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Simultaneous determination of malachite green and its metabolite leucomalachite green in eel plasma using post-column oxidation

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Abstract

A rapid HPLC method with solid-phase extraction (SPE) clean-up for malachite green (MG) and leucomalachite green (LMG) in eel plasma was developed. MG and LMG were extracted with a buffered methanolic solution. The extract was subjected to aromatic sulphonic acid SPE. MG and LMG were eluted from the SPE column with methanol after a treatment with ammonia gas. The reconstituted eluate was analyzed on a Chromspher B column with acetonitrile–ion-pair buffer (pH 4.0) (6:4, v/v) as the mobile phase and detection at 610 nm after post-column oxidation with PbO_2. The average recoveries for MG and LMG over the linear range of applicability (20–2500 ng/ml) were 82 ± 1% and 83 ± 1%, respectively. The limits of quantification were 5.0 μg/l for MG and 0.9 μg/l for LMG.

1. Introduction

Over the last decade, the fish farming industry has shown a shift towards intensive culture systems [1]. Eel farming is the most important sector in the Dutch fish farming industry with an annual production in 1993 of approximately 1000 metric tons (Board for Fish and Fishery Products in the Netherlands, pers. commun., 1994). As a result of this shift the use of therapeutic products has increased [2].

Malachite green (MG) is a triphenylmethane dye which has a broad spectrum of fungicidal and ectoparaciticidal activities [3,4]. No equally effective alternative for MG has been found despite intensive research [5,6]. Because of its potential carcinogenicity, mutagenicity and teratogenicity [7,8] it is very unlikely that legislation authorities in the countries of the European Union will authorise MG for use in fish [9]. The low cost of MG and its proven efficacy, however, enhance the risk of ongoing use of MG in the fish farming industry. The knowledge on biotransformation and pharmacokinetics of MG in eel and on residue profiles after MG treatment is scarce. In catfish [10] and rainbow trout [11] MG is primarily metabolized to leucomalachite green (LMG) which is stored in fat. The elimination rate constant of LMG from rainbow trout muscle is strongly dependent on the fat content [11].

Several methods for the determination of MG and LMG in fish tissue have been published.

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Two methods determine the amount of LMG indirectly. A fish muscle tissue extract is cleaned up by silica gel SPE followed by splitting of the cleaned sample and oxidizing half of the sample with lead(IV)oxide before HPLC analysis [11,12]. In 1991, an in-line lead(IV)oxide post-column oxidation reactor was introduced which opened the possibility for the simultaneous HPLC analysis of MG and LMG with Vis detection [13]. This system was applied to the analysis of residues of MG and LMG in water after a simple and rapid SPE procedure on diol SPE-columns [14] and recently also to the analysis of residues of MG in the eggs, the fry and the muscle tissue of adult rainbow trout after extraction of homogenates with 1% (v/v) of acetic acid in acetonitrile or in methanol, partitioning of the extracts with chloroform and reconstitution in mobile phase [15]. Roybal et al. [16] also used this in-line post-column oxidation reactor [13] for the simultaneous determination of MG and LMG in catfish after sample clean-up with propyl sulphonic acid and alumina SPE columns. However, they reported a short lifetime of the in-line post-column oxidation reactor.

In aqueous solutions MG and LMG can be photo-oxidative demethylated to N,N′,N″-tri- and/or N,N′-dimethylated compounds [17]. Klein et al. [12] found chromatographic peaks in cleaned fish muscle extracts which they associated with demethylated degradation products of MG and LMG.

Only one method of analysis for MG in rainbow trout serum has been published [18]. Serum was buffered and MG was partitioned into 1-pentanol and the concentration was estimated by means of scanning spectrophotometry. However, a very low recovery of MG (19%) was reported and no metabolites were incorporated into the method.

The aim of the present study, therefore, was the development of a simple and non-laborious procedure for the determination of MG and LMG in eel plasma to be used for pharmacokinetic studies of MG in eel. The analytical goals were (1) the adaptation of the chromatographic conditions for the resolution of MG, LMG and their assumed demethylated degradation products, (2) the optimization of the in-line lead(IV)oxide post-column oxidation reactor with respect to dimensions, PbO_2 content and lifetime, and (3) the development of a rapid SPE-based sample clean-up procedure for eel plasma with high recoveries for LMG and MG.

