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Analytica Chimica Acta 529 (2005) 173–177
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Determination of residues of malachite green in finfish by liquid chromatography tandem mass spectrometry

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Received 31 May 2004; received in revised form 4 August 2004; accepted 4 August 2004
Available online 1 September 2004

Abstract

A liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of leuco-malachite green (LMG) in various fish tissues is described. LMG, which is the primary metabolite of the parasiticide and fungicide malachite green (MG), is the targeted analyte to reveal abuse of this veterinary drug in fish. After extraction using McIlvaine buffer and acetonitrile, the extract was purified on an aromatic sulphonic acid solid-phase extraction column. After conversion of LMG into MG by post-column oxidation with PbO_2, the effluent was analysed by LC-MS/MS in the multiple reaction monitoring (MRM) mode. The MS–MS trace *m/z* 329 → *m/z* 313 was used for quantification of LMG and, for salmon, gave an averaged decision limits (CCα; α 1%) and detection capability (CCβ; β 5%) of 0.11 and 0.18 $\mu g\,kg^{-1}$, respectively, with a measurement each of three consecutive days. The last values were comparable for those for MG. Other traces were used to collect sufficient identification points to establish the identity of this prohibited veterinary drug, which was achieved at CCβ and higher. These values were comparable for other tested species, including pangasius, tilapia, trout and Victoria perch. Recoveries ranged from 66% in trout at 0.4 $\mu g\,kg^{-1}$ to 112% in pangasius at 0.1 $\mu g\,kg^{-1}$. Three out of nineteen samples including pangasius, salmon, shrimps and trout bought in local shops, revealed detectable amounts of residues, i.e. in excess of CCα, and were considered non-compliant. The findings demonstrate the suitability of the presented analytical method to detect residues of malachite green in various aquatic species at relatively low residue levels.

Keywords: Residue analysis; Veterinary public health; Food safety; Veterinary drugs; Mariculture; Aquaculture

1. Introduction

Malachite green (MG) is a popular, but potentially carcinogenic, mutagenic and terotogenic ectoparasiticide, fungicide and antiseptic used in fish farming [1,2]. Its public health-threatening (illegal) use in edible fish species, such as in trout and eel, has been recognized since 1933 [2]. When absorbed by the fish, the major part of MG is rapidly reduced to its non-chromophorous metabolite leuco-malachite green (LMG) [3–5]. For this reason, LMG is the prevalent residue found in residue analyses in treated aquatic animals.

The drug acquired recently much attention from residue analysts, as it was found in cultured Atlantic salmon and hake (cf. weekly EC Rapid Alert Reports for Food and Feed 2003/2004). We described a liquid chromatography (LC) method with detection at 620 nm for the determination of residues of MG, which was validated meeting the quality criteria published in Commission Decision 93/256/EC [6]. This method gave a limit of detection at 1 $\mu g\,kg^{-1}$, whereas the European minimum required performance limit (MRPL), a quality parameter for residue laboratories, is set at 2 $\mu g\,kg^{-1}$ (Commission Decision 2004/25/EC). Reference [6] gives an overview of methods described for detection of residues of MG. Recently, in addition to work described here, a gas chromatography mass spectrometry (GC–MS) [7] and another liquid chromatography tandem mass spectrometry (LC-MS/MS) method [8] were presented at the fifth edition of the

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doi:10.1016/j.aca.2004.08.009

EuroResidue Conference. Like the method presented here, both GC–MS and LC-MS/MS methods had favourable performance securing the detection and identity of the residues of MG at concentrations far below the settled MRPL.

Samples considered suspected by LC analysis [6], were confirmed by LC-MS/MS analysis. The present study reports the validation of this LC-MS/MS method for the determination of residues of MG in salmon, trout, pangasius, tilapia and Victoria perch meeting the quality criteria of Commission Decision 2002/657/EC.

2. Experimental

2.1. Reagents and chemicals

All chemicals used were of analytical grade unless stated otherwise. Acetic acid, acetonitrile, ascorbic acid, citric acid monohydrate, lead(IV)oxide, di-sodium hydrogen phosphate dihydrate, were purchased from Merck (Darmstadt, Germany). Ammonium hydroxide 25% (m/v), dichloromethane, methanol (HPLC-grade), sodium acetate, sodium perchlorate monohydrate, *para*-toluenesulfonic acid (*p*-TSA) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Brilliant green and *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) were from Aldrich (Steinheim, Germany), and celite from Acros (Geel, Belgium). Leucomalachite green was delivered by Sigma Chemical Company (St. Louis, MO, USA) and malachite green oxalate (Vetrenal reference standard) by Riedel-de-Haën (Seelze, Germany). Aromatic sulfonic-acid-bonded SPE columns (3 ml, 500 mg) were purchased from J.T. Baker. Water was of Milli-Q quality (Millipore, Bedford, USA).

