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Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

A LC–MS–MS method is presented to analyse simultaneously the metabolites of four nitrofuran antibacterial agents, furazolidone, furaltadone, nitrofurazone and nitrofurantoin in animal muscle tissue. Sample clean-up and analyte enrichment was performed by solid-phase extraction (SPE) with a polystyrene sorbent following combined hydrolysis of the protein-bound drug metabolites and derivatisation of the homogenised tissue with 2-nitrobenzaldehyde. Limits of detection of 0.5–5 ng g^{-1} tissue and limits of determination of 2.5–10 ng g^{-1} tissue were achieved using electrospray ionisation in positive mode. Analyte identification and quantification was performed according to EU guidelines, using multiple reaction monitoring (MRM) with one precursor ion and two product ions as identifiers. The use of an internal standard in combination with the simplified sample preparation led to a sensitive and reliable analysis method. The yield of the derivatisation reaction was between 66 and 74% and the recovery of SPE reached 92–105% for all values between 10 and 500 ng g^{-1}. The developed analytical protocol has been applied to contaminated tissue samples of furazolidone- and furaltadone-treated pigs and allowed unequivocal identification and quantification of the metabolites. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Mass spectrometry; Furazolidone; Furaltadone; Nitrofurazone; Nitrofurantoin; Antibiotics

1. Introduction

The four drugs furazolidone, furaltadone, nitrofurazone and nitrofurantoin (Fig. 1) belong to the group of nitrofuran antibacterial agents (antibiotics), which have been widely used in the form of food additives for the treatment of gastrointestinal infections (bacterial enteritis caused by *Escherichia coli* and *Salmonella*) in cattle, pigs and poultry.

Following data which classified furazolidone as a mutagenic and genotoxic drug, legislation was enforced to remove this and similar compounds from the market. Since data concerning the toxicity of furaltadone, nitrofurazone and nitrofurantoin were insufficient, the European Union (EU) prohibited the use of nitrofuran antibiotics in food-producing animals by listing them in Annex IV of the Council Regulation 2377/90 [1]. Furazolidone was moved to Annex IV in June 1995 because the manufacturers were not able to provide any favourable data to

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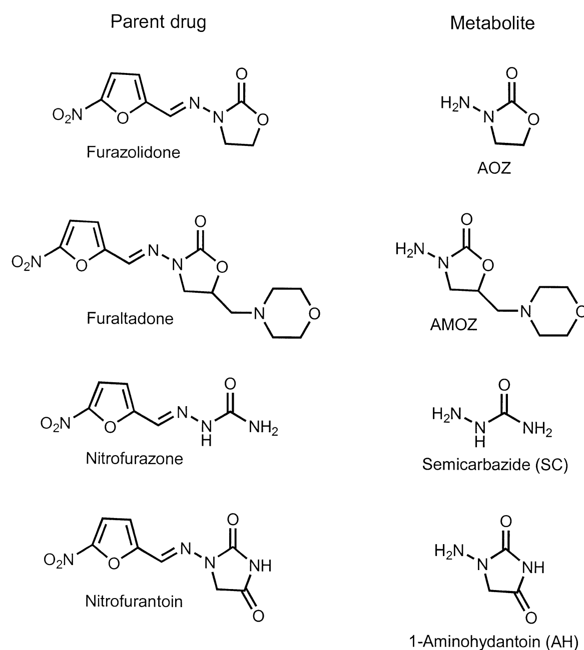

Fig. 1. Structures of the nitrofuran antibiotics and their free metabolites. (AOZ = 3-amino-2-oxazolidinone, AMOZ = 3-amino-5-morpholinomethyl-2-oxazolidinone).

justify further use. As a consequence, with effect from 1 January 1997, the application of all nitrofurans to food-producing animals in the EU is no longer allowed.

Because no maximum residue limits (MRLs) have been set, the goal of analytical methods has to be to reach a lowest possible detection limit. A detection of the drugs at ''any'' concentration should be sufficient to point to a violation of the law. Nevertheless, it has to be noted that contamination may not only occur from deliberate, direct misuse of the drugs but also from contaminated feed, environmental contamination (housing at the slaughterhouse) or transfer between animals [2].

