Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 359–365

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Analytical strategy for the confirmatory analysis of the non-steroidal anti-inflammatory drugs firocoxib, propyphenazone, ramifenazone and piroxicam in bovine plasma by liquid chromatography tandem mass spectrometry

Geraldine Dowling*, Edward Malone

The State Laboratory, Backweston Laboratory Complex, Young's Cross, Celbridge, Co. Kildare, Ireland

ARTICLE INFO

Article history:
Received 10 January 2011
Received in revised form 1 May 2011
Accepted 16 May 2011
Available online 23 May 2011

Keywords:
Basic non-steroidal anti-inflammatory drugs
Plasma
Liquid chromatography tandem mass spectrometry
Method validation
Animal

ABSTRACT

A sensitive and selective method for the simultaneous determination of non-steroidal anti-inflammatory drugs in bovine plasma was developed. Confirmatory analysis was carried out by liquid chromatography coupled with an electrospray ionisation tandem mass spectrometer (LC-ESI–MS/MS). Target compounds were acidified in plasma and extracted with acetonitrile. Sodium chloride was added to assist separation of the plasma and acetonitrile mixture. The acetonitrile extract is then subjected to liquid–liquid purification by the addition of hexane. Accuracy of the methods in plasma was between 93 and 102%. The precision of the method for the basic NSAIDs in plasma expressed as % RSD, for the within-laboratory reproducibility was less than 10%. Decision limit (CCα values) and detection capability (CCβ) values were established. The methods were validated according to Commission Decision 2002/657/EC.

1. Introduction

Firocoxib (FIRO), propyphenazone (PROPY), ramifenazone (RAMI), and piroxicam (PIROXI) are non-steroidal anti-inflammatory drugs (NSAIDs) and their structures are illustrated in Fig. 1. Residues of NSAIDs in food are a cause for concern and studies have shown that the second most prescribed class of drugs after microbials is NSAIDs [1]. Dairy farmers and veterinarians are using NSAIDs in dairy animals more frequently [2] and studies have shown that their increased use [3] poses a threat to human health as permitted residue levels are being violated [4]. The European Council recommend rigorous control of NSAIDs in food producing animals [5] because of the health effects in humans such as aplastic anaemia, gastrointestinal disorders, agranulocytosis [6] and changes in renal function [7]. Long term exposure to PBZ has caused kidney tumors in mice and liver tumors in rats [8]. In recent years the COX-II inhibitor class of NSAIDs has been implicated in cardiovascular harm in humans [9,10]. According to EU law, all drugs for veterinary use need to be included in Annex of Commission Regulation (EU) No. 37/2010 [11]. This regulation establishes lists of allowed substances that have a fixed maximum residue limit, MRL or need no MRL (Annex, Table 1).

FIRO has been included in Annex, Table 1 and has an MRL set in equine tissues only. Substances that have no MRL established are prohibited for use in food producing animals. FIRO has no MRL established in bovine species. It is anticipated that due to the large increase in NSAID use in recent years that this substance may be used to treat food producing animals other than equines. Off label application of veterinary products is illegal. FIRO has been shown to be comparable in efficacy to the NSAIDs meloxicam and carprofen [12] and also been shown to be comparable in efficacy to the NSAID phenylbutazone [13]. In the case of carprofen and meloxicam these substances are licensed for use in horses and cattle in the EU, therefore it cannot be excluded that FIRO would not be used in cattle. There are no MRL's set in plasma as is not an edible matrix. PROPY, RAMI and PIROXI are considered as prohibited substances also and are not included in "Annex Table 1 or 2" therefore have no maximum residue limit (MRL) established. The recommended minimum concentration for phenylbutazone and oxyphenbutazone in plasma is set at 5 ng ml^{-1} [14] and this target level was investigated in this study. The widespread use of NSAIDs presents a threat to the consumer if food containing residues enter the food chain so there is a need for the development of analytical tools to monitor compliance with legislation in the EU in a variety of animal tissues.

* Corresponding author.
E-mail address: Geraldine.Dowling@statelab.ie (G. Dowling).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.05.029