2. Experimental

2.1. Reagents and chemicals

All organic solvents were HPLC grade. All chemicals were analytical grade unless otherwise stated. Water was purified via Milli-Q Plus (Millipore, Bedford, MA, USA). Methanol, acetonitrile, citric acid monohydrate, lead(IV)oxide, L(+)-ascorbic acid and sodium hydroxide were from Merck (Darmstadt, Germany). Acetic acid (99–100%), sodium acetate trihydrate (HPLC grade) and sodium perchlorate monohydrate were from J.T. Baker (Phillipsburgh, NY, USA). Celite (high-purity analytical grade) was from Janssen Chimica (Beerse, Belgium). Anhydrous ammonia gas was from Union Carbide (Nieuw Vennep, Netherlands). LMG and sodium 1-pentane sulphonate were from Sigma (St. Louis, MO, USA). MG oxalate Vetranal (98%) was from Riedel-de Haën (Seelze, Germany).

An MG stock solution (1 mg/ml) was prepared by dissolving 100 mg of MG in acetonitrile and adjusting to 100 ml with acetonitrile. Working solutions containing 1, 10 or 100 μg/ml of MG were prepared by diluting the stock solution in acetonitrile. MG calibration standards for HPLC were prepared in the range of 0.020–2.5 μg/ml by diluting a working solution in the "anti-oxidative" mobile phase (see below). A LMG stock solution (1 mg/ml) was prepared by dissolving 100 mg of LMG in acetonitrile and adjusting to 100 ml with acetonitrile. Working solutions containing 1, 10 or 100 μg/ml of LMG were prepared by diluting the stock solution in acetonitrile. LMG calibration standards for HPLC were prepared in the range of 0.020–2.5 μg/ml by diluting a working solution in the "anti-oxidative" mobile phase (see below). For a study of the HPLC resolution of MG, LMG and

their assumed demethylated degradation products, solutions containing these compounds were obtained by exposing a standard solution of MG (1 μg/ml) and/or LMG (1 μg/ml) in mobile phase to sunlight at room temperature for one week.

An ascorbic acid stock solution (1 mg/ml) was prepared by dissolving 100 mg of L(+)-ascorbic acid in 100 ml of methanol.

A citric acid buffer solution (0.1 *M*, pH 3.0) was prepared by dissolving 5.26 g of citric acid monohydrate in 225 ml of water, adjusting the pH to 3.0 with 1 *M* sodium hydroxide and adjusting the volume to 250 ml with water. A methanol–citric acid 3:1 (v/v) mixture was prepared by adding 750 ml of methanol to 250 ml of the citric acid buffer. A protein denaturation and/or SPE conditioning solution (containing 10 μg/ml of ascorbic acid) was prepared by adjusting 1 ml of the ascorbic acid stock solution to 100 ml with the methanol–citric acid 3:1 (v/v) mixture.

Bakerbond sulphonic acid (SO_3H) disposable SPE columns (1 ml, 100 mg) were from J.T. Baker. Just before use the columns were activated by passing 1 ml of methanol and conditioned by passing 1 ml of SPE conditioning solution. After conditioning, the columns should not be allowed to run dry.

A sodium acetate–ion-pair buffer (25 m*M* sodium acetate, 25 m*M* sodium 1-pentane sulphonate and 50 m*M* sodium perchlorate; pH 4.0) was prepared by dissolving 4.36 g of sodium 1-pentane sulphonate, 3.40 g of sodium acetate trihydrate and 7.02 g of sodium perchlorate monohydrate in 950 ml of water, adjusting the pH to 4.0 with acetic acid and adjusting the volume to 1000 ml with water. The HPLC mobile-phase solvent was prepared by adding 600 ml of acetonitrile to 400 ml of the acetate–ion-pair buffer, pH 4.0. The HPLC mobile-phase solvent was filtered and degassed before use. "Anti-oxidative" mobile phase (containing 10 μg/ml ascorbic acid) was prepared by adjusting 1 ml of the ascorbic acid stock solution to 100 ml with mobile phase.

A 25% PbO_2 mixture was prepared by vortexing 1 g of lead(IV)oxide with 3 g of Celite for 1 min. A 50% PbO_2 mixture was prepared by vortexing 3 g of lead(IV)oxide with 3 g of Celite for 1 min.

2.2. Plasma samples

Blank eels were caught in the river Meuse by electrical fishing. Blank eel blood samples were taken from the vena caudalis and were processed to plasma within 2 h. All plasma samples were frozen and stored at −80°C.