Nitrofuran-type antibiotics are characterised by their rapid metabolism with half-lives in vivo in the range of not more than a number of hours. Therefore, the detection of the parent drugs in animal tissue is generally not very likely and useful. While most of the methods published in the literature still rely on their analysis [3–8], some research groups have focused on the detection of metabolites. Studies conducted with ^{14}C-labelled furazolidone have shown that protein-bound residues are formed, some of which contain the intact side chain AOZ (3-amino-2-oxazolidinone) [9–11]. It is possible to release these residues from the proteins under moderately acidic conditions. Later, the existence of analogous metabolites has been reported for the other three nitrofuran-derived drugs as well [12,13]. The free side chains and metabolites, respectively, corresponding to the drugs are shown in Fig. 1.

Due to the higher stability and longer residence time (between 4 and 9 days half-life time) of the bound metabolites, their detection is still possible when concentrations of the parent drugs have fallen below their detection limits. Respective analysis methods described in the literature [2,13–17] are based on the determination of the 2-nitrobenzaldehyde imine-type derivatives (Schiff bases) of the metabolites employing UV and mass spectrometric detection. Thermospray ionisation [2,14–16] or atmospheric pressure chemical ionisation (APCI) [17] were used for hyphenated LC–MS techniques. So far, only AOZ [2,14–16] and AMOZ (3-amino-5-morpholinomethyl-2-oxazolidinone, see Fig. 1) [17] have been target analytes involving also mass spectrometric detection. McCracken et al. reported on the application of a LC–MS method for routine analysis of a series of porcine kidney samples in Northern Ireland [15]. Of 200 examined samples in 1995, 32 (16%) contained detectable amounts of AOZ demonstrating that illegal use of these drugs occurs and routine monitoring is necessary.

No LC–MS method for the detection of residues of nitrofurazone and nitrofurantoin metabolites has been published so far. Therefore, it was our goal to develop a fast and simple analysis protocol enabling the simultaneous and unambiguous detection of the metabolites of four nitrofuran compounds. After a one-step extraction/derivatisation procedure, further sample preparation was simplified by introducing solid-phase extraction (SPE) replacing the time-consuming and more error-prone liquid–liquid extraction (LLE) applied in other methods. In combination with the use of an internal standard this led to lower limits of quantification and increased robustness of the whole procedure. Tandem mass spectrometry provided the highest degree of certainty in analyte identification and was employed in accordance with European Union guidelines.

2. Experimental

2.1. Chemicals and solvents

With the exception of semicarbazide (SC), the metabolites under investigation are not commercially available. Several milligrams of AOZ and AMOZ were provided by the Bundesanstalt für Tierseuchenbekämpfung (Federal Research Centre for Animal Diseases, Mödling, Austria) while 1-aminohydantoin (AH) was synthesised according to the three-step synthesis of Jack [18]. Semicarbazide hydrochloride (p.a. grade) was bought from Fluka (Buchs, Switzerland). Stock solutions were prepared in methanol, wrapped in aluminum foil and stored at 4°C for a maximum of 2 months.

Methanol (HPLC gradient grade), ethyl acetate (HPLC grade) and dimethyl sulfoxide (DMSO, spectroscopy grade) were supplied by Merck (Darmstadt, Germany), hydrochloric acid (pro analysi grade) by Riedel-de Haën (Seelze, Germany). Water was double-distilled prior to use. 2-Nitrobenzaldehyde (2-NBA), 4-nitrobenzaldehyde (4-NBA), both puriss. p.a., and ammonium acetate (p.a.) were obtained from Fluka (Buchs, Switzerland). Di-potassium hydrogen phosphate trihydrate (p.a.) was bought from Merck, sodium hydroxide (pharmaceutical grade) from Riedel-de Haën. Nitrogen for solvent evaporation (purity$>$99.0%) was produced with a nitrogen generator from Whatman (Haverhill, MA, USA).

2.2. Synthesis of 4-nitrobenzaldehyde semicarbazone (used as internal standard)

1.0 g (9.0 mmol) of semicarbazide hydrochloride and 5 g of sodium acetate were dissolved in 50 ml of a mixture of water and dimethyl sulfoxide (95:5, v/v). 0.5 g (3.3 mmol) of 4-nitrobenzaldehyde was added to this solution in small portions. After stirring for 60 min at room temperature, the semicarbazone, a yellow precipitate, was filtered off, washed thoroughly with water–DMSO (95:5, v/v) to remove the excess of semicarbazide and dried in vacuo. The product was examined by mass spectrometry and ^{1}H-NMR.