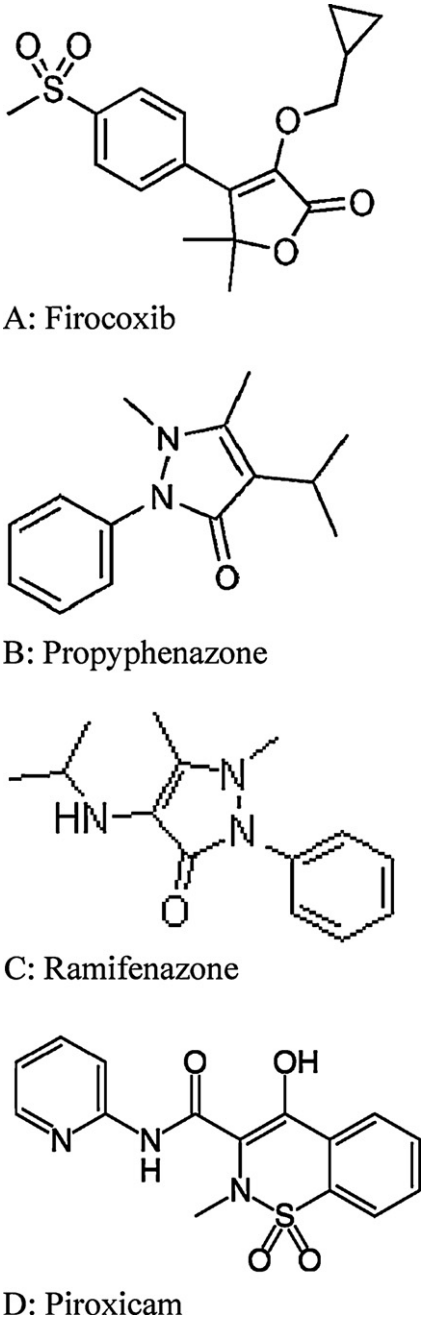

Fig. 1. Structures of FIRO, PROPY, RAMI and PIROXI.

Table 1
LC gradient profile for determination of FIRO, PROPY, RAMI and PIROXI.

Time (min)	Component A (%)	Component B (%)
0.0	90	10
1.0	90	10
3.5	85	15
7.5	35	65
9.5	35	65
11.0	90	10
15.0	90	10

Component A: water containing 0.001 M acetic acid/acetonitrile (90/10 (v/v)).
Component B: acetonitrile.

In Ireland plasma is one of the target matrices chosen to identify the misuse of NSAIDs in animal production. The advantages of using plasma as a target matrix in regulatory control are that it is an easy matrix for analysis, can identify the presence of drugs in live animals prior to slaughter and residues can be found in this matrix for a long time (personal communication with the CRL). Methods have been reported for the analysis of NSAIDs in plasma in the literature by LC–UV [15,16,18–24], GC–MS [19,25–28], LC–MS [17,23,25,29–31] and capillary electrophoresis [32]. The majority of methods that have been cited to date have been developed in equine plasma or serum alone or in combination with other matrices [17–20,22,25–27,30–32] with limits of detection ranging from 0.1 ng ml^{-1} to 5 μg ml^{-1}. Other methods exist for the determination of NSAIDs in bovine plasma or serum but the limits of detection range from 20 ng to 3.4 μg ml^{-1} [15,21,22,24,29,30] or in rat serum [23]. Only two methods are available in equine plasma to date capable of meeting the requirement to monitor at 5 ng ml^{-1} in plasma. A method by Luo et al. [17] for a single residue had a limit of detection of 0.1 ng ml^{-1} for FLU. A multi-residue method by Gonzalez et al. [27] had a limit of detection of 5 ng ml^{-1} for IBP, FLU, DCF, TLF but KPF, MFN and PBZ were also included in this study with higher limits of detection ranging from 10 to 25 ng ml^{-1} achieved. Most of the methods developed to determine veterinary drugs in animal products are focused on antibacterials and, although, they are also widely used, other veterinary drugs such as the NSAIDs have been less determined [33,34].

The limited methods that are available for the analysis of NSAIDs in food producing animal plasma concentrate on the analysis of acidic NSAIDs. There are no methods available for the determination of FIRO and the basic NSAIDs PROPY, RAMI and PIROXI in bovine plasma. It is necessary to develop an analytical strategy for these prohibited substances in bovine plasma. Overall there are no analytical tools in the literature for the confirmatory analysis of basic NSAIDs in plasma of food producing animals. The objective of this study was to develop an analytical strategy for the determination of FIRO and the basic NSAIDs in bovine plasma that meets the EU target levels set and validate according to Commission Decision 2002/657/EC [35]. In this study plasma was acidified and extracted with acetonitrile. Sodium chloride was added. Extracts were defatted with hexane and analysed by liquid chromatography tandem mass spectrometry. The developed procedure was suitable for the purification of FIRO and 3 basic NSAIDs in bovine plasma. The method was comprehensively validated according to Commission Decision 2002/657/EC. This is the first time that a suitably sensitive method for the analysis of the FIRO and the basic NSAIDs in bovine plasma is available to the best of our knowledge.

