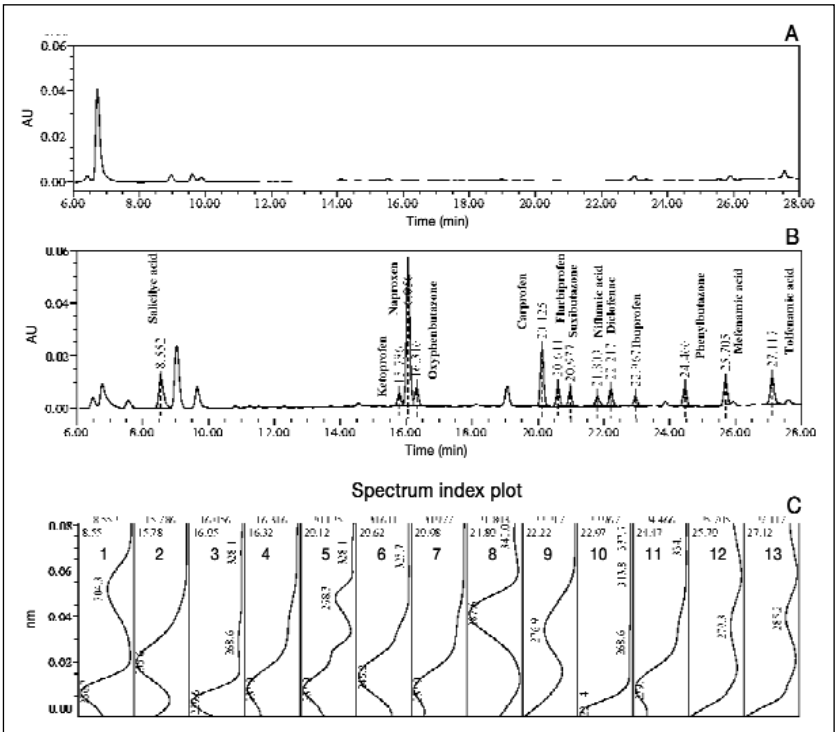

Figure 4. The 230 nm chromatogram of a blank plasma sample (A) and a plasma sample spiked at 0.100 µg/mL of each NSAID (B), with the respective UV–vis spectra (C). Analyte numbers for spectrum are: salicylic acid, 1; ketoprofen, 2; naproxen, 3; oxyphenbutazone, 4; carprofen, 5; flurbiprofen, 6; suxibutazone, 7; niflumic acid, 8; diclofenac, 9; ibuprofen, 10; phenylbutazone, 11; mefenamic acid, 12; and tolfenamic acid, 13.

Results

HPLC–DAD analysis of NSAID standards

The NSAIDs studied belong to different chemical classes, as can be seen in Figure 1. Apart from salicylic acid and phenylbutazone and its metabolite (oxyphenbutazone), they show apolar structures with acidic functional groups. Their chromatographic separation was achieved using a C12 stationary phase HPLC column with non-polar end-capping, characterized by high selectivity for non-polar hydrophobic compounds. The method developed was able to completely separate all the 13 analytes, and the linearity of detector response was verified in both solvent and matrix within the concentration range of 0.25–2.0 µg/mL. The retention times and the relative maxima in the UV–vis spectra of all compounds are summarized in Table I. Different wavelengths can be chosen for selective identification of the analytes on the basis of their characteristic relative absorbance maximum. An example is shown in Figure 2, where the chromatograms at 230, 243, 254, and 280 nm of a standard mix at 1.0 µg/mL are reported. The presence of each drug can be confirmed on the basis of the retention time and its own UV–vis spectrum (Figure 3), according to the performance characteristics required by the European Council Decision 2002/657/EC (22) for permitted veterinary drugs. This decision has the purpose of harmonizing methods performed in laboratories involved in the official control of food and animal products, and it requires that in HPLC–DAD-based methods, the analyte should show a tolerance range within ± 2.5% in the relative retention time and within ± 2 nm in the relative maxima in the UV–vis spectrum.