For spiking studies blank eel plasma samples were spiked with the working solution at levels of 20, 50, 100, 200, 1000 and 2500 μg/l at least 15 min before extraction by the procedure described below.

To obtain real plasma samples a blank adult eel was given an injection (0.25 mg of MG/kg of body weight) of a physiological saline solution containing 201 mg/l of MG oxalate in the vena caudalis. Blood samples (1 ml) from the vena caudalis were taken 0.28, 0.7, 1.7 and 3.8 h after injection and processed to plasma.

2.3. Apparatus and chromatographic conditions

The instruments used were a KS 500 mechanical shaker (IKA-Labortechnik, Janke and Kunkel, Staufen, Germany), a Vibrofix VF 1 vortex mixer (IKA Labortechnik), a Centra-8R centrifuge (IEC, Needham, MA, USA), a Reacti-Therm III heating module and a Reacti-Vap III evaporator (Pierce, Rockford, IL, USA) and an SPE-21 column processor (J.T. Baker).

The HPLC system consisted of a P100 HPLC pump (Spectra Physics, San Jose, CA, USA), a PROMIS II autosampler (Spark, Emmen, Netherlands) with a 7010 air-actuated sampling valve (Rheodyne, Cotati, CA, USA) and a 100-μl sample loop. Detection was performed with a 783A HPLC monitor equipped with a tungsten lamp operated at 610 nm and a 10-μl HPLC flow cell (Applied Biosystems, Foster City, CA, USA). A ChromSep cartridge holder system contained a stainless-steel guard column (10 × 2.1 mm I.D.) packed with pellicular 40-μm reversed-phase particles and an analytical glass column (100 × 3.0 mm I.D.) packed with 5 μm

Chromspher B (Chrompack, Bergen op Zoom, Netherlands). A PEEK guard column (10 × 2.0 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) was dry-packed with Celite–lead(IV)oxide 75:25 (w/w) and fitted in a guard column cartridge holder. This assembly served as in-line post-column oxidation reactor. An All-tech (Deerfield, IL, USA) threaded stainless-steel column (50 × 4.6 mm I.D.) with 2-μm stainless-steel-in-teflon frits was hand-packed with Celite–lead(IV)oxide 1:1 (w/w) and placed between the HPLC-pump and the autosampler serving as an in-line pre-injection "guard" oxidation reactor. Freshly packed PbO_2 oxidation reactors should be flushed with methanol for 10 min prior to use.

The system was operated at ambient temperature. The injection volume was 25 μl. Samples were eluted isocratically at a flow-rate of 0.6 ml/min. Peak areas were calculated with a Data Jet integrator (Spectra-Physics).

2.4. Sample preparation

Extraction

An amount of 100 μl of eel plasma was pipetted into a 6-ml disposable polypropylene centrifuge tube and 2.5 ml of protein denaturation solution was added. The tube was capped, shaken on a vortex mixer for 10 s and placed on a mechanical shaker for 10 min (500 rpm). The suspension was centrifuged for 10 min at 4400 *g*.

Clean-up

The supernatant was carefully decanted onto a conditioned sulphonic acid SPE column. The pellet was rinsed with 1 ml of SPE conditioning solution and the rinse liquid was also passed through the SPE column. The SPE column was washed with 1 ml of water and 500 μl of methanol and was allowed to run dry. The SPE column was dried in a stream of nitrogen gas for 30 min. Immediately after drying a stream of anhydrous ammonia gas was passed through the SPE column. MG and LMG were eluted with 500 μl of methanol.

The eluate was evaporated to dryness in a stream of nitrogen at 37°C. The residue was dissolved in 250 μl of the "anti-oxidative" mobile phase. This solution was vortexed for 10 s and used for HPLC analysis.

Chromatography

Aliquots of the sample and standard solutions (25 μl) were injected into the HPLC system. Samples were eluted isocratically at a flow-rate of 0.6 ml/min.

4. Results and discussion

4.1. Chromatography

Typical chromatograms of standard solutions of both MG and LMG, blank eel plasma extract and spiked eel plasma extract are shown in Fig. 1A to C.