2. Experimental

2.1. Materials and reagents

Water, acetonitrile, n-hexane, acetic acid, hydrochloric acid (37%), (HiPerSolv grade) were obtained from BDH (Merck, UK). PIROXI was purchased from Sigma (Sigma Aldrich, Ireland). RAMI, d_3-RAMI and PROPY were obtained as a gift from The Community Reference Laboratory for NSAIDs in the EU in Germany. FIRO was obtained as a gift from Merial (Saint-Vulbas, France). d_6-FIRO was obtained from Witega (Witega, Germany). Primary stock standard solutions (stable for 6 months) were prepared in ethanol at a concentration of 1 mg ml^{-1}. Intermediate single standard solutions (stable for 6 months) were prepared in methanol at a concentration of 10 μg ml^{-1}. FIRO, PROPY, RAMI and PIROXI standard fortification solution for plasma (stable for 6 months) was prepared in methanol at a concentration of 500 ng ml^{-1} from the 10 μg ml^{-1} intermediate stock solution. Internal standard fortification solution for plasma

Table 2
MS/MS parameters for determination of FIRO, PROPY, RAMI and PIROXI.

Compound	Transition	Declustering potential (V)	Collision energy (eV)	Collision cell exit potential (V)
FIRO	337 > 130 (strong)	100	45	21
	337 > 283 (weak)	100	13	21
PROPY	231.2 > 189 (strong)	130	15	10
	231.2 > 56 (weak)	130	15	15
RAMI	245.9 > 55.9 (strong)	140	50	12
	245.9 > 124.9 (weak)	140	22	14
PIROXI	332.1 > 95.2 (strong)	150	19	14
	332.1 > 121.1 (weak)	150	17	14
d_6-FIRO	343.2 > 136 (strong)	140	45	21
d_3-RAMI	248.9 > 59.1 (strong)	140	50	14

Note: Matrix matched curves were used for quantification of all compounds.
d_6-FIRO was used as internal standard (I.S.) for FIRO, PROPY and PIROXI and d_3-RAMI was used as I.S. for RAMI.

containing d_3-RAMI and d_6-FIRO was prepared at a concentration of 1.25 μg ml^{-1}. All standards were stored at 4 °C in the dark. Injection solvent was water:acetonitrile (90:10 (v/v)).

2.2. LC–MS/MS conditions

The LC consisted of a Shimadzu LC equipped with a LC-20AD XR Binary pump, SIL-20AC autosampler and a CTO-20A column oven (Shimadzu Corp., Japan). The NSAIDs were chromatographed on a 1.8 μm Agilent Eclipse Plus C_{18} column (3.0 mm × 50 mm) (Agilent, Ireland) and the column temperature was maintained at 55 °C. A gradient was applied with water containing 0.001 M acetic acid (A) and acetonitrile (B) (90:10 (v/v)) (Table 1). The total run time was 15 min. The injection volume was 10 μl. The mass spectrometer used was a QTRAP 5500 with a TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software. The described LC–MS/MS system was shown to be suitable for the analysis of NSAIDs in plasma (Fig. 2).

2.3. MS/MS parameters

The analysis was performed using positive ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. The collision voltages were optimised as shown (Table 2). Each transition was performed with a 13 ms dwell time and a pause time of 3 ms. The MS/MS detector conditions were as follows: ion mode electrospray positive; curtain gas 45 psi; ion spray voltage 4400 V; temperature 650 °C; ion source gas one 70 psi; ions source gas two 70 psi; interface heater on; entrance potential 10 V; Resolution Q1 unit; Resolution Q2 unit; CAD gas = high.

2.4. Plasma samples

Plasma obtained for use as negative controls was separated into 50 ml aliquots and stored at −20 °C. The plasma was analysed in previous batches and plasma found to contain no detectable residues of NSAIDs were used as negative controls.

2.5. Sample extraction and clean-up

2.5.1. Plasma extraction

Plasma samples (5 ml) were aliquoted into 50 ml polypropylene tubes. The plasma aliquots (5 ml) were fortified with internal standard at levels corresponding to 15 ng ml^{-1} by adding a 60 μl portion of a 1.25 μg ml^{-1} mix solution of d_6-FIRO and d_3-RAMI. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng ml^{-1} by adding 50, 75 and 100 μl portions of a 500 ng ml^{-1} solution of FIRO, PROPY, RAMI and PIROXI. After fortification, samples were held for 15 min prior to extraction. Hydrochloric acid (500 μl, 1 M) was added and plasma samples were left to stand at room temperature (10 min). Acetonitrile (5 ml) and sodium chloride (2 g) were added and the samples were vortexed (30 s), centrifuged (4333 × *g*, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. Hexane (5 ml) was added and the samples were vortexed (30 s) and centrifuged (4333 × *g*, 10 min, 4 °C). The hexane layer was removed and the samples were partially evaporated to approximately 1 ml under nitrogen at 60 °C before being transferred to 1.5 ml micro-centrifuge vials. The samples were centrifuged (25,155 × *g*, same centrifuge but interchangeable rotor depending on size of tube, 15 min, 4 °C). The supernatant was transferred to a clean glass test-tube and evaporated to dryness under nitrogen at 60 °C. The samples were re-dissolved in 200 μl of water:acetonitrile (90:10 (v/v)) and vortexed (1 min). An aliquot (10 μl) was injected on the LC column.

