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Development and validation of a method for fipronil residue determination in ovine plasma using 96-well plate solid-phase extraction and gas chromatography–tandem mass spectrometry

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ABSTRACT

Fipronil, a phenylpyrazole insecticide introduced for pest control on a broad range of crops, undergoes a reinforcement of the regulation within the European Union (2007/52/EC directive) due to its potential effects on environment and human health. In order to assess the plasmatic concentrations of fipronil residues (sulfone, sulfide, fipronil, desulfinyl and amide) in ovine, a methodology based on gas chromatography coupled with tandem mass spectrometry (GC–MS/MS) was developed and validated according to the European standard (2002/657/EC). The proposed method allows a large number of samples to be treated concurrently ($n = 80$) using a reduced sample amounts (0.2 mL), and consents to reach a level of quantification of 0.1 pg μL^{-1}. The sample preparation consisted of a single solid-phase extraction (SPE) purification on a 96-well plate filled with a styrene-divinyl-benzene phase. Linearity was demonstrated all along the investigated range of concentrations, i.e. from 0.25 to 2000 pg μL^{-1}, with coefficient of determination (R^2) from 0.977 to 0.994, depending on target analytes. Calculated decision limit (CCα) and detection capability (CCβ) for fipronil, sulfone and sulphide were in the range 0.05–0.16 and 0.28–0.73 pg μL^{-1} respectively.

1. Introduction

Fipronil, (±)-5-amino-1-(2,6-dichloro – α,α,α – trifluoro-*p*-tolyl)-4-trifluoromethylsulfinyl-pyrazole-3-carbonitrile, is a phenylpyrazol insecticide, for which a market authorization was delivered in France in 1994 (Rhône-Poulenc Agro). This molecule is used against culture pests, clickbeetles, grasshoppers, ants, pets fleas, etc. Nevertheless, a reinforcement of the regulation concerning the use of fipronil in the European Union was edited in the 2007/52/EC directive [1], authorizing its use only as seed treatment. In France its utilisation as a phytosanitary product has been forbidden but it remains one of the most commonly used veterinary insecticide. In insects, fipronil is actually recognised to act as a potent blocker of the gamma-aminobutyric acid (GABA)-regulated chloride channel in the central nervous system. In rats, fipronil causes in high dosage a transient increase in neuronal excitability, but this effect is usually mild and temporary [2]. In human and mammals the very low potency of fipronil for GABA ionotropic receptors [3] contributes to the very low risk of this compound as a neurotoxic although few case reports of acute intoxications are associated [4]. A strong genotoxic effect was also reported on mucosal epithelial cells taken from tonsil tissue [5]. More recently, concerns about fipronil-related potential endocrine disruption have been raised in particular on reproduction Ohi et al. [6] and thyroid [7]. Fipronil was also demonstrated to inhibit the testosterone 6β-hydroxylation of testosterone in human liver microsomes [8]. As far as thyroid is concerned, there are some debates about the relevance of toxicological evaluations for the human health risk analysis. Indeed, most investigations were conducted in rats which present a physiological scheme of thyroid regulation as different from humans. The potential thyroid disruption of fipronil had therefore to be re-evaluated in a more relevant animal model. Investigation using sheep was thus initiated within the frame of the research program proposed by the French Ministry of Ecology entitled "National Research Program on Endocrine Disruptors". These studies were related to simultaneous assessment of fipronil exposure and potential impact on thyroidal and corticosurrenal functions in the ewe.

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In order to investigate the pharmacokinetics of fipronil residues excretion in plasma, a dedicated analytical method had to be developed as prerequisite, such method according to our knowledge was never described before. Some methodologies reaching as low limits of detection as 100 pg g^{-1} were recently developed involving different analytical techniques such as enzyme-linked immunosorbent assay (ELISA) for water [9], gas chromatography–electron capture detection (GC–ECD), gas chromatography–mass spectrometry (GC–MS) [10], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [11,12] for more complex matrices such as pollen and honey or gas chromatography–tandem mass spectrometry (GC–MS/MS) for milk and vegetables [13]. As regards sample preparation of these biological samples, SPE on different stationary phases and liquid–liquid extractions are commonly carried out [9–14]. Other more anecdotic sample preparations were studied such as solid-phase micro-extraction (SPME) [15] or matrix solid-phase dispersion (MSPD) [16]. The latter was rapidly given up because it is efficient only on solid matrices such as honeybees; whereas classical SPE are more adapted for biological fluids. Even if the SPME approach is the least time consuming, the quantities of plasma available are often limited (around 200 μL for little species such as rodents) and do not allow the use of this technique since the SPME fibre is only partially immersed in the sample. Our purpose was then to develop and validate a procedure authorizing the simultaneous handling of a large quantity of samples (often several hundred samples per pharmacokinetic study) in a reasonable amount of time (around one day) with good performances in terms of sensitivity and quantification. Finally, small quantities of fitted adsorbent [13] were selected for fipronil residues purification in plasma, authorising the preparation of more than 80 samples per day on 96-well plates.