The conditions used as a starting point for the optimization of the chromatography of MG, LMG and their assumed demethylated degradation products [17] are based on those described by Abadi [19] for standard solutions of triphenylmethane dyes and Allen and Meinertz [13] for standard solutions of MG and LMG. Sodium 1-pentane sulphonate and sodium perchlorate were used as binary electrolyte system in the mobile phase, acetonitrile was used as organic modifier and *di*potassium hydrogen phosphate crystals were used for pH adjustment. A 10% lead(IV)oxide–Celite in-line post-column oxidation reactor (30 × 4.6 mm I.D.) was employed for the simultaneous detection of MG and LMG. The chromatographic system was optimized with respect to resolution of the analytes, effective column efficiency and in-line post-column oxidation reactor lifetime.

Several column packing materials (Chromspher C-8 and C-18, Nucleosil 5 C-18, Hypersil ODS, Microspher C-18, LichroSorb RP-8 and Chromspher 5B (all from Chrompack), PLRP-S (Polymer Laboratories, Amherst, MA, USA) and PRP-1 (Hamilton, Bonaduz, Switzerland)) were tested for effective column efficiency and resolution of MG, LMG and their assumed demethylated degradation products. Nucleosil 5

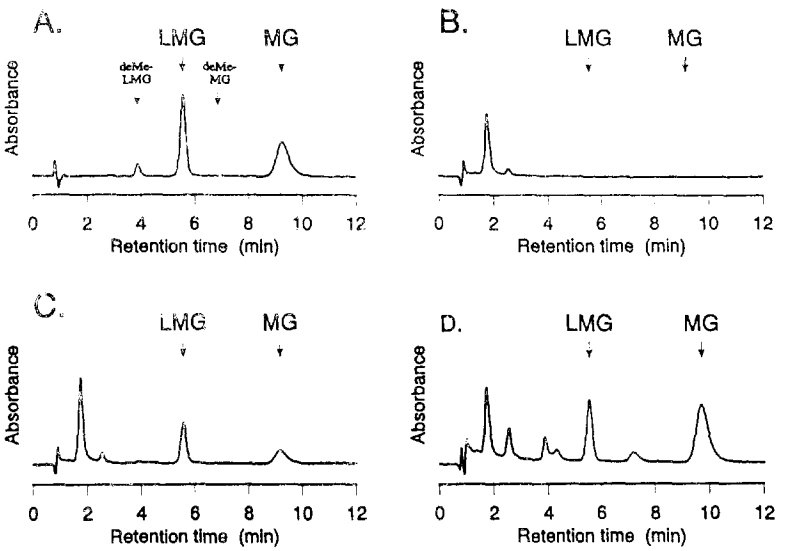

Fig. 1. Typical chromatograms of: (A) a standard solution of both malachite green (MG; 50 μg/l) and leucomalachite green (LMG; 50 μg/l); (B) a blank eel plasma extract (dilution factor of 2.5); (C) a spiked (50 μg/l MG and 50 μg/l LMG) eel plasma extract (dilution factor of 2.5); (D) a plasma extract of an eel treated with malachite green (contains 74 μg/l of LMG and 230 μg/l of MG). Blood was drawn 0.28 h after an injection of 0.25 mg/kg of bodyweight of malachite green in the vena caudalis. For chromatographic conditions see Section 2.3. Attenuation = 4.

C-18, LichroSorb C-8 and Chromspher 5B showed the best effective column efficiency and resolution of these compounds.

The HPLC mobile-phase composition was further optimized using a Chromspher 5B column (100 × 3.0 mm I.D.). The optimum concentrations in the binary electrolyte system of the mobile phase appeared to be 25 m*M* sodium 1-pentane sulphonate and 50 m*M* sodium perchlorate. However, pH adjustment with K_2HPO_4 crystals caused poor repeatability of the retention behaviour of MG and LMG. Furthermore, the required pH (3.5–4.5) of the mobile phase is outside the effective pH buffering range of phosphate. To overcome these problems, citric acid and acetate were tested as buffering agent in the aqueous ion-pair solution of the mobile phase. Citric acid caused clogging of the in-line post-column oxidation reactor. Sodium acetate gave no problems in this respect. The best resolution of MG, LMG and the demethylated degradation products is obtained when the pH of the aqueous 25 m*M* acetate–ion-pair buffer is adjusted to pH 4.0 and an organic modifier content of 60% of acetonitrile is used.