2.6. Matrix-matched calibration

Matrix extracted calibration curves were prepared and used for quantification. Control plasma previously tested and shown to contain no residues was prepared as above (Section 2.4). One control plasma sample was used for each calibration standard level. Plasma samples (5 ml) were aliquoted into 50 ml polypropylene tubes. Individual plasma samples were fortified with internal standard at levels corresponding to 15 ng ml^{-1} by adding a 60 μl portion of a 1.25 μg ml^{-1} mix solution of d_6-FIRO and d_3-RAMI. Plasma samples were fortified at levels corresponding to 0, 2, 5, 7.5, 10 and 20 ng ml^{-1} by adding 0, 20, 50, 75, 100 and 200 μl portions of a 500 ng ml^{-1} standard solution of FIRO, PROPY, RAMI and PIROXI.

After fortification, plasma samples were held for 15 min prior to the extraction procedure as described above (Section 2.5). Calibration curves of plasma were prepared by plotting the response factor as a function of analyte concentration (0–20 ng ml^{-1}) to quantify samples.

2.7. Method validation

For estimation of accuracy, blank plasma samples were fortified with FIRO, PROPY, RAMI and PIROXI at 5, 7.5 and 10 ng ml^{-1}. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions for each matrix. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was calculated. For unauthorised substances the decision limit (CCα) of the method was calculated according to the calibration curve procedure using the intercept (value of the signal, *y*, where the concentration, *x* is equal to zero) and 2.33 times the standard error of the intercept for a set of data with 6 replicates at 3 levels. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα.

