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Use of an immobilized enzyme reactor for the analysis of residues of 17α-methyltestosterone in trout by high-performance liquid chromatography

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ABSTRACT

We have recently observed in trout that 48 h after ingestion of a single dose of [^{14}C]-17α-methyltestosterone ([^{14}C]-17-MT), 25% of the radioactivity was still in the carcass, corresponding to metabolites of 17-MT. These compounds have no appreciable chromophore, fluorophore or electrophore, therefore the usual detection systems were not satisfactory for their analysis. Consequently a method was developed for high-performance liquid chromatographic separation and detection of two of the major tissue metabolites of 17-MT: 5α-androstane-17α-methyl-3α,17β-diol and 5β-androstane-17α-methyl-3α,17β-diol. A column of immobilized 3α-hydroxysteroid dehydrogenase was prepared and used for detection. The NADH produced from 3-hydroxysteroids by this immobilized enzyme reactor was monitored fluorimetrically. The detection limit of this method, as obtained from the calibration curve, was at the picomole level; the limit of quantification in muscle was 1 μg/kg, at a signal-to-noise ratio of 4.

INTRODUCTION

It has been demonstrated that 17α-methyltestosterone (17-MT) has a growth-promoting effect in fish if given in appropriate dose [1–3]. In addition, this androgen has been widely used to induce sex reversal in fish thereby producing monosex stocks [3–5]. The aim here is to produce fish of the faster-growing sex or to eliminate the incidence of precocious sexual maturation, *e.g.* genotypic males in salmonids.

Thus the use of 17-MT in fish culture shows promise. However, use of this steroid is still illegal in most European countries. Therefore, analytical methods for its detection and quantification from tissues of treated animals must be developed.

Using high-performance liquid chromatography (HPLC), 17-MT can be determined in tissues above the 50 μg/kg level [6]. However, owing to metabolism [7], the residual amount of the parent compound in tissues is often below 10 μg/kg, especially 24 h or more after administration. Therefore, for more effective monitoring of the presence of 17-MT residues, a method with a detection limit well below 1 μg/kg is necessary; alternatively, a more persistent metabolite, as a proof of the use of 17-MT, must be determined.

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We have observed in trout that, 48 h after ingestion of a single dose of [^{14}C]-17-MT, 25% of the radioactivity was still in the carcass, corresponding to metabolites of 17-MT [7]. Recently, we demonstrated the presence of 5α-androstane-17α-methyl-3α,17β-diol and 5β-androstane-17α-methyl-3α,17β-diol in tissues of trout treated with [^{3}H]-17-MT [8]. These metabolites persisted in the muscle after the elimination of the parent compound. Because these biotransformation products have no appreciable chromophore, fluorophore or electrophore, the usual detection systems were not satisfactory for their analysis.

The aim of this study was to develop a reliable HPLC method for the separation and detection of 17-MT residues. For detection of these metabolites, we prepared a post-column reactor containing 3α-hydroxysteroid dehydrogenase (3α-HSD), and studied the conditions necessary for quantitative analysis by mean of a fluorometric detector.

EXPERIMENTAL

Chemicals, glassware and solvents

All glassware was silanized with a solution of dimethyldichlorosilane in toluene before use. All solvents, unless otherwise specified, were of analytical grade. Water was filtered on activated charcoal, purified by reverse osmosis and filtered over a 0.2-μm filter before use. Sep-Pak silica cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.), and Lipidex 5000 was from Packard Instruments (Rungis, France). 3α-HSD and nicotinamide-adenine dinucleotide (NAD) were purchased from Sigma Chimie SARL (L'Isle d'Abeau, France).

Steroids

17α-Methyltestosterone (4-androstene-17α-methyl-17β-ol-3-one) was obtained from Sigma, and 5α-androstan-17α-methyl-3α,17β-diol (5α-17-MTD) was purchased from Steraloids (Wilton, NH, U.S.A.). 5β-Androstane-17α-methyl-3α,17β-diol (5β-17-MTD) was synthesized from 5β-androstane-17α-methyl-17β-ol-3-one (Steraloids) by sodium borohydride reduction, which led to a mixture of 3α- and 3β-diols [9]. The two epimers were separated by reversed-phase HPLC [9], and the 3α-diol was detected by the 3α-HSD system described below.