Subsequently, the size and PbO_2 content of the in-line post-column oxidation reactor were optimized with respect to lifetime and LMG-to-MG conversion performance. The relatively large size (32 × 4 mm I.D. = 402 μl) of the post-column oxidation reactor used by Allen and Meinertz [13] is probably the cause of band broadening of the MG and LMG peaks. Reduction of the size of the reactor to 10 × 2.1 mm I.D. (16.5 μl) gave much sharper peaks for MG and LMG but the PbO_2 in the reactor depleted rapidly. Increasing the PbO_2 content from 10 to 25% markedly prolonged the lifetime of the reactor. Furthermore, as suggested by J.E. Roybal (pers. commun.), the acetonitrile in the mobile phase contains oxidizable substances (e.g. ketones and aldehydes in the order of 10 mg/l; source: Merck), which may also deplete the PbO_2 reactor. Placing a "guard" oxidation reactor (50% PbO_2 in Celite; 50 × 4.6 mm I.D.) between HPLC-pump and autoinjector elimi-

Table 1
Regression coefficients for calibration line and recovery line ($y = A_0 + A_1 x$) of MG and LMG in eel plasma

	A_0^a	A_1^a	r^2	S.E.	N
Calibration					
LMG	−2161 ± 2072	610 ± 8	0.9964	7585	96
MG	−1567 ± 700	551 ± 4	0.9990	2566	96
Recovery					
LMG	n.s.	83 ± 2%	0.9966	0.153	41
MG	n.s.	82 ± 1%	0.9994	0.045	41

A_0 = constant of regression line; A_1 = slope of regression line; r^2 = correlation coefficient of regression line; S.E. = standard error of regression line; N = number of replicates for regression line; n.s. = omitted, not significant.
[a] Mean ± 95% confidence limit.

nates all oxidizable interferences in the mobile phase before the actual analytical process and thus prolongs the lifetime of the post-column oxidation reactor to more than 800 injections.

The performance of the post-column oxidation reactor with respect to the LMG-to-MG conversion was also assessed. Since MG is the actual chemical entity which is detected at a wavelength of 610 nm, the detector response of equimolar solutions of LMG and MG should theoretically be identical in case of a quantitative LMG-to-MG conversion. The calculated regression coefficients of the calibration lines for MG and LMG and their 95% confidence limits are presented in Table 1. The values of the regression coefficients of the calibration lines for MG and LMG are almost identical. Thus, the 25% PbO_2, 10 × 2.1 mm I.D., in-line post-column oxidation reactor shows an almost quantitative conversion of LMG to MG under the optimized chromatographic conditions.

In Table 2 the chromatographic parameters of MG, LMG and their assumed demethylated degradation products for the optimized chromatographic system are presented.

As already mentioned, it is known that, in aqueous solutions, MG and LMG can be photo-oxidative demethylated to N,N′,N″-tri- and/or N,N′-dimethylated compounds [17]. Therefore, the stability of MG and LMG in standard solutions in mobile phase was investigated at room temperature. MG proved to be stable but LMG proved to be unstable. In the course of 13 h the peak areas of LMG calibration solutions decreased approximately 25%, while the peak areas of the assumed demethylated degradation

Table 2
Chromatographic parameters of MG, LMG and their assumed demethylated degradation products for the optimized chromatographic system (for chromatographic conditions see Sect. 2.3)

Chromatographic parameter	Symbol	deMe-LMG		LMG		deMe-MG		MG
Mobile phase hold-up time	t_0 (min)	0.85		0.85		0.85		0.85
Retention time	$t_{R,i}$ (min)	3.89		5.56		6.91		9.19
Retention factor	k'_i	3.58		5.54		7.28		9.94
Effective column efficiency	$N_{eff,i}$	2400		3230		2200		2000
Asymmetry factor	$A_{s,i}$	1.56		1.20		1.44		1.57
Column selectivity	$\alpha_{i,j}$		1.54		1.31		1.37	
Column resolution	$Rs_{i,j}$		6.26		3.79		3.70	

deMe-LMG = assumed demethylated degradation product of leucomalachite green; LMG = leucomalachite green; deMe-MG = assumed demethylated degradation product of malachite green; MG = malachite green.

products of LMG increased. A standard solution of LMG in mobile phase stored at 4°C did not show this photo-oxidative demethylation. A standard solution of LMG in "anti-oxidative" mobile phase was also stable at room temperature. The presence of ascorbic acid in standard solutions of MG and LMG influenced neither the stability of MG nor the chromatography of MG and LMG, nor the stability of the in-line post-column oxidation reactor.