2.3. LC–MS–MS analysis

The LC–MS–MS system consisted of an Agilent Series 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) connected to a PE Sciex API 365 triple quadrupole mass spectrometer (PE Sciex Instruments, Thornhill, Canada) equipped with an Ion Sprayer (pneumatically assisted electrospray) interface. The column used was an Inertsil® ODS-3, 150×3 mm I.D., 5 μm (GL Sciences, Tokyo, Japan) connected to an Inertsil® ODS-3 pre-column, 10×3 mm I.D., 5 μm. A mixture of methanol and water (50:50, v/v) with 10 m*M* ammonium acetate was used as mobile phase at a flow rate of 0.5 ml min^{-1} and at a temperature of 25°C. The solvent flow was split in a ratio of 1:50 prior to MS detection.

MS detection was performed in the positive ion mode using multiple reaction monitoring (MRM). The precursor/product ion combinations are listed in Table 1. The dwell time for each fragmentation pathway was 300 ms. The electrospray voltage was set to 5600 V and the collision energy to 15 eV. Nitrogen was used as collision gas.

2.4. Sample preparation

10 g of minced muscle tissue were homogenised in approximately 90 ml 0.125 *M* aqueous hydrochlo-

Table 1
Parent/fragment ion combinations used for multiple reaction monitoring[a]

Compound	Parent ion (m/z)	Fragment ions (m/z)
2-NBA-SC	209.0	166.0, 192.0
2-NBA-AOZ	236.0	134.0, 149.0
2-NBA-AH	249.0	134.0, 178.0
2-NBA-AMOZ	335.0	262.0, 291.0
4-NBA-SC (internal standard)	209.0	192.0

[a] ESI, positive ion mode.

ric acid using an Ultra Turrax® T25 (IKA Labortechnik, Staufen, Germany) at 24000 rpm. The homogenate was transferred to a 100-ml volumetric flask and the volume adjusted to 100 ml with 0.125 *M* HCl. Ten ml of the homogenate were transferred to a 24-ml glass vial with screw cap and 400 μl of a freshly prepared 2-nitrobenzaldehyde solution (50 m*M* in DMSO) were added. The reaction mixture was kept for 16 h (overnight) in a waterbath at 37°C.

After this period of time, samples were removed and allowed to cool to room temperature. A pH-value of about 7.4 was adjusted by addition of 12.5 ml of 0.1 *M* aqueous dipotassium hydrogen phosphate and 1.25 ml of 0.8 *M* aqueous sodium hydroxide. After this step, 100 μl of a freshly prepared solution of the internal standard 4-nitrobenzaldehyde semicarbazone (20 μg ml^{-1} in DMSO) were added. The homogenised sample solution (about 24 ml) was filtered through a type 589 paper filter (Schleicher & Schüll, Dassel, Germany) and afterwards washed with 3 ml of water. The filtrate was applied to a 200-mg LiChrolut® EN SPE column (Merck, Darmstadt, Germany) preconditioned with 3 ml of ethyl acetate, followed by 3 ml of methanol and 5 ml of water. After sample loading the column was washed with 2.5 ml of water and dried by sucking through air until the sorbent completely changed its colour from brown to orange. Elution of the analytes was achieved with 3 ml of ethyl acetate. The eluate was evaporated under a stream of nitrogen and the residue redissolved in 250 μl of mobile phase. A 50-μl volume of this solution was injected into the LC–MS–MS system. During the whole procedure, direct exposure of the sample solutions to light was avoided to prevent any photodegradation.

2.5. Determination of limits of detection (LOD) and quantification (LOQ), linear range

Samples for the generation of calibration curves were prepared by adding aliquots of blank tissue homogenate to the residue of evaporated standard solutions of AOZ, AMOZ, AH and SC. Linearity was tested in the range of 1–800 ng g^{-1}. The limits of detection were determined in dedicated experiments in the range of 0.25–5 ng g^{-1} tissue.