HPLC apparatus

The HPLC system consisted of LKB pump equipment (Bromma, Sweden) and a Rheodyne 7125 loop injection valve (50 μl). A 740 Spectra-Physics pump (Spectra-Physics, San Jose, CA, U.S.A.) was linked to the system via a post-column 950-μl static mixing-chamber (Touzart & Matignon, Vitry-sur-Seine, France). Peaks were detected using a FS 970 fluorometer (Schoeffel Instruments, Westwood, NJ, U.S.A.) at excitation and emission wavelengths of 340 and 470 nm, respectively. Peak areas were integrated using a Shimadzu Model CR 3A in-

tegrating recorder. When necessary, peaks were monitored by UV detection with an LKB 2158 UV detector equipped with a 254-nm UV filter.

HPLC conditions

An isocratic elution system was developed to resolve the steroid compounds (Fig. 1). It consisted of two on-line columns: a C_8 Hypersil column (5 μm particle size, 150 mm × 4.6 mm I.D.) from Interchim (Montluçon, France) and a C_{18} Nucleosil column (5 μm particle size, 150 mm × 4.6 mm I.D.) from SFCC (Neuilly, France) protected by a guard column (25 mm × 3.8 mm I.D.) packed with ODS 2 (5 μm). The metabolites of 17-MT were separated at ambient temperature with methanol–water (75:25) as mobile phase at a flow-rate of 0.8 ml/min.

The eluate from the column was mixed with the NAD solution pumped at a flow-rate of 1.2 ml/min just before the immobilized enzyme reactor (IMER). The reagent solution, which was freshly prepared according to Kamada *et al.* [10], contained 20 m*M* pyrophosphate buffer (pH 9) and 0.6 m*M* NAD.

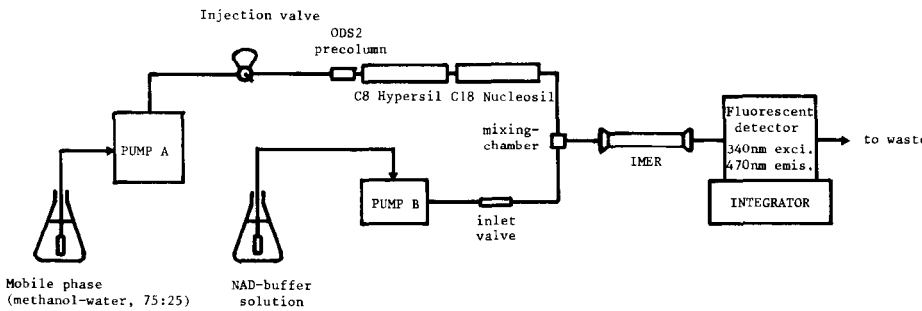

Fig. 1. Schematic diagram of the combined IMER–HPLC system.

Preparation of the IMER

3α-HSD was immobilized on the Chrom Sep IMER by flushing it with the enzyme solution, as described by the supplier (Chrompack, Middelburg, The Netherlands). Briefly, the IMER was connected directly to the injector equipped with a 100-μl loop, and a mobile phase consisting of 0.01 *M* KH_2PO_4 (pH 7.8) was pumped at a flow-rate of 0.8 ml/min through the injector and the IMER. Ten 100-μl samples of an enzyme solution consisting of 25 U of 3α-HSD dissolved in 1 ml of 0.01 *M* KH_2PO_4 adjusted to pH 7.8 were injected onto the IMER. The IMER was equilibrated for 20 min with the mobile phase and then disconnected from the injector.

Animal treatment

Immature rainbow trout (*Oncorhynchus mykiss*) (mean weight 180 g) were force-fed a gelatin capsule containing 1.60 g of feed and 40 μg of 17-MT. Control fish were fed a pelleted commercial fish diet. Groups of three animals were killed by cervical dislocation after 12, 24 and 48 h. A 10-g piece of latero-dorsal muscle was removed from each animal and homogenized using a blender.