Clean up procedure

The procedure for the clean up of NSAIDs from serum and plasma was optimized to maximize analyte recoveries. To develop the method, several samples spiked at 0.050, 0.100, and 0.150 µg/mL of each drug were purified while changing some experimental parameters, such as the time of hydrolysis, SPE column rinsing and elution volumes, SPE drying time, and evaporation temperature. As a consequence of these trials, the SPE elution volume and the cold evaporation of SPE eluate were recognized as critical points. Indeed, the recoveries of analytes ranged between 13.3% and 75.8% when an SPE elution volume of 2.5 mL was tested, and recoveries ranged between 28.9% and 77.6% after SPE eluate drying at 40°C. These recovery values improved to a range between 75.7% and 104.2% when SPE elution by 3 mL and drying without heating were

introduced in the method. The time of acid hydrolysis was already recognized as a critical parameter by other authors (14). The clean-up procedure was simple and rapid, allowing many samples to be processed in relatively little time.

HPLC–DAD analysis of spiked samples

The HPLC–DAD chromatography of spiked serum and plasma samples proved the method was able to recover all the analytes and to separate them for subsequent qualitative and quantitative analysis. The chromatograms were complex, but their interpretation was accomplished by analyzing different time ranges. Figure 4 reports the 230 nm chromatogram of a blank plasma sample and a plasma sample spiked at 0.100 µg/mL of each NSAID; the chromatographic analysis at selective wavelengths, corresponding to the relative maxima in NSAID spectra, can help in the most difficult cases. The confirmation of all drugs was successfully performed in all spiked samples, on the basis of the UV–vis spectrum analysis, as described in Figure 4. During method development, it was observed that haemolysis traces in serum could seriously affect the HPLC separation by causing interfering peaks in the middle of the chromatogram. The UV–vis spectrum analysis usually eliminated these interference, but a proper procedure to obtain serum after blood sampling would be strongly recommended. When properly drawn, serum and plasma samples showed no significant matrix components in the chromatograms that interfered with the analytes.

Table II. Method Repeatability (*n* = 6) and Within Laboratory Reproducibility (*n* = 6) in Terms of Mean Recoveries, Calculated Over all the Results, Standard Deviations (SDs), and Percentage Coefficient of Variation (CVs%) at Each NSAID Spiking Level