2.6. Recovery experiments

For recovery experiments, 2-NBA derivatives were synthesised in analogy to the synthesis of 4-nitrobenzaldehyde semicarbazone as described above. 2-NBA-AOZ and 2-NBA-AMOZ were prepared using microscale techniques due to the limited amounts of available substance. Accordingly, the derivatives have not been isolated, for structure identification MS data were taken into consideration. The experiments were carried out in duplicate at analyte concentrations of 10, 50, 100, 250 and 500 ng g^{-1}.

Derivatisation efficiency in the tissue matrix was determined by adding the underivatised analytes (metabolites) to blank tissue homogenate before the derivatisation step and adding the corresponding amounts of 2-NBA derivatives to identical samples after the derivatisation procedure. Recovery of solid-phase extraction was determined by adding the 2-NBA derivatives before and after the SPE step.

2.7. Contaminated tissue

In the course of an animal feeding study at the Bundesanstalt für Tierseuchenbekämpfung (Mödling, Austria), furazolidone and furaltadone were administered in therapeutic doses to one pig per investigated drug. For these studies, 3.5 g of each drug were dissolved in 10 ml ethanol and over a period of 3 days, 1-ml aliquots of the solutions were added to pig feed rations (approximate dosage: 5 mg per kg body weight per portion). Three to four rations were offered to the pigs for three consecutive days. After the end of the third day, the pigs were killed and muscle samples collected. Samples were stored at −20°C prior to analysis.

3. Results and discussion

3.1. Method development

Several crucial aspects regarding sample preparation, chromatography and MS detection had to be considered during the development of this assay.

All metabolites have molecular masses between 75

and 201 g mol^{-1}. Due to highly abundant mass spectrometric background noise in this mass range, a low ionisation efficiency of the analytes and their non-specific fragmentation behaviour (predominatly loss of ammonia, water or carbon dioxide), the MS detection sensitivity is relatively poor. Consequently, derivatisation of the free amino group of the target analytes was carried out (see Fig. 2) to achieve compounds with more favourable properties. 2-Nitrobenzaldehyde (2-NBA), which has been described by various authors [2,3,9–17] requires a relatively long reaction time of about 15 h. To circumvent this time-consuming step, several other aromatic aldehydes (pyridine-3-carboxaldehyde, 2,4-dinitrobenzaldehyde, 2-hydroxy-5-nitrobenzaldehyde) were, therefore, examined by us to increase the detection sensitivity and/or to shorten the reaction time. None of these derivatising agents gave, however, any significant improvement. Thus, 2-NBA was used for all further experiments. The extraction/derivatisation procedure was found to be completed after a reaction time of approximately 15 h, no further increase of analyte signals was observed after this period. The one step procedure of simultaneous hydrolysis of tissue-bound metabolites and derivatisation was favoured over a two step routine due to its simplicity but it was not directly transferable and applicable to liver tissue due to the entirely different matrix as preliminary experiments revealed. At this point, it should be noted that the described method quantitates the sum total of free and bound metabolites and no differentiation was made. From a pharmacokinetic point of view, this aspect would be of interest but for the aim of this work only the identification of any metabolite as identifier for the use of nitrofuran antibiotics was in focus.

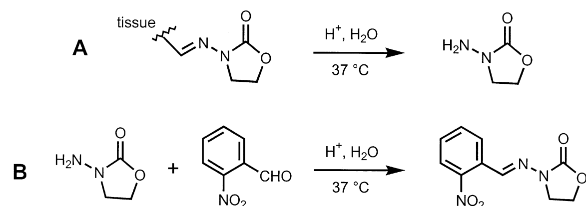

Fig. 2. Release of the side chains from the protein-bound metabolites (A) and simultaneous derivatisation with 2-nitrobenzaldehyde (B) in the course of sample preparation; example: furazolidone.

In contrast to previous methods [2,14–17], sample clean-up was mainly performed in one step by solid-phase extraction replacing the liquid–liquid extraction protocol. Overall, this resulted in a significant reduction of analysis time and solvent consumption. In addition, SPE is less error-prone and is ideally suited for automatisation. A polystyrene–divinylbenzene copolymer was used as sorbent material which enables a strong and quite selective retention of the nitro-aromatic derivatives (π–π-interaction) while most of the matrix compounds are more weakly retained. Quantitative desorption of the analytes from the SPE column was achieved with 3 ml of ethyl acetate as eluent.