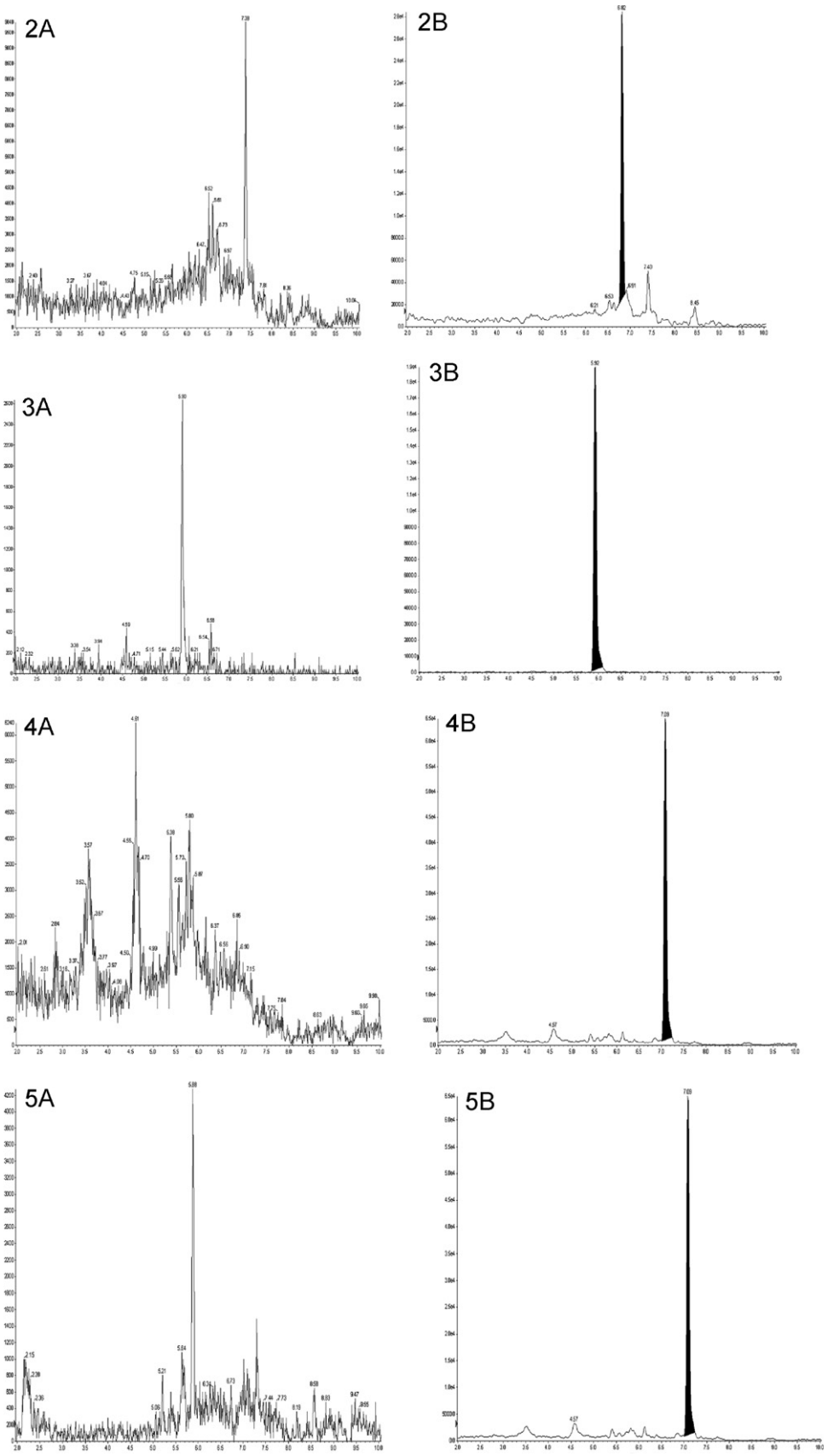

Fig. 2. Chromatograms of negative controls (2A–5A) and negative control fortified with 2 ng ml^{-1} of FIRO (2B), PROPY (3B), RAMI (4B) and PIROXI (5B).

Table 3
Intra- and inter-assay variation for accuracy of FIRO, PROPY, RAMI and PIROXI in plasma.

Analyte	Fortification level (ng ml^{-1})	Accuracy (%)	Within run CV (%)	Between run CV (%)	Total CV (%)
FIRO	5	95	5.2	6.7	8.5
	7.5	98	3.5	0.0	3.5
	10	102	4.5	3.4	5.7
Combined variance	5, 7.5, 10				8.2
PROPY	5	98	6.5	0.0	6.5
	7.5	98	5.6	3.1	6.5
	10	101	4.2	2.7	5.0
Combined variance	5, 7.5, 10				6.0
RAMI	5	101	8.1	9.8	12.7
	7.5	97	4.0	2.5	4.7
	10	98	4.1	0.0	4.1
Combined variance	5, 7.5, 10				8.2
PIROXI	5	94	9.0	1.3	9.1
	7.5	93	7.1	6.2	9.4
	10	99	8.9	0.0	8.9
Combined variance	5, 7.5, 10				9.2

3. Results and discussion

3.1. Preliminary experiments

The MS/MS method was developed to provide confirmatory data for the analysis of FIRO, PROPY, RAMI and PIROXI. The ionisation of all NSAIDs was studied in positive mode. The optimum conditions (polarity mode, declustering potential, collision energy, collision cell exit potential) were determined for each drug and the best diagnostic ions for MS/MS analysis were obtained and can be seen in Table 2. For a method to be deemed confirmatory 4 identification points must be obtained. This is achieved by monitoring one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion) which is a suitable confirmatory method in accordance with 2002/657/EC [35].

The LC method developed in this study was based on a method developed at the author's laboratory for the determination of NSAIDS in plasma and milk [36]. Chromatographic tests were carried out to evaluate the suitability of the 1.8 μm Agilent Eclipse Plus C_{18} column (3.0 mm × 50 mm) and the LC mobile phase utilised in the previous study with the new NSAIDs evaluated in this study. The tests showed that this LC method was suitable for analysis of the target compounds. The extraction and centrifugation procedure utilised in this study was based on a method developed by Malone et al. [37] in milk. In this study it was evaluated for the analysis of the new basic substances in plasma and the procedure produced extremely clean extracts. The extraction procedure was found to be satisfactory in the extraction of the target NSAIDs from plasma in this study. The criteria for relative retention times and ion ratios were examined for all samples and standards used for the validation study. The average ion ratios were 0.70 for FIRO, 0.09 for PROPY, 0.25 for RAMI and 0.30 for PIROXI. The values for these were in agreement with the EU requirements for all the analytes investigated in this study. In terms of relative retention time, the analyte peaks in samples were found to be within 2.5% tolerance when compared with standards. Furthermore two transition ions were monitored for each of the four analytes. The most intense ion was used for quantitation. All ion ratios were within the tolerances as required by EU criteria when compared with standards. The primary advantage of the developed analytical strategy is the ability to analyse for FIRO and the basic NSAIDs in bovine plasma matrix that meets the requirements of EU legislation for NSAIDs. There is a lack of analytical methods meeting EU legislation requirements for NSAIDs selected in this study. This is the first time that FIRO, PROPY, RAMI and PIROXI residues have been purified from bovine plasma and the methodology developed is capable of meeting the level of 5 ng ml^{-1} set for phenylbutazone and oxyphenbutazone in plasma. This target level was selected for validation. Additionally there are no LC–MS methods available for the analysis of FIRO and basic NSAIDs simultaneously within a single injection that meet the stringent validation requirements according to Commission Decision 2002/657/EC as a quantitative confirmatory method. The advantage of the developed LC–MS/MS method in this study is that the quantitation and confirmation can be carried out using a single analytical technique and single injection according to Commission Decision 2002/657/EC [35]. A further advantage of the developed LC–MS/MS method described in this study for plasma is that compared with previously published methods, the values determined for the decision limit (CCα) for the substances studied in bovine plasma are available by LC–MS/MS in the literature to date to the best of our knowledge.