The present article describes the developed sample preparation procedure and subsequent GC–MS/MS measurement method for determination of fipronil and its main metabolites at trace levels in ovine plasma, as well as the main validation results obtained according to the 2002/657/EC [17].

2. Experimental

2.1. Material and reagents

Residue analysis grade methanol was supplied by Carlo-Erba (Val de Reuil, France). Solvents used for gas chromatography (ethylacetate, toluene and tetrahydrofuran) were taken from Fluka (St. Quentin Fallavier, France). Ultrapure water (>14 MΩ) was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Fipronil and its metabolites (desulfinyl, sulfone and sulphide derivatives) were purchased from Accustandard (New Haven, CT, USA). Amide product was acquired from CIL (Sainte Foy la Grande, France) and tebufenpyrad from Riedel de Häen (Hanover, Germany). 1-(4-Chlorophenyl)-3-methyl-1H-pyrazol-5-ylamine (pyrazole 82), 5-amino-1-phenylpyrazole-4-carbonitrile (pyrazole 83), 5-amino-3-methyl-1-phenylpyrazole (pyrazole 86) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) (Fig. 1). Manifold for 96-well plate was from Supelco-Sigma–Aldrich (Saint Quentin Fallavier, France). Solid-phase extraction well plates (SPE-96) Atoll XC (30 and 70 μm, 30–150 mg, styrene-divinylbenzene polymer) were from Interchim (Montluçon, France).

2.2. Plasma collection

Control blood samples were collected via jugular venipuncture from seven ewes of 4–5 years old. All samples were centrifuged at 3200 *g* at 4 °C for 10 min and plasma was harvested and pooled. Resulting mixed plasma sample was constituted by 4 mL from ewe no. 1, 4 mL from ewe no. 3, 16 mL from ewe no. 4, 2 mL from ewe no. 5, 6 mL from ewe no. 7, 6 mL from ewe no. 9 and 50 mL from ewe no. 10. Plasma samples were stored at −20 °C pending analysis.

2.3. Sample preparation

The present sample preparation procedure was inspired by the method previously developed by Le Faouder et al. for fipronil residue determination in milk and animal feeds [13]. However, significant improvements and adaptations have been introduced for its application to ovine plasma. The ATOLL XC 96-well solid phase extraction plate was conditioned with 2 mL methanol and 2 mL ultrapure water. Then, 200 μL of the previously centrifuged (3200 *g*, 4 °C) plasma samples as well as 20 μL of the internal standard (pyrazole 83) solution at 5 μg/mL were loaded. The plate was washed with 600 μL of methanol/water 70:30 (v/v). High vacuum (50 kPa) was subsequently applied to dry the column completely. Analytes were eluted with 2 × 1 mL methanol. Extract samples were evaporated to dryness at 45 °C under a nitrogen stream and reconstituted in 400 μL ethylacetate prior to be transferred into a 1 mL GC-vial. A second evaporation to dryness was then carried out (45 °C, under N_2), before final reconstitution with 20 μL of an external standard (pyrazole 86) solution of 1 μg/mL for a direct injection in GC–MS/MS. At least six-spiked control and one blank control sample were included in each analysis batch. Spiked samples were used to build an appropriate calibration curve for quantification. Blank sample was used to control the absence of any carry over.

2.4. Gas chromatography–tandem mass spectrometry measurement

For GC–MS/MS analysis, a gas chromatograph (Agilent, 6890 Series) with split/splitless injector and a programmable oven was coupled to a Quattro-II triple quadrupole analyzer (Waters, Micromass) operating in electron ionisation mode. Gas chromatography was performed on a non-polar ZB5MS column (30 m × 0.25 mm, 0.25 μm) purchased from Phenomenex (Le Pecq, France). The injector (splitless mode) and transfer line temperature were set at 250 and 320 °C, respectively. Injection volume was 2 μL. The oven temperature was increased from 75 °C (3 min) to 210 °C (0 min) at 20 °C/min, then to 225 °C (0 min) at 5 °C/min and finally to 320 °C (7 min) at 20 °C/min. Mass spectra were recorded in the multiple reactions monitoring (MRM) acquisition mode using two diagnostic signals for each target analyte (Table 1).

3. Results and discussion

Even if five fipronil residues were included in the present method, the present study focused mainly on fipronil, sulphide and sulfone compounds as they are the most likely to be found in ovine plasma.

3.1. Optimisation of the purification on 96-well plate SPE

According to the previous investigations [13], we demonstrated that one efficient phase for extracting fipronil residues from complex biological matrices was the Atoll XC. One reason explaining this observation was its very high retention surface (1500 m^2/g), one of the highest available on the current market, which guarantees a good adsorption of both fipronil and its more polar residues (amide, sulphide, desulfinyl and sulfone). Indeed, Kowick et al. (2006) have evaluated the log Kow value for sulfone to 3.7. In parallel, a log Kow equal to 4 was mentioned in the patent for fipronil, this value remaining controversial. Other log Kow values were reported in the