Several reversed-phase columns were evaluated for HPLC. In view of the inherent matrix effects on the electrospray ionisation process of the analytes of interest, the Inertsil® column exhibited the best separation characteristic for analytes and the majority of the matrix components with the given mobile phase composition. Besides, the mixture of methanol and water (50:50, v/v) with 10 m*M* ammonium acetate which was chosen for the experiments gave the best compromise with regard to short analysis time and sufficient chromatographic separation for 2-NBA semicarbazone and 4-NBA semicarbazone which was used as internal standard (IS). Acetonitrile as organic modifier showed no advantages concerning sensitivity or stability of MS detection while the addition of ammonium acetate improved the intensity of the MS response significantly.

Atmospheric pressure chemical ionisation (APCI) has previously been used as interface [17]. However, due to its poor sensitivity this ionsation technique was not applicable to the API 365 instrument used in this study. Thermal degradation of the analytes which may be attributed to the design of the ion source might be a reason for this observation. As a consequence, electrospray ionisation was chosen which is commonly known to be more influenced by coeluting matrix compounds compared to APCI. Because of its structural similarity to the analytes and similar chromatographic behaviour, 4-NBA semicarbazone was used as a suitable internal standard in order to compensate for variations of the MS

response which may be caused by coeluting matrix compounds and day-to-day performance differences. A disadvantage of this internal standard protocol is, however, that 4-NBA semicarbazone had to be added after the derivatisation step with 2-NBA followed by the pH adjustment of the sample solution to pH 7.4 to avoid any side-reaction with the 2-NBA derivatives which might occur under acidic conditions, e.g. exchange of the 2-nitrophenyl group with the 4-nitrophenyl group due to equilibrium reactions. While this compromise does not compensate analyte loss due to incomplete extraction, the high recoveries and derivatisation yields of further sample preparation steps limit a further loss and ensure sufficient overall sensitivity of the method (see below). 4-NBA semicarbazone exhibits a distinctly lower sensitivity than its 2-NBA counterpart under the experimental conditions (voltage settings, MRM transition used) which made it necessary to add relatively large amounts equivalent to 2 $\mu g\ g^{-1}$ tissue. The amount of internal standard was chosen so that similar signal areas were obtained for the I.S. and 100 $\mu g\ kg^{-1}$ 2-NBA-SC.

Generally, ionisation in positive ion mode gave better results compared to the negative ion mode. To reach highest sensitivity, optimal MS–MS parameters proved to be quite different for the four analytes. Since MS sensitivity was found to be lowest for the AH and SC derivatives, parameter sets were optimised in view of these compounds, accepting a decrease in sensitivity of 20–30% for AOZ and AMOZ derivatives. Substance identification and quantification was performed by multiple reaction monitoring (MRM) selecting one parent ion and two product ions for each analyte, which is in accordance with the EU guidelines for unambiguous positive identification and quantification of analytes [19].

3.2. Method evaluation and discussion

The method detection limits for AOZ and AMOZ, set at a signal-to-noise ratio of 3, reached by the presented analysis protocol (0.5 $ng\ g^{-1}$, see also Table 2) were comparable to those already reported in the literature [14,17]. The sensitivity for the AH and SC analytes, which, to our knowledge, have not been described before, were significantly lower, but still in the low ppb range. As previously noted, MS settings were not optimised for AOZ and AMOZ, so that even higher sensitivity for these analytes may be feasible (see above). Limits of quantification, which were set at the lower end of the linear range, were 2.5 $ng\ g^{-1}$ for AOZ and AMOZ and 10 $ng\ g^{-1}$ for AH and SC, with linear ranges from the limit of quantification up to 800 $ng\ g^{-1}$. Linearity of the detector response was observed up to 800 ppb.

Previously reported limits of quantification for AOZ and AMOZ (although different definitions for the LOQ were used) were 10 $ng\ g^{-1}$ [14,17]. The improved data of the present method indicate the usefulness of an internal standard and of the highly efficient sample clean-up by using selective SPE sorbent materials.