Sample preparation

17-MT and biotransformation products were extracted from muscle as previously described [7]. Samples (1–5 g) were homogenized in 10–50 ml of chloroform–methanol (2:1, v/v). Water (0.2 volume) was added to the extract and, after centrifugation at 1800 *g* for 10 min, the organic phase was evaporated *in vacuo*. The aqueous phase was extracted again with two volumes of chloroform. The extract was taken to dryness. The residues were dissolved in acetonitrile and, after partition with isooctane and elution through a Lipidex 5000 column and a Sep Pak silica cartridge, they were analysed by the reversed-phase HPLC system.

Identification of 17-MT and related diol metabolites

Identification of 17-MT, 5α-17-MTD and 5β-17-MTD on HPLC profiles was established by comparing the retention times with authentic reference compounds. Confirmation by GC and GC–MS analyses were obtained for each of these compounds [8].

RESULTS AND DISCUSSION

IMER performance

This assay for 17-MT residues depends on the oxidation of 3α-hydroxysteroids promoted by specific nicotinamide nucleotide-dependent hydroxysteroid dehydrogenase. The first step of this method is the enzymic oxidation of 17-MT diol metabolites, namely 5α-17-MTD and 5β-17-MTD, with the 3α-HSD IMER.

The standard calibration curve of 5α-17-MTD, obtained by the spectrofluorimetric detection of the NADH produced, showed a linear response in the range 5–200 ng (correlation coefficient, 0.9984; regression equation, $y = 0.015x + 0.001$), suggesting that the enzyme was not saturated at these concentrations. The intra-assay variation [(S.D./mean) × 100] ranged from 2.3 to 9.1% ($n = 3$). When the IMER was washed with NAD solution for 20 min before storage at 4°C, no significant change of its initial activity was observed after one month when four tissue samples were assayed every day.

Detection of 5α-17-MTD and 5β-17-MTD in muscle

The efficiency and reproducibility of the extraction and purification procedure were evaluated by adding standards (5α-17-MTD and 5β-17-MTD) to 5-g samples of rainbow trout muscle. The recovery was quantitated by comparison with

peak areas of unextracted steroid standards and was found to bc 91 ± 10 and 94 ± 9% for 5α-17-MTD and 5β-17-MTD, respectively.

Owing to the detrimental effect on the IMER activity of a high proportion of organic modifier in the mobile phase [11], the HPLC method used in a previous study for the separation of 17-MT metabolites [7] was not selected. With the system developed in this study, the standard 3α-hydroxysteroids were completely separated, and unknown fluorescent compounds detected in control samples did not interfere with 5α- and 5β-17-MTD (Fig. 2). Retention times of 21.2 and 22.6 min were obtained for 5β-17-MTD and 5α-17-MTD, respectively.

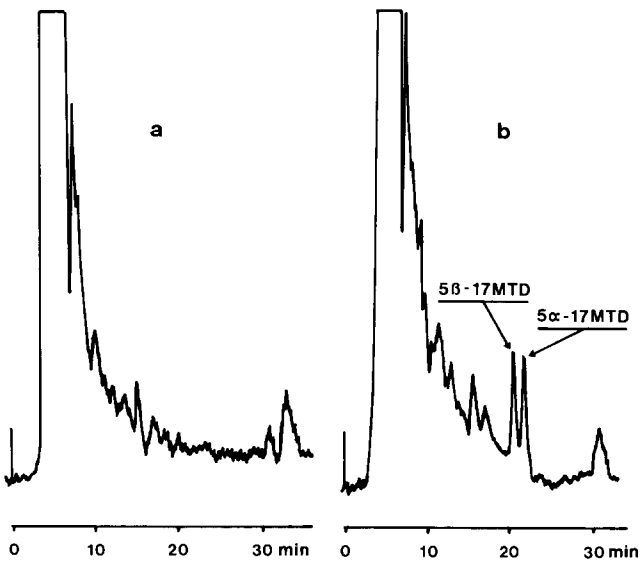

Fig. 2. Chromatograms of muscle extracts from (a) control fish and (b) a muscle sample (5 g) fortified with 25 ng of 5α-17-MTD and 5β-17-MTD. Two in-line columns (C_8 Hypersil, 5 μm, 150 mm × 4.6 mm I.D., and C_{18} Nucleosil, 5 μm, 150 mm × 4.6 mm I.D.) protected by an ODS2 guard column were used. Mobile phase, methanol–water (75:25); flow-rate, 0.8 ml/min.

