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Analytica Chimica Acta 586 (2007) 383–393
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Validation of a method for the detection and confirmation of nitroimidazoles and the corresponding hydroxy metabolites in pig plasma by high performance liquid chromatography–tandem mass spectrometry

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Received 19 June 2006; received in revised form 18 December 2006; accepted 8 January 2007
Available online 13 January 2007

Abstract

Nitroimidazoles (Ronidazole, Dimetridazole, Metronidazole, Ipronidazole) and their hydroxy metabolites are banned substances with antibiotic and anticoccidial activity. They are suspected to be carcinogenic and mutagenic. Since nitroimidazoles showed an inhomogeneous distribution and a rapid degradation in incurred muscle samples, plasma is the preferred target matrix for residue analysis. The analytical method of Polzer et al. [J. Polzer, C. Stachel, P. Gowik, Anal. Chim. Acta 521 (2004) 189] was adapted for liquid chromatography–tandem mass spectrometry detection and was validated in house according to the Commission Decision 2002/657/EC. The method is specific for all nitroimidazole except for Ipronidazole and its metabolite, due to interferences at their retention times in chromatograms of blank plasma and reagents samples. The absence of a matrix effect enables the use of a (linear) calibration curve in solution for quantitation. The apparent recovery (obtained after correction with a deuterated internal standard) is between 93% and 123%, except for the metabolite of Metronidazole (58–63%). The repeatability (CVr = 2.49–13.39%) and intralaboratory reproducibility (CVRW = 2.49–16.38%) satisfy the Horwitz equation. The obtained values for the detection capacity (CCβ) range from 0.25 to 1 μg L−1, while values obtained for the decision limit (CCα) are below CCβ.

Keywords: Nitroimidazoles; Plasma; Liquid chromatography–tandem mass spectrometry; Validation; Commission Decision 2002/657/EC

1. Introduction

Ronidazole (1-methyl-2-[(carbamoyloxy)methyl]-5-nitroimidazole, RNZ), Dimetridazole (1,2-dimethyl-5-nitroimidazole, DMZ), Metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, MNZ), Ipronidazole (2-isopropyl-1-methyl-5-nitroimidazole, IPZ) and their corresponding hydroxy metabolites 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI, the metabolite of both RNZ and DMZ), 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (MNZ-OH) and 1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole (IPZ-OH) are banned substances with antibiotic and anticoccidial activity. They were used to prevent and treat histomoniasis and coccidiosis in poultry. They have been also used for the treatment of genital trichomoniasis in cattle and haemorrhagic enteritis in pigs. They are suspected to be carcinogenic and mutagenic. Therefore, RNZ, DMZ and MNZ have been included in Annex IV of Council Regulation 2377/90/EC [1], whereas IPZ is a non-authorized substance for veterinary purposes. Many methods for the determination and confirmation of nitroimidazoles in different matrices, mainly in meat and eggs, have been published [2–12], either using LC–MS/(MS) or GC–MS/(MS). Recent studies on the stability and homogeneity of nitroimidazoles in incurred muscle samples have been carried out by the BVL (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Berlin, Germany) which is the Community Reference Laboratory (CRL) responsible for nitroimidazoles [13,14]. It was shown that the analyte distribution in turkey muscle was not homogeneous; moreover a rapid decline in analytes at storage above 4 °C was observed. For plasma and retina samples however, the analytes were stables during storage under the same conditions, found in considerably higher concentrations and could be detected for a longer period of time after withdrawal of the medication. Therefore, plasma and retina have been recommended as target matrices for the residue control of nitroimidazoles, especially in poultry. All the studies performed by the CRL, employing

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doi:10.1016/j.aca.2007.01.009

gas chromatography–mass spectrometry (GC–MS), required derivatisation prior to analyses. In the course of the applied silylation, RNZ and HMMNI formed an identical derivative and it was therefore not possible to distinguish between these two compounds. As far as we know, no methods coupling extraction of plasma samples and LC–MS/MS detection have been published to confirm nitroimidazoles. Such a method, using the extraction protocol of the CRL [13] and a confirmatory analysis by liquid chromatography coupled to atmospheric pressure chemical ionisation mass spectrometry (LC–APCI-MS/MS), has been validated according to the Commission Decision 2002/657/EC [15] and is presented here. We used pig plasma to perform this validation study due to the difficulty to obtain large amounts of poultry plasma.