A McIlvaine solution at pH 3.0 was prepared by mixing 18.9 ml of 0.2 M sodium hydrogen phosphate and 81.1 ml of 0.1 M citric acid, whereas these volumes were 62.5 and 37.5 ml, respectively, to obtain a McIlvaine solution at pH 6.0. Fish and shrimps were bought at the fish market and local stores.

2.2. Extraction

Fish samples were processed as described with minor modifications [6]. Briefly, 2 g homogenized fish tissue material was weighed in a 50-ml tube, and 2 ml McIlvaine buffer pH 3, 100 μl of 1 M *para*-toluenesulfonic acid, 50 μl of 1 mg ml^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride, and 12 ml acetonitrile were added. Extraction was supported on a platform shaker at 500 rpm for 10 min. After centrifugation at 3500 × *g* at 15 °C and for 10 min, the supernatant was collected and the residue re-extracted with 2 ml McIlvaine buffer pH 6.0 and 12 ml acetonitrile. Following centrifugation at the same speed at 15 °C for 10 min, supernatants were combined and mixed with 6 ml dichloromethane to remove water. The organic phase was passed through an aromatic sulfonic acid SPE column (J.T. Baker, 500 mg, 3 ml), which was conditioned with 2 ml acetonitrile/dichloromethane 80/20 (v/v) prior to use. The analyte-containing column was washed with 1.5 ml methanol and dried in a stream of nitrogen gas. Elution of the analytes was obtained with 4 ml of a mixture containing 90% (v/v) methanol, 5% (v/v) of 1 mg ml^{-1} ascorbic acid and 5% (v/v) of 25% (m/v) aqueous NH_4OH, which was prepared just before use. The collected eluate was dried under a stream of nitrogen gas at ambient temperature. Finally, residual material was dissolved in 500 μl of a mixture of 50 mM acetate buffer pH 4.5/acetonitrile 40/60 (v/v).

2.3. LC-MS/MS

Chromatography of 10-μl samples was performed on a Phenomenex Luna C_{18} column 150 mm × 2.0 mm packed with 3 μm reversed phase column material. The mobile phase consisted of 50 mM acetate buffer pH 4.5/acetonitrile 25/75 (v/v) and was pumped at 200 μl min^{-1}. An in-line post-column reactor (20 mm × 2.0 mm) filled with a mixture of PbO_2 and celite in a weight ratio of 3:1 converted the lipophilic LMG into the cation MG. The eluent was then introduced into the electrospray ionization (ESI) chamber of the mass-spectrometer (Sciex API 365). The ESI interface operated at an ionization voltage of 5500 V and a temperature of 400 °C. The entrance, declustering and focussing potentials were set at −9, 40 and 180 V, respectively. Tandem MS analysis was performed using the multi reaction monitoring (MRM) mode. The collision energy (CE) was set separately for each product-ion trace monitored: *m/z* 329.3→ *m/z* 313.0 (CE 45 V), *m/z* 329.3→ *m/z* 208.0 (CE 55 V), 329.3→ *m/z* 165.0 (CE 75 V). For quantification purposes, trace *m/z* 329.3→ *m/z* 313.0 was used, while the other traces confirmed the presence of LMG. In [9] a representation of the chemical structures of MG and LMG and fragmentation of MG in the mass-spectrometer is shown.

2.4. Evaluation of data

Concentration of residues of MG was determined using brilliant green as internal standard as described [6]. The linear regression analysis of peak area against concentrations of the spiked samples, which were prepared as described above, gave the following description of the curve:

$$Y_{\text{peak area}} = aX_{\text{concentration of analyte}} + b \quad (1)$$

Through which decision limit (CCα) for prohibited substances, as MG/LMG, at α 1% was obtained [10]:

$$\text{CC}\alpha = b + 2.33\text{S.D.}_b \quad (2)$$

wherein S.D.$_b$ is the standard deviation of *b*.

Detection capability (CCβ) at β 5% was then calculated as:

$$\text{CC}\beta = \text{CC}\alpha + 1.64\text{S.D.}_{\text{CC}\alpha} \quad (3)$$