Although amounts as low as 1 ng could easily be distinguished from noise, a realistic limit appeared to be 5 ng for the quantification of these steroids, with good resolution of 5α-17-MTD and 5β-17-MTD peaks. When compared with other studies in which an IMER was used for steroid detection, the present work shows a sensitivity similar to that obtained by Lam *et al.* [12] with pregnanediols, androsterone and etiocholanolone, but higher than that described for bile acids [13].

Analysis of muscle extracts from 17-MT-treated fish

Fig. 3 shows typical chromatograms of muscle extracts obtained from trout treated with 40 μg of 17-MT. In contrast with several studies on steroid residues, enzymic deconjugation of the samples was not applied (see Experimental), be-

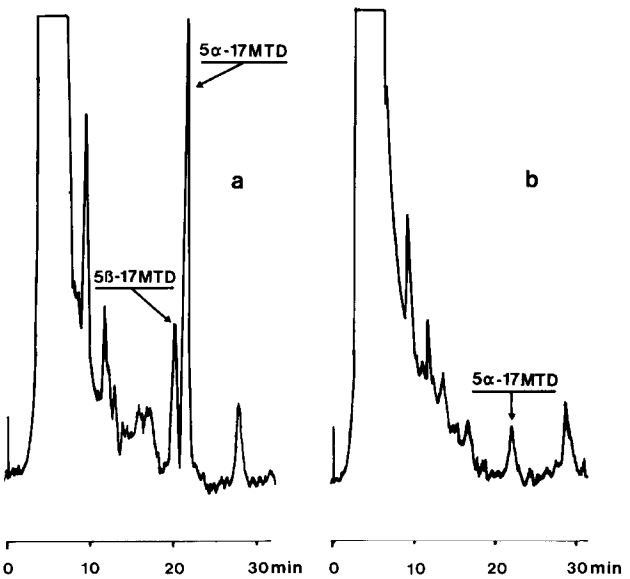

Fig. 3. Chromatograms of muscle extracts from rainbow trout dosed *per os* with 40 μg of 17-MT. Analysis carried out (a) 12 h and (b) 48 h after treatment. HPLC conditions as in Fig. 2.

cause in trout muscle 17-MT metabolites are mainly present as free compounds [7].

In the 12-h sample (Fig. 3) the peaks for 5β-17-MTD and 5α-17-MTD were clearly separated, and identified by comparison with the authentic samples. The other peaks that appeared in the chromatogram correspond to unknown fluorescence compounds or endogenous 3α-hydroxysteroids existing in muscle (see Fig. 2a) or to unidentified 17-MT metabolites. This chromatogram shows that 5β-17-MTD is a minor biotransformation product and 5α-17-MTD is a major metabolite.

After 48 h, no trace of 5β-17-MTD was detected, but 5α-17-MTD was still present (Fig. 3b). The 5α-17-MTD concentrations, as detected with the IMER system, were as follows: 4.2 ± 1.4 and 2.6 ± 1.1 μg/kg, 24 and 48 h after dosing, respectively. It must be noted that under the same experimental conditions the residues of unchanged 17-MT found in muscle were below 1 μg/kg as early as one day after treatment [7].

CONCLUSIONS

The high sensitivity of fluorescence detection of NADH, combined with the specificity of the IMER loaded with 3α-HSD, allows a rapid determination of traces of 5β-17-MTD and 5α-17-MTD. Owing to its relative persistence in tissues of trout treated with 17-MT, this latter compound can be used as an indicator

and eventually a proof of the administration of 17-MT. Since the detection limit can be estimated to be in the range 1–5 μg/kg, which is the concentration range in muscle residues 72 h after treatment, the technique reported here is a convenient control system for up to three days post-application.

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