3.2. Validation study

Validation of the method in plasma was according to procedures described in Commission Decision 2002/657/EC [35] covering specificity, calibration curve linearity, recovery (accuracy), precision, decision limit (CCα) and detection capability (CCβ).

3.2.1. Specificity

The technique of LC–MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, plasma samples were fortified with analytes and internal standards and non-fortified samples were also analysed. No interfering peaks were observed at the retention time of the analytes. To further test specificity in plasma, samples were also fortified with 5.0 ng ml^{-1} of flunixin (FLU), naproxen (NAP), diclofenac (DCF), niflumic acid (NIFLU), phenylbutazone (PBZ), oxyphenbutazone (OXYPHEN) and suxibuzone (SUXI). No interfering peaks were observed at the retention window of the analytes.

3.2.2. Linearity of the response

The linearity of the chromatographic response in plasma was tested with matrix matched curves using 6 calibration points in the concentration range of 0–20 ng ml^{-1} when fortified with FIRO, PROPY, RAMI and PIROXI. Overall the regression coefficients (r^2) were ≥0.98.

3.2.3. Accuracy

The accuracy was determined using bovine plasma fortified at 5.0, 7.5 and 10.0 ng ml^{-1} with FIRO, PROPY, RAMI and PIROXI. Mean corrected recoveries (n = 18) determined in three separate assays in plasma (Table 3) were between 93 and 102%.

Table 4
Calculated CCαand CCβ values for plasma.

	CCα (ng ml^{-1})	CCβ (ng ml^{-1})
FIRO	0.67	1.14
PROPY	0.77	1.31
RAMI	0.88	1.50
PIROXI	1.35	2.31

3.2.4. Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility for FIRO, PROPY, RAMI and PIROXI in bovine plasma was less than 10% (Table 3). No deuterated analogue was available for PROPY and PIROXI in our laboratory at the time of carrying out this work. d_6-FIRO was used as I.S. for FIRO, PROPY and PIROXI and d_3-RAMI was used as I.S. for RAMI. A one-way analysis of variance was carried out at each of the fortification levels to separate out estimates for within run, between run and total variance of the method and the results are shown in Table 3. Commission Decision 2002/657/EC states that the precision for quantitative methods for mass fractions lower than 100 ng ml^{-1}, the application of the Horwitz equation gives unacceptable high values. Therefore, the RSD values for concentrations lower than 100 ng ml^{-1} shall be as low as possible.

3.2.5. CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1% is applied. The detection capability (CCβ) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of $1-\beta$, where $\beta = 5\%$. In the case of non MRL substances CCα is the concentration corresponding to the intercept +2.33 times the standard error of the intercept. CCβ is the concentration corresponding to the signal at CCα +1.64 times the standard error of the intercept (i.e. the intercept +3.97 times the standard error of the intercept). Blank plasma was fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng ml^{-1} set for FIRO, PROPY, RAMI and PIROXI. CCα and CCβ were calculated in plasma using the intercept (value of the signal, *y*, were the concentration, *x* is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (5.0, 7.5 and 10.0 ng ml^{-1}). CCα values of 0.67, 0.77, 0.88 and 1.35 ng ml^{-1} were determined for FIRO, PROPY, RAMI and PIROXI respectively. CCβ values of 1.14, 1.31, 1.50 and 2.31 ng ml^{-1} were determined for FIRO, PROPY, RAMI and PIROXI respectively (Table 4).

3.3. Measurement uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [38]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different plasma sourced from different animals it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method in plasma was estimated at 25, 19, 18 and 27% for FIRO, PROPY, RAMI and PIROXI. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

4. Conclusions

A fast, simple, sensitive and selective LC–MS/MS method for the determination FIRO, PROPY, RAMI and PIROXI in bovine plasma has been developed. There is no published method available to the best of our knowledge for the simultaneous determination of FIRO and the basic NSAIDs in bovine plasma. This is the first time that a method is available that meets the minimum requirements of 5 ng ml^{-1} for FIRO, PROPY, RAMI and PIROXI. There is no published method available to the best of our knowledge for the simultaneous determination FIRO, PROPY, RAMI and PIROXI in bovine plasma that extracts using acetonitrile/sodium chloride and washes extracts with hexane in a single injection with validation according to Commission Decision 2002/657/EC [35] and with the measurement uncertainty described. This methodology shows that suitable sensitivity was obtained and that the method performs very well in terms of accuracy and within-laboratory reproducibility. The objective of the work to anticipate the requirements of the future where risks could occur due to the administration of basic NSAIDs by developing a method to monitor for these NSAIDs simultaneously has been achieved.

