Talanta 99 (2012) 238–246

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Analysis of trenbolone acetate metabolites and melengestrol in environmental matrices using gas chromatography–tandem mass spectrometry

Jed A. Parker, Jackson P. Webster, Stephanie C. Kover, Edward P. Kolodziej *

Department of Civil and Environmental Engineering, University of Nevada, Reno, MS 258, 1664 N. Virginia Street, Reno, NV 89557, USA

ARTICLE INFO

Article history:
Received 19 November 2011
Received in revised form
20 May 2012
Accepted 22 May 2012
Available online 2 June 2012

Keywords:
Trenbolone
Trendione
Steroid hormones
Endocrine disruption
Growth promoters

ABSTRACT

Studies demonstrate that exposure to steroid hormones in receiving waters can adversely impact reproduction of aquatic organisms. In particular, exogenous steroid hormones widely used as growth promoters in animal agriculture are of high concern, yet no gas chromatography–tandem mass spectrometry (GC/MS/MS) analytical methods for the detection of these compounds in complex environmental matrices is described in the literature. This study utilizes analytical methods based upon *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide-iodine (MSTFA-I_2) derivatization for the analysis of metabolites of trenbolone acetate (TBA), including 17α-trenbolone, 17β-trenbolone, and trendione, and melengestrol acetate in receiving waters and surface soils associated with animal agriculture. Results suggest method detection levels of 0.5–1 ng/L for the trenbolone metabolites, while detection of melengestrol is qualitative only. Isotope dilution methods employing d3-17β-trenbolone were used to improve steroid quantification. Method recoveries in spiked samples collected from a variety of representative receiving waters generally ranged from 80–120% with consistent and low standard deviation (generally < 10%) for replicate analysis. Analysis of a storm water runoff sample from a commercial confined animal feeding operation (CAFO) that used TBA implants detected 17β-trenbolone and trendione at concentrations of 31 and 52 ng/L, respectively. Analysis of surface soils at a commercial CAFO using TBA implants detected 17α-trenbolone at concentrations between 4–6 ng/g dry weight. Method development efforts suggested that the concentration of I_2 in MSTFA, the removal of I_2 from sample extracts after derivatization, and the use of Florisil clean-up to reduce organic matter matrix were vital aspects of steroid hormone quantification at low (< 30 ng/L) concentrations in complex environmental matrices.

1. Introduction

The occurrence of steroid hormones that can act as endocrine disrupting compounds in surface waters has been linked to feminization, altered morphology, and reproductive disruption in fish upon exposure to steroid hormones [1–3]. Of particular interest are potent exogenous steroid hormones that have the potential to cause catastrophic, population-wide effects due to disruption of reproductive function in sensitive aquatic organisms. For example, a population of fathead minnows (*Pimephales promelas*) continuously exposed to 5 ng/L ethynyl estradiol over 3 years neared local extinction due to disrupted spermatogenesis in male fish, suggesting that exogenous steroids can pose a substantial ecological risk in affected receiving waters [4].

Androgens also have demonstrated endocrine disrupting potential at similar low concentrations, although these steroids are not as well studied as the estrogens [5–7]. Sources of endogenous and exogenous androgens into the natural environment include wastewater treatment plant effluent [8], pulp and paper mill effluent [9], and animal agriculture sources such as confined animal feeding operations (CAFOs) [10,11]. In particular, the synthetic androgen 17β-trenbolone, a potent anabolic steroid, is of considerable interest as an environmental pollutant due to its widespread use and potential for endocrine disruption in sensitive species of fish.

For example, over 90% of beef cattle raised in the United States receive at least one growth implant, and the vast majority of these implants contain trenbolone acetate (TBA) [12]. TBA use is widespread in cattle production because of demonstrated increases in weight gain and carcass quality of TBA-implanted cattle [13]. After implantation, TBA is metabolized to 17β-trenbolone (the most biologically potent compound), 17α-trenbolone and

Abbreviations: CAFOs, confined animal feeding operations; GC/MS, gas chromatography–mass spectrometry; GC/MS/MS, gas chromatography–tandem mass spectrometry; LC, liquid chromatography; MGA, melengestrol acetate; MDLs, method detection limits; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide; MRM, multiple reaction monitoring mode; S/N, signal-to-noise; SPE, solid phase extraction; TBA, trenbolone acetate; TMS, trimethylsilyl

* Corresponding author. Tel.: +1 775 682 5553; fax: +1 775 784 1390.
E-mail address: koloj@unr.edu (E.P. Kolodziej).