NSAID	Spiking concentration (µg/mL)	Mean percentage recovery	Repeatability (*n* = 6)		Within laboratory reproducibility (*n* = 6)	
			SD (µg/mL)	CV %	SD (µg/mL)	CV%
Salicylic acid	0.100	49.3	0.011	22.3	0.010	20.3
Ketoprofen	0.050	89.4	0.0010	2.3	0.009	11.3
Naproxen	0.050	90.1	0.006	14.5	0.008	14.5
Oxyphenbutazone	0.050	77.8	0.0011	2.8	0.0007	1.8
Carprofen	0.050	72.5	0.002	4.6	0.004	10.6
Flurbiprofen	0.050	80.2	0.0007	1.9	0.0008	1.78
Suxibutazone	0.050	76.8	0.0007	1.8	0.0017	4.3
Niflumic acid	0.050	86.5	0.007	15.8	0.018	18.2
Diclofenac	0.050	88.6	0.0014	2.9	0.0014	3.5
Ibuprofen	0.050	79.6	0.007	16.7	0.004	9.7
Phenylbutazone	0.050	99.0	0.002	4.0	0.007	15.8
Mefenamic acid	0.050	78.8	0.002	5.8	0.006	16.0
Tolfenamic acid	0.050	87.5	0.002	1.5	0.016	10.5
Salicylic acid	0.200	54.9	0.016	14.6	0.019	17.3
Ketoprofen	0.100	91.2	0.0010	1.2	0.013	14.2
Naproxen	0.100	101.4	0.005	4.8	0.012	11.5
Oxyphenbutazone	0.100	74.8	0.0007	0.95	0.011	15.0
Carprofen	0.100	87.6	0.0017	2.0	0.008	8.4
Flurbiprofen	0.100	93.6	0.002	2.0	0.014	16.0
Suxibutazone	0.100	86.0	0.003	2.8	0.014	17.0
Niflumic acid	0.100	100.2	0.005	4.7	0.007	7.8
Diclofenac	0.100	88.2	0.0008	0.86	0.010	11.8
Ibuprofen	0.100	91.1	0.004	4.3	0.004	4.0
Phenylbutazone	0.100	77.6	0.0014	1.9	0.016	18.2
Mefenamic acid	0.100	86.0	0.002	2.3	0.007	8.6
Tolfenamic acid	0.100	85.8	0.0010	1.1	0.013	16.8
Salicylic acid	0.300	36.3	0.007	6.4	0.009	8.3
Ketoprofen	0.150	94.4	0.003	2.1	0.019	14.0
Naproxen	0.150	92.9	0.007	7.4	0.004	3.0
Oxyphenbutazone	0.150	93.0	0.008	5.6	0.03	19.7
Carprofen	0.150	81.5	0.0015	1.2	0.02	21.9
Flurbiprofen	0.150	92.9	0.004	2.7	0.005	4.9
Suxibutazone	0.150	91.6	0.003	2.1	0.016	11.1
Niflumic acid	0.150	92.4	0.015	11.2	0.011	7.2
Diclofenac	0.150	82.0	0.0018	1.3	0.009	8.4
Ibuprofen	0.150	104.5	0.013	8.5	0.04	24.2
Phenylbutazone	0.150	73.6	0.004	3.8	0.008	7.8
Mefenamic acid	0.150	80.6	0.004	3.9	0.008	6.3
Tolfenamic acid	0.150	87.5	0.0018	1.5	0.016	10.5

Method performances

Method repeatability (six replicates in a day) and within laboratory reproducibility (six replicates over three different days) for each NSAID are reported in Table II, in terms of standard deviations and percentage coefficients of variation. As is shown, all NSAIDs studied were recovered from samples, with the percentage mean values ranging between 72.5% and 104.5%, without significant differences among the sub-classes of each compounds. Only salicylic acid showed a lower mean recovery at all spiking levels (ranging from 36.3% to 54.9%) because of its major structural polarity, which probably should require a different SPE purification strategy, such as ion exchange rather than reversed-phase. The within-laboratory reproducibility results were comparable to the within day repeatability data, accounting for the ability of different laboratory analysts to perform the test method. The analysis of 10 serum and 10 plasma blank samples, from bovine, pig, and equine, as well as the absence of significant matrix interference, proved the method specificity.

Conclusion

This work describes, for the first time, a multiresidue HPLC–DAD method for the simultaneous determination of 13 NSAIDs in animal plasma and serum samples. The NSAIDs tested belong to different sub-classes of these drugs, allowing a wide range of possible treatments in farm animals to be controlled. The method was in-house validated, below and above the action residue limit indicated by the European Council in serum and plasma, and some analytical performance param-

eters were calculated. For all NSAIDs, apart from salicylic acid, the mean recoveries from spiked samples were very satisfactory, even at concentrations below the physiological levels described in the literature for some animals. Even if the recovery of salicylic acid was relatively low, it was enough for Italian monitoring programs, considering the action residue limit of 0.100 µg/mL set by the European Council. The method is rapid and specific, and repeatability and within laboratory reproducibility data account for its reliability. Moreover, the confirmation of the drugs was accomplished by the DAD analysis of the UV–vis spectra according to the requirements of Decision 2002/657/EC (22), proving the multi-residue method developed is fit for the purposes of the official control laboratory.

Acknowledgments

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