Acknowledgements

This work was funded by The State Laboratory, Co. Kildare in Ireland. The authors would like to thank suppliers of donated analytical standards and staff at The State Laboratory, Ireland for their practical assistance.

References

[1] S.F. Sundlof, J.B. Kaneene, R.A. Miller, National survey on veterinarian – initiated drug use in lactating dairy cows, J. Am. Vet. Med. Assoc. 207 (1995) 347–352.
[2] U.S. Code of Federal Regulations, 21 CFR 520.1720 and 522.1720, 1988.
[3] M. Kopcha, J.B. Kaneene, M.E. Shea, R. Miller, S. Alwynelle, A.S. Ahl, Use of non-steroidal anti-inflammatory drugs in food animal practice, J. Am. Vet. Med. Assoc. 201 (1992) 1868–1872.
[4] G.W. Smith, J.L. Davis, L.A. Tell, A.I. Webb, J.E. Riviere, Extra-label use of non-steroidal anti-inflammatory drugs in cattle, J. Am. Vet. Med. Assoc. 232 (2008) 697–701.
[5] SANCO 2000. European Commission-Reference Laboratory for Residues of Veterinary Drugs. Workshop Manual: NSAIDs and Validation According to SANCO 1805/2000, Berlin, 07–09 May 2001.
[6] P. Taylor, in: A. Gilman, T. Rall, A. Nies, P. Taylor (Eds.), The Pharmacological Basis of Therapeutics, Pergamon Press, NY, 1990, pp. 131–149.
[7] P. Taylor, in: A. Gilman, T. Rall, A. Nies, P. Taylor (Eds.), The Pharmacological Basis of Therapeutics, McGraw-Hill, Singapore, 1992, pp. 638–681.
[8] F. Kari, J. Buchner, J. Haseman, S. Eustis, H. Huff, Long-term exposure to the anti-inflammatory agent phenylbutazone induces kidney tumors in rats and liver tumors in mice, Jpn. J. Cancer Res. 86 (1995) 252–263.
[9] V.P. Staa, L. Smeeth, I. Persson, J. Parkinson, H.G.M. Leufkems, What is the harm-benefit ratio of COX-II inhibitors, Int. J. Epiedemiol. 37 (2008) 405–413.
[10] M. Debabrata, Non-steroidal anti-inflammatory drugs and the heart: what is the danger? Congest. Heart Fail. 14 (2008) 75–82.
[11] European Commission, Council Regulation EU No. 37/2010 of 22 December 2009, Off. J. Eur. Union L15 (2010) 1–72.
[12] EMEA/V/C/082 – European Public Assessment Report (EPAR) – PREVICOX (last updated in February 2007).
[13] M.Y. Doucet, A.L. Bertone, D. Hendrickson, F. Hughes, C. MacAllister, S. McClure, C. Reinemeyer, Y. Rossier, R. Sifferman, A.A. Vrins, G. White, B. Kunkle, R. Alva, D. Romano, P.D. Hanson, Comparison of efficacy and safety of paste formulations of firocoxib and phenylbutazone with naturally occurring osteoarthritis, J. Am. Vet. Med. Assoc. 232 (2008) 91–97.
[14] SANCO 2007. CRL Guidance Paper (7 December 2007) CRLs View on State of the Art Analytical Methods for National Residue Control Plans.
[15] E.J.I. De Veau, Determination of non-protein bound phenylbutazone in bovine plasma using ultrafiltration and liquid chromatography with ultraviolet detection, J. Chromatogr. B 721 (1999) 141–145.
[16] V. Kvaternick, T. Malinski, J. Wortmann, J. Fischer, Quantitative HPLC-UV method for the determination of firocoxib from horse and dog plasma, J. Chromatogr. B 854 (2007) 313–319.
[17] Y. Luo, J.A. Rudy, C.E. Elboh, R.L. Soma, F. Guan, J.M. Enright, D.S. Tsang, Quantitation and confirmation of flunixin in equine plasma by liquid chromatography–quadrupole time-of-flight tandem mass spectrometry, J. Chromatogr. B 801 (2004) 173–184.