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http://dx.doi.org/10.1016/j.talanta.2012.05.046

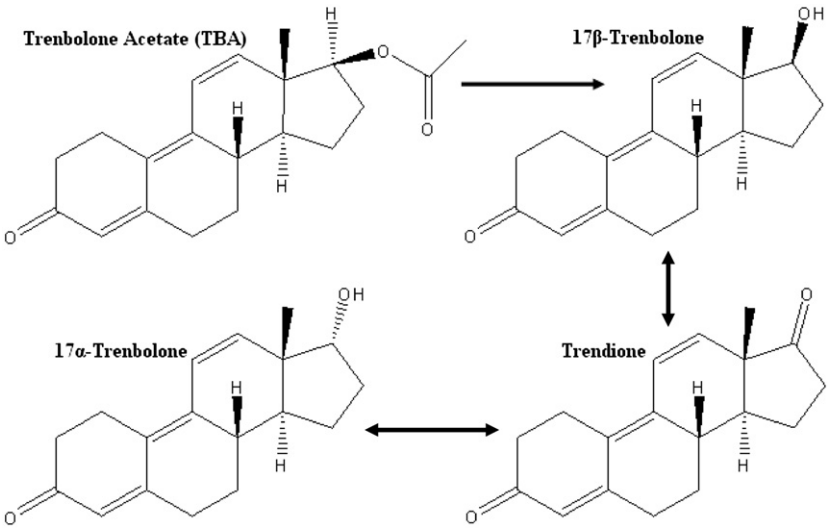

Fig. 1. Primary pathway of trenbolone acetate (TBA) metabolism in cattle. Figure adapted from Khan et al. [14].

trendione (Fig. 1) [14]. Of these metabolites, 17α-trenbolone accounts for ~95% of the total trenbolone mass excreted by the implanted animal; thus this compound is expected to dominate occurrence in the aquatic environment, although transformations between the different metabolites likely occur [14]. Exposure to low concentrations (10–30 ng/L) of either 17α-trenbolone and 17β-trenbolone can result in significant reductions in fecundity in some species of fish [10,11]. Similarly, other growth promoters such as melengestrol acetate (MGA) are used in animal agriculture, though not as widely as TBA, and metabolites of MGA such as melengestrol also are likely contaminants in receiving waters near animal agriculture operations utilizing growth promoters [14].

While the discharge of untreated CAFO runoff directly into surface waters is prohibited, the widespread use of growth promoters and the potential for incidental CAFO runoff discharges suggest that TBA metabolites such as trendione, 17α-trenbolone and 17β-trenbolone along with other steroidal contaminants linked to animal agriculture can occur in receiving waters. TBA metabolites have been detected in receiving waters at ecologically relevant concentrations via overland flow, CAFO effluent discharges, and runoff from fields receiving animal manure [14,15]. The ubiquitous use of TBA and MGA growth promoters suggests that assessing the environmental fate of these exogenous steroids and their metabolites is a critical aspect of evaluating the risk these compounds pose to sensitive aquatic organisms.

To date, gas chromatography–mass spectrometry (GC/MS) analytical methods have been used to detect trenbolone metabolites in biological matrices (*e.g.* urine and tissue) [16,17]. Analytical methods for the detection of these compounds in environmental matrices (*e.g.* water and solids) often utilize liquid chromatography (LC) methods due to the simpler sample processing steps required for LC along with difficulties associated with thermal instability and problematic derivatization of trenbolone for GC analysis [10,14,18–20]. One study used a simple GC/MS method (no derivatization) for the confirmatory analysis of 17α-trenbolone and 17β-trenbolone in an agricultural receiving water, though with very poor sensitivity and variable performance [10]. However, no gas chromatography–mass tandem spectrometry (GC/MS/MS) analytical methods for detecting low concentrations of the metabolites of TBA and MGA in complex environmental matrices such as receiving waters and solids samples (*e.g.* soil, manure) have been published to date. GC/MS/MS can be advantageous due to its reduced costs in comparison to other analytical instruments and sometimes offers better chromatographic resolution of analytes in difficult sample matrices. Tandem mass spectrometry also offers improved sensitivity, especially in the very heterogeneous, high suspended solids, high organic carbon matrices typical of field sampling efforts. In comparison, liquid chromatography–tandem mass spectrometry methods are often preferred choices for steroid analysis due to their ability to analyze compounds without using a preparatory derivatization step, but can experience substantial matrix effects.

The objective of this research was to apply GC/MS/MS analytical methods to quantitatively detect trace levels of the metabolites of TBA and MGA in environmental samples such as receiving waters and solids. While GC/MS analytical methods for analysis of 17β-trenbolone and 17α-trenbolone in biological matrices exist [16,17], the unique aspects of this method include optimization for aqueous and soil samples and the incorporation of trendione and melengestrol as analytes. No published GC/MS/MS analytical method exists for these compounds. The basic derivatization procedure for trenbolone was first presented by Maume et al. and Marchand et al. [16,17]. Optimization of this derivatization method to quantify exogenous steroid metabolites at low concentrations in complex environmental matrices employed C18 solid phase extraction followed by Florisil cleanup, *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide (MSTFA)-I_2 derivatization, and subsequent GC/MS/MS analysis.

2. Materials and methods

2.1. Materials

All steroids and standards used in these studies were obtained at the highest possible commercially available purity. The steroids melengestrol (4,6-pregnadien-6-methyl-16-methylene-17-OL-3,20-dione) and 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) were purchased from Steraloids. Inc. (Newport, R.I., USA). Deuterated 17β-trenbolone (d3-17β-hydroxy-estra-4,9,11-trien-3-one) was obtained from the Bank of Reference Standards (RIVM, Netherlands). 17α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one) was purchased from NMI (Pymble, NSW, AU). Trendione (estra-4,9,11-trien-3,17-dione) was synthesized from 17β-trenbolone using the protocol outlined in Khan et al. [14], although this compound is recently