Since the method described in this contribution may not yet be directly applicable as a reference method, further method evaluation was not performed. The authors are well aware that for its legal use in certified laboratories, the procedure will have to be further validated according to internationally accepted guidelines [19,20] which was, however,

Table 2
Method performance data for muscle tissue samples

2-NBA derivative of . . .	AOZ	AMOZ	SC	AH
Typical retention factor[a]	1.1	1.8	1.2	0.9
Limit of detection [$ng\ g^{-1}$]	0.5	0.5	3.0	5.0
Limit of quantification [$ng\ g^{-1}$]	2.5	2.5	10.0	10.0
Linear range [$ng\ g^{-1}$]	2.5–800	2.5–800	10.0–800	10.0–800
Mean reaction efficiency[b] [%, $n=10$]	74.2±5.9	71.8±7.2	66.3±5.2	68.3±1.4
Mean SPE recovery[b] [%, $n=10$]	96.6±4.8	102.2±4.9	91.8±6.8	105.4±4.0

[a] Dead time 2:20 min.
[b] Within the range measured.

beyond our focus at this stage. In addition, this would imply the synthesis of more reference standard materials of all analytes, possibly including deuterated congeners as internal standards.

The total ion chromatogram (TIC) of a spiked sample containing 10 ng g^{-1} of each analyte, together with the TIC of a blank sample, is shown in Fig. 3.

Fig. 4 shows individual MRM chromatograms of the four analyte derivatives (at the 10 ng g^{-1} level) and the I.S. No interfering matrix signals were observed for all MRM transitions. As Fig. 4A shows,

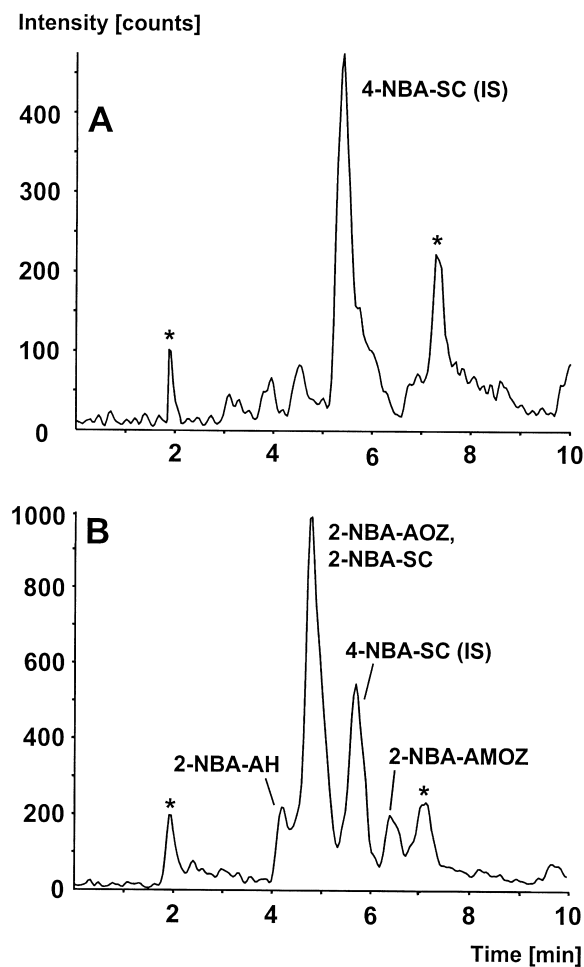

Fig. 3. Total ion chromatograms of (A) a blank sample and (B) a spiked muscle tissue sample containing 10 ng g^{-1} of each analyte. An asterisk (*) denotes major matrix signals.