[18] G.E. Hardee, J.W. Lai, J.N. Moore, Simultaneous determination of flunixin, phenylbutazone, oxyphenbutazone, gamma hydroxyphenylbutazone in equine by high performance liquid chromatography with application to pharmacokinetics, J. Liq. Chromatogr. 5 (1982) 1991–2003.
[19] L.M. Neto, M.H. Andraus, M.C. Salvadori, Determination of phenylbutazone and oxyphenbutazone in plasma and urine samples of horses by high performance liquid chromatography and gas chromatography–mass spectrometry, J. Chromatogr. B: Biomed. Appl. 678 (1996) 211–218.
[20] E. Grippa, L. Santini, G. Castellano, M.T. Gatto, M.G. Leone, L. Saso, Simultaneous determination of hydrocortisone, dexamethasone, indomethacin, phenylbutazone and oxyphenbutazone in equine serum by high performance liquid chromatography, J. Chromatogr. B: Biomed. Sci. Appl. 738 (2000) 17–25.
[21] P. Jedziniak, T. Szprengier-Juszkiewicz, M. Olejnik, J. Jaroszewski, Determination of flunixin and 5-hydroxyflunixin in bovine plasma with HPLC–UV method development, validation and verification, Bull. Vet. Inst. Pulawy 51 (2007) 261–266.
[22] P. Gowik, B. Julicher, S. Uhlig, Multi-residue method for non-steroidal anti-inflammatory drugs in plasma using high performance liquid chromatography–photodiode array detector: method description and comprehensive in-house validation, J. Chromatogr. B 716 (1998) 221–232.
[23] M.C. Quintana, L. Ramos, M.J. Gonzalez, M.H. Blanco, L. Hernandez, Development of a solid phase extraction method for the simultaneous determination of corticoids and tranquilizers in serum samples, J. Sep. Sci. 27 (2004) 53–58.
[24] M. Fiori, M. Farne, C. Civitareale, A. Nasi, L. Serpe, P. Gallo, The use of bovine serum albumin as a ligand in affinity chromatographic clean-up of non-steroidal anti-inflammatory drugs from bovine plasma, Chromatographia 60 (2004) 253–257.
[25] A.K. Singh, Y. Yang, U. Mishra, K. Granley, Simultaneous analysis of flunixin, naproxen, ethacrynic acid, indomethacin, phenylbutazone, mefenamic acid and thiosalicylic acid in plasma and urine by high-performance liquid chromatography and gas chromatography–mass spectrometry, J. Chromatogr. B: Biomed. Appl. 568 (1991) 351–361.
[26] S. Hines, C. Pearce, J. Bright, P. Teale, Development and validation of a quantitative gas chromatography–mass spectrometry confirmatory method for phenylbutazone in equine plasma, Chromatographia 59 (2004) S109–S114.
[27] G. Gonzalez, R. Ventura, A.K. Smith, R. de la Torre, J. Segura, Detection of non-steroidal anti-inflammatory drugs in equine plasma and urine by gas chromatography–mass spectrometry, J. Chromatogr. A 719 (1996) 251–264.
[28] P. Jassaud, D. Guieu, D. Courtot, B. Barbier, Y. Bonnaire, Identification of tolfenamic acid metabolite in the horse by gas chromatography–mass spectrometry, J. Chromatogr. 573 (1992) 136–140.
[29] I.R. Miksa, M.R. Cummings, R.H. Poppenga, Multi-residue determination of anti-inflammatory analgesics in sera by liquid chromatography–mass spectrometry, J. Anal. Toxicol. 29 (2005) 95–104.
[30] F. Vinci, S. Fabbrocino, M. Fiori, L. Serpe, P. Gallo, Determination of 14 non-steroidal anti-inflammatory drugs in animal serum and plasma by liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 3412–3420.
[31] Y. You, C.E. Uboh, L.R. Soma, F. Guan, X. Li, J.A. Rudy, J. Chen, Screening, quantitation and confirmation of phenylbutazone and oxyphenbutazone in equine plasma by liquid chromatography tandem mass spectrometry, J. Anal. Toxicol. 33 (2009) 41–50.
[32] X. Gu, M. Meleka-Boules, C.L. Chen, D.M. Ceska, D.M. Tiffany, Determination of flunixin in equine urine and serum by capillary electrophoresis, J. Chromatogr. B: Biomed. Sci. Appl. 692 (1997) 187–198.
[33] C. Blasco, C.M. Torres, Y. Pico, Progress in antibacterial analysis of residues in food, TRAC Trends Anal. Chem. 26 (2007) 895–913.
[34] V. Andreu, C. Blasco, Y. Pico, Analytical strategies to determine quinoline residues in food and the environment, TRAC Trends Anal. Chem. 26 (2007) 895–913.
[35] Commission Decision (2002/657/EC) of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Commun. L221 (2002) 8–36.
[36] G. Dowling, E. Malone, T. Harbison, S. Martin, Analytical strategy for the determination of non-steroidal anti-inflammatory drugs in plasma and improved analytical strategy for the determination of authorised and nonauthorised non steroidal anti-inflammatory drugs in milk by LC/MS/MS, Food Addit. Contam. 27 (2010) 962–982.
[37] E.M. Malone, G. Dowling, C.T. Elliott, D.G. Kennedy, L. Regan, Development of a rapid multi-class method for the confirmatory analysis of anti-inflammatory drugs in bovine milk using liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1216 (2009) 8132–8140.
[38] SANCO/2004/2726/Rev 1 Guidelines for implementation of Commission Decision 2002/657/EC.