2. Experimental

2.1. Chemicals and reagents

All analytical standards of nitroimidazoles, including deuterated substances, were provided by the CRL (BVL, Berlin, Germany). Pig plasma was purchased from Centre d'Economie Rurale (CER, Laboratoire d'Hormonologie, Marloie, Belgium). Water was of Milli-Q quality (Millipore Corp., Bedford, MA, USA). All organic solvents were of LC analytical grade and purchased from VWR (Leuven, Belgium). A sodium chloride/potassium dihydrogen phosphate buffer ($NaCl/KH_2PO_4$) was prepared by dissolving 5.84 g of NaCl (VWR, p.a. quality) and 13.61 g of KH_2PO_4 (VWR, p.a. quality) in 950 mL of water, adjusted to pH 3 with 25% hydrochloric acid (HCl) and filled up to 1000 mL with water. For the enzymatic hydrolysis, Protease Type XVIII (EC no. 232-642-4) from Sigma (Bornem, Belgium) was used at a concentration of 80 mg mL^{-1} in 0.002 mol L^{-1} HCl. The solid phase extraction (SPE) step was performed using Chromabond XTR-cartridges® 45 mL, 8300 mg from Macherey-Nagel (Filter Service, Eupen, Belgium). The LC mobile phase used to reconstitute the dried extracts before injection was prepared by mixing 93% of mobile phase A (0.1% acetic acid in water) with 7% of mobile phase B (acetonitrile). Individual stock standard solutions at 1 mg mL^{-1} in methanol were prepared and stored at 4 °C for 1 year. Individual intermediate standard solutions (10 and 1 μg mL^{-1}) and working standard solutions (mixture of (deuterated) nitroimidazoles) were prepared in the LC mobile phase (93:7) and stored at 4 °C for 6 months.

2.2. LC–MS/MS instrumentation

A quaternary delivery LC pump (HP 1100, Hewlett-Packard) linked to an automatic sampler (Gilson 231 XL, Gilson, The Netherlands) and coupled to a Quattro II triple quadrupole mass spectrometer (Micromass Inc., Manchester, UK) was used for the measurements. The separation was achieved using a reversed phase (C18) Genesis column from Jones Chromatography (4 μm; 250 mm × 3 mm I.D.) provided by Grace Vydac (USA) and maintained at 30 °C using an oven. The chromatographic separation was performed in a gradient mode using water acidified with 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B), at a flow rate of 0.6 mL min^{-1}. The initial conditions (0–16 min) were 93% acidified water and 7% acetonitrile. Then the conditions changed to 30% acidified water and 70% acetonitrile between 16 and 21 min and these proportions were maintained up to 25 min. Finally, the conditions returned to the initial ones in 2 min (25–27 min), and were maintained until the chromatographic run of 30 min ended. The ionisation mode used was the positive APCI mode, with the corona pin discharge

Table 1
Monitored transitions in MRM mode, collision energies, cone voltages and retention times of nitroimidazoles, hydroxynitroimidazoles and deuterated internal standards (IS)

Analyte	Parent ion (*m/z*)	Daughter ions (*m/z*)	Collision energy (eV)	Cone voltage (V)	Retention time (min)
MNZ-OH	188	144	15	25	6.05
	188	**126**	20	25	
HMMNI	158	**140**	15	20	8.23
	158	110	20	20	
MNZ	172	**128**	15	25	9.12
	172	82	20	25	
RNZ	201	**140**	15	20	11.06
	201	110	20	20	
DMZ	142	**96**	20	30	13.32
	142	81	25	30	
IPZ-OH	186	**168**	15	20	22.28
	186	122	20	20	
IPZ	170	**124**	20	25	23.46
	170	109	25	25	
HMMNI-d3 (IS)	161	143	10	15	8.14
MNZ-d3 (IS)	175	131	15	20	9.07
RNZ-d3 (IS)	204	143	15	20	10.97
DMZ-d3 (IS)	145	99	15	25	13.09
IPZ-OH-d3 (IS)	189	171	20	15	22.28
IPZ-d3 (IS)	173	127	15	20	23.46

Bold figures refer to the daughter ion transitions giving the most intense response and used for quantitation.