4.2. Sample pretreatment

The sample pretreatment which had to be developed should be applicable in pharmacokinetic studies. In pharmacokinetic studies, the maximum blood volume which can be withdrawn from an adult eel per sampling time is approximately 1 ml (corresponding to approximately 0.5 ml of plasma). Therefore, the plasma test portion for the sample pretreatment was limited to 100 μl.

Simple deproteinization of plasma with 2% trichloroacetic acid (1:1, v/v), 6% perchloric acid (1:1, v/v), 10% phosphoric acid (1:1, v/v) or acetonitrile (1:9, v/v), subsequent centrifugation and direct injection of the (diluted) deproteinized plasma supernatant resulted in unsatisfactory recoveries of MG and LMG. Acidification or alkalinization of plasma and subsequent extraction with ethyl acetate also yielded low recoveries.

Therefore an SPE clean-up was introduced. Because of the ionic character of MG, ion exchange was the mechanism of choice. An ammonia gas treatment of an aromatic sulphonic acid SPE column charged with MG and LMG followed by elution with methanol, as earlier used for the determination of sulphonamides in swine tissue [20], showed only marginal losses of LMG and MG. The ammonia gas treatment probably deprotonates MG and LMG, thus disrupting the electrostatic interaction with the sulphonic acid SPE column and permitting the elution with a small volume of methanol. Plasma protein denaturation with 2.5 ml of a 3:1 (v/v) mixture of acetonitrile and 0.1 *M* of citric acid, pH 3.0, followed by clean-up on an aromatic sulphonic acid SPE column resulted in low recoveries of LMG and MG. The high acetonitrile content impeded the retention of MG and LMG on the SPE column. Replacing acetonitrile for methanol improved the retention of MG and LMG on the SPE column very much. Therefore, plasma protein was denaturated with 2.5 ml of a 3:1 (v/v) mixture of methanol and 0.1 *M* of citric acid, pH 3.0, followed by clean-up on an aromatic sulphonic acid SPE column.

Due to solubility problems of MG and LMG the methanol eluate had to be reconstituted in 250 μl of "anti-oxidative" mobile phase, thus introducing a dilution factor of 2.5 in comparison with the plasma test portion.

In the course of this study it was found that eel plasma cannot be frozen and/or kept at −20°C without loss of LMG and MG. When freshly prepared blank eel plasma was spiked and submitted to a single −20°C freeze–thaw cycle, approximately 60% of the amount of LMG and 10% of the amount of MG disappeared. No influence on LMG or MG content, however, was observed when freshly prepared spiked eel plasma was submitted to several consecutive −80°C freeze–thaw cycles.

4.3. Spiking studies

Recovery experiments were carried out in six replicates with blank eel plasma spiked at levels of 20, 50, 100, 200, 1000 and 2500 μg/l for both MG and LMG. MG and LMG were added simultaneously to the blank eel plasma. The results for the mean recoveries are shown in Table 3. A good recovery at all levels investigated and a low standard deviation for repeatability were attained.

In order to assess a possible constant error of the developed method a linear-regression procedure was applied ($y = A_0 + A_1 \cdot x$) to the results of the recovery experiments for LMG and MG as was outlined by Wernimont [21]. The calculated regression coefficients (with SPSS for Windows, Version 6.0) for the recovery and their 95% confidence limits are presented in Table 1. There was no statistical support for a constant

Table 3
Mean recovery of malachite green and leucomalachite green from spiked eel plasma

Spiking level (μg/l)	Leucomalachite green			Malachite green		
	Theoretical concentration (μM)	Measured concentration (μM)	Recovery (%)	Theoretical concentration (μM)	Measured concentration (μM)	Recovery (%)
20[a]	0.061	0.052 ± 0.002	86 ± 4	0.043	0.035 ± 0.003	80 ± 7
50[b]	0.151	0.129 ± 0.008	85 ± 5	0.108	0.086 ± 0.009	80 ± 8
100[b]	0.303	0.256 ± 0.008	85 ± 3	0.215	0.171 ± 0.005	80 ± 2
200[b]	0.605	0.533 ± 0.018	88 ± 3	0.431	0.384 ± 0.007	89 ± 2
1000[b]	3.026	2.394 ± 0.162	79 ± 5	2.153	1.786 ± 0.073	83 ± 3
2500[b]	7.562	6.302 ± 0.339	83 ± 5	5.382	4.428 ± 0.084	82 ± 2

For reasons of comparability of malachite green and leucomalachite green, concentrations are given in both μg/l and μM.
[a] $N = 5$.
[b] $N = 6$.

error in the developed method neither for MG nor for LMG.