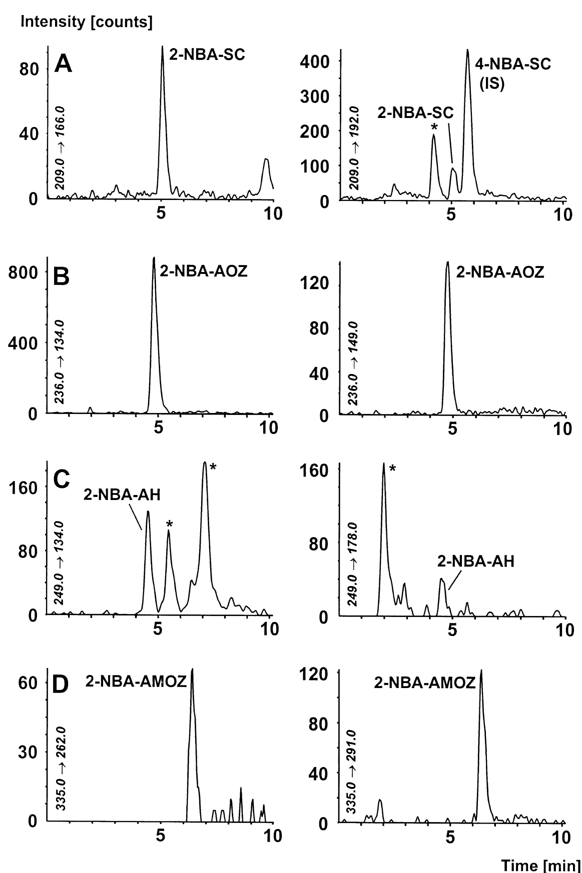

Fig. 4. MRM chromatograms of a spiked muscle tissue sample containing 10 ng g^{-1} of each analyte. (A) SC derivative and internal standard, m/z 209.0→166.0 (left) and 209.0→192.0 signal (right); (B) AOZ derivative, m/z 236.0→134.0 (left) and 236.0→149.0 signal (right); (C) AH derivative, m/z 249.0→134.0 (left) and 249.0→178.0 signal (right); (D) AMOZ derivative, m/z 335.0→262.0 (left) and 335.0→291.0 signal (right). An asterisk (*) denotes major matrix signals.

the internal standard is well separated from the analyte 2-NBA-SC derivative. This is necessary because the fragmentation pattern of the two isomers in tandem MS is very similar and the same precursor/product ion combination (m/z 209.0→192.0) was used for both compounds. In contrast, no interferences were observed for the other analytes so that their complete chromatographic separation is not required.

It was found in preliminary experiments that the analytes themselves did not influence each others'

signals even at concentrations differing by two orders of magnitude. Therefore, the (remaining) matrix constituents eluting in the region of the analytes are mainly responsible for some ion suppression effects which were observed but were very well compensated by the internal standard.

Data about the reaction efficiency and solid-phase extraction recovery are collected in Table 2, indicating that almost no loss of the four analytes occurs during the SPE step. In contrast, the derivatisation reaction exhibits yields around 70% and no statistically significant variations at different concentration levels could be observed.

At this point, it has to be noted that the extraction efficiency of the metabolites of the nitrofuran-type antibiotics from contaminated tissue samples could not be fully examined as we could only validate our method by spiking tissue samples. However, after conducting experiments using ^{14}C-labelled furazolidone, Hoogenboom et al. reported [9,10] that only a small fraction of the analytes could be released from authentic samples. This finding points to much higher actual concentrations in the contaminated tissue and, despite the limited bioavailability of the metabolites, overall method sensitivity is therefore of great importance. Efforts to increase the extraction yield which were undertaken, e.g. increasing temperature and reaction time, did not prove to be successful and resulted mainly in an increase of interferences.

3.3. Application of the method to contaminated pig tissue samples

In addition to the examination of the method performance for spiked samples, the assay was also applied to real world samples for two of the analytes, AOZ and AMOZ.

In the course of an animal feeding study (see also Experimental section), furazolidone and furaltadone were administered in therapeutic doses to pigs over a period of 3 days. After the pigs had been slaughtered, muscle samples were collected, stored at −20°C and analysed by the developed LC–MS–MS method. Due to the scope of the feeding study, tissue samples of nitrofurazone- and nitrofurantoin-treated pigs (leading to free and bound SC and AH metabolites) were not available to us; however, a comparison with literature data could be made with the samples we obtained.

Chromatograms of respective meat samples of the pigs are shown in Fig. 5. Both AOZ and AMOZ could be identified unambiguously and quantified at levels of 100 and 30 ng g^{-1}, respectively. The determined content of the AOZ metabolite is of the same magnitude as the concentration values obtained by McCracken et al. [15,16] when similar amounts of furazolidone were applied, considering that no discrimination between free and bound metabolites was made in our work.