For the assessment of the limits of detection and of quantification plasma of eight blank eels was analyzed following the developed method and the peak height in a 10% window around the chromatographic peak of the analytes was measured. The average peak height and its standard deviation in a 10% window around the chromatographic peak of the analytes were calculated. There was no interference from the plasma matrix. The limit of detection was set as the average general peak height plus 3 times its standard deviation. The limit of quantification was set as the average general peak height plus 6 times its standard deviation. These values were converted to μg/kg and corrected for the recovery. The limits of detection and quantification were determined to be 3.5 and 5.0 μg/l, respectively, for MG, and 0.6 and 0.9 μg/l, respectively, for LMG. These values are corrected for the average recoveries.

4.4. Real eel plasma samples

As an illustration of the applicability of the described method, plasma samples of an eel taken 0.28, 0.7, 1.7 and 3.8 h after an intravenous injection of 0.25 mg/kg of MG were analyzed. The amounts found were 230, 113, 53 and 29 μg/l of MG and 74, 70, 39 and 15 μg/l of LMG, respectively. In plasma samples drawn more than 4 h after the intravenous injection neither MG nor LMG was detectable. A chromatogram of eel plasma drawn 0.28 h after the intravenous injection is shown in Fig. 1D.

In the chromatograms of plasma samples of the treated eel peaks of MG, LMG and their assumed demethylated degradation products are found. In spiked plasma samples these demethylation products were also present but the amounts were much smaller, which supports the assumption that the presence of the assumed demethylated degradation products of MG and LMG in plasma of treated eel is a result of biotransformation or biodegradation of MG in eel.

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References

[1] E.-M. Bernoth, Dtsch. Tierärztl. Wochenschr., 98 (1991) 312.
[2] D. Manz, J. Tögel, J. Nilz and F. Lutz, Tierärtzl. Umsch., 46 (1991) 543.
[3] M. Beeke, Ph.D. Thesis, Tierärztliche Hochschule, Hannover, 1988.
[4] D.J. Alderman, J. Fish Dis., 8 (1985) 289.
[5] T.A. Bailey, Aquaculture, 38 (1984) 97.
[6] R.A. Schnick, Prog. Fish Cult., 50 (1988) 190.
[7] S.K. Khanna, G.B. Singh and C.R. Krishna Murti, J. Food Sci. Technol., 17 (1980) 95.
[8] G. Werth and A. Boiteux, Arzneim. Forsch., 17 (1967) 155.
[9] E.-M. Bernoth, J. Vet. Med. B, 37 (1990) 401.
[10] W.E. Poe and R.P. Wilson, Prog. Fish Cult., 45 (1983) 228.
[11] K. Bauer, H. Dangschat, H.-O. Knöppler and J. Neudegger, Arch. Lebensm. Hyg., 39 (1988) 97.
[12] E. Klein, M. Edelhäuser and R. Lippold, Dtsch. Lebensm. Rundsch., 87 (1991) 350.
[13] J.L. Allen and J.R. Meinertz, J. Chromatogr., 536 (1991) 217.
[14] J.L. Allen, J.R. Meinertz and J.E. Gofus, J. Assoc. Off. Anal. Chem., 75 (1992) 646.
[15] J.L. Allen, J.E. Gofus and J.R. Meinertz, J. AOAC Int., 77(3) (1994) 553.
[16] J.E. Roybal, A.P. Pfenning, R.K. Munns, D.C. Holland, J.A. Hurlbut and A.R. Long, J.AOAC Int., (1994) in press.
[17] R. Bangert, W. Aichele, E. Schollmeyer, B. Weimann and H. Herlinger, Melliand Text. Ber., 5 (1977) 399.
[18] D.J. Alderman and R.S. Clifton-Hadley, J. Fish Dis., 16 (1993) 297.
[19] S.L. Abadi, J.Chromatogr., 255 (1983) 101.
[20] N. Haagsma and C. van de Water, J. Chromatogr., 333 (1985) 256.
[21] G.T. Wernimont, in W. Spendley (Editor), Use of Statistics to Develop and Evaluate Analytical Methods, Association of Official Analytical Chemists, Arlington, 1987, Ch. 3, p. 64.