The half-life of AOZ residues in vivo has been determined to be between 4 and 9 days [10],

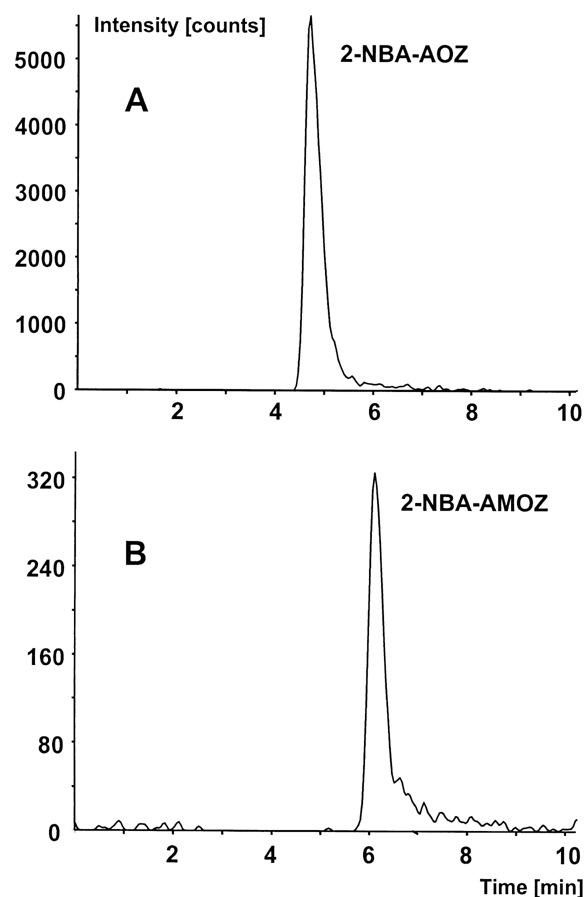

Fig. 5. MRM chromatograms of contaminated porcine tissue samples from an animal feeding study containing 100 ng g^{-1} AOZ (A, *m*/*z* 236.0→134.0 signal) and 30 ng g^{-1} AMOZ (B, *m*/*z* 335.0→291.0 signal).

depending on the nature of the tissue, with the highest stability in muscle tissues. Judging from these data and from the detection limits of the present method, the illegal use of this group of antibacterial drugs should be detectable at least for several weeks after the last administration depending on the tissue and the individual analyte.

Furthermore, it has been reported that AOZ concentrations in liver tissue are several times higher than in muscle tissue [10,11,15,16]. This indicates that detection of nitrofuran type antibiotics would be possible over an even longer period of time. Since the nature of the liver matrix is considered to be different and/or more difficult for sufficient isolation of the metabolites and removal of possibly interfering matrix constituents, the developed LC–MS–MS method needs to be tested in more detail for its applicability also to this matrix. This is a matter for further investigations in our laboratory.

However, the sensitivity of the present method and its straightforward clean-up procedure, which may have to be modified for liver tissue samples, may qualify this assay as a suitable tool for the determination of nitrofuran antibiotic residues in muscle tissue, especially considering that there are cases where liver samples might not be readily available for analysis.

4. Conclusion

A sensitive LC–MS–MS method has been established which allows the simultaneous detection of four nitrofuran antibacterial drugs in animal muscle tissue via their metabolites. In its course, the bound analytes (metabolites) are released from tissue by acidic hydrolysis and simultaneously derivatised using 2-nitrobenzaldehyde. The introduction of an analyte-selective solid-phase extraction step greatly facilitated an efficient sample clean-up. Detection limits of the analytes ranged from 0.5 to 5 ng g^{-1}, and linearity of the detector response was excellent between the limit of determination up to 800 ng g^{-1}. Electrospray ionisation tandem mass spectrometry was used for detection and two fragment ions per analyte have been used as additional identifiers taking EU regulations into account.

A simple sample preparation protocol including solid-phase extraction and the addition of an internal standard has led to a sensitive and robust method. The analysis of muscle tissue samples from animals treated with furazolidone and furaltadone allowed unequivocal identification of the AOZ and AMOZ metabolites at concentrations which were expected from literature values and which were approximately two orders of magnitude above the method's detection limit.

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