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Development of a Fast Screening and Confirmatory Method by Liquid Chromatography–Quadrupole-Time-of-Flight Mass Spectrometry for Glucuronide-Conjugated Methyltestosterone Metabolite in Tilapia

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ABSTRACT: This paper describes the development of a fast method to screen and confirm methyltestosterone 17-*O*-glucuronide (MT-glu) in tilapia bile. The method consists of solid-phase extraction (SPE) followed by high-performance liquid chromatography–mass spectrometry. The system used was an Agilent 6530 Q-TOF with an Agilent Jet stream electrospray ionization interface. The glucuronide detected in the bile was characterized as MT-glu by comparison with a chemically synthesized standard. MT-glu was detected in bile for up to 7 days after dosing. Semiquantification was done with matrix-matched calibration curves, because MT-glu showed signal suppression due to matrix effects. This method provides a suitable tool to monitor the illegal use of methyltestosterone in tilapia culture.

KEYWORDS: *methyltestosterone-17-O-glucuronide, tilapia bile, LC-QTOF*

INTRODUCTION

The exogenous androgen methyltestosterone (MT) is commonly used in newly hatched tilapia fry for sex reversal.[1−3]

Production of all-male populations of tilapia by feeding androgenic steroids to the fry is practiced in many countries. In the United States, MT is not approved for use in fish. There is, however, the possibility that fish farmers use MT for sex reversal in fish to have fast-growing all-male populations for economic gains. Therefore, there is a need to develop methods for the detection and confirmation of MT residues in different fish matrices for monitoring unapproved MT use.

Previously, our laboratory has developed an analytical method suitable for the detection of the parent drug MT in fish.[4] However, MT undergoes rapid elimination from the tissues of tilapia after 24 h. Analytical schemes for monitoring of xenobiotics in fish should be designed to detect metabolites in addition to the parent compound. We hypothesize that a significant portion of MT residues may persist longer and at higher concentrations as the glucuronide metabolite in fish, and thus the metabolite could serve as a better marker residue for monitoring purposes than the parent compound MT.

Early reports of glucuronidation in fish are conflicting due to low activities and sensitivity of assays used. However, in the past two decades a large number of compounds have been detected as glucuronides in fish, confirming that glucuronidation is an important route of metabolism in fish.[5] Analysis of glucuronides is generally performed by GC-MS or LC-MS determination of the free aglycones before and after hydrolysis. Risk of incomplete hydrolysis, isomerization, increased sample processing time, and loss of potentially useful information on the specific metabolite profile are disadvantages of the hydrolysis procedure.

In 1988 Cravedi reported indirect evidence for the presence of ^{14}C-labeled MT glucuronides in bile of rainbow trout by hydrolysis and detection of the aglycone MT.[6] However, to our knowledge, up to now there is no direct mass spectroscopic confirmatory evidence for the existence of an intact MT glucuronide in fish tissue. At present such methods have been used only for a few glucuronides of endogenous androgens and of androgenic anabolic steroids (AAS) in humans.[7−11] This is partly due to a lack of commercially available reference standards of AAS glucuronides. These standards are necessary in the development of direct analytical methods.

In this work we directly analyzed fish bile for methyltestosterone 17-*O*-glucuronide (MT-glu) without hydrolysis. To overcome the lack of commercially available standards, we chemically synthesized and purified MT-glu and characterized it by NMR and MS. The pure synthesized MT-glu standard was used for qualitative/semiquantitative analysis of fish bile.

Application of liquid chromatographic (LC) separation interfaced by soft ionization techniques, such as electrospray ionization (ESI), with tandem mass spectrometry (MS/MS) offers an effective analytical tool for the direct monitoring of AAS glucuronides. In the current study we developed rapid and sensitive LC-MS and LC-MS/MS methods for screening and confirmation of MT-glu within bile of fish, which would enable more efficient and extensive sampling to monitor the use of MT in fish.

In general, the determination of xenobiotic residues is performed in edible tissues of fish such as muscle and skin. However, we found it attractive to also analyze bile, which often contains high concentrations of metabolites. Bile is a good source of biotransformation products and could be useful as an

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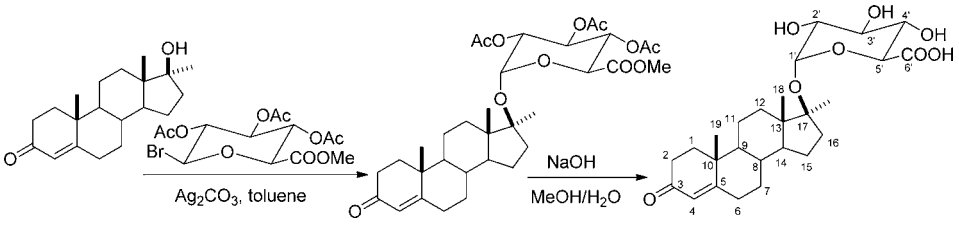

Figure 1. Synthesis of MT-glu.

aid in both residue monitoring studies and determining the metabolic profile of a chemical.

MATERIALS AND METHODS

Reagents and Supplies. LC-grade water used in preparing solutions was purified in-house with a Milli-Q Plus water system. Methanol (MeOH), acetonitrile (ACN), hexane, and ethyl acetate (EtOAc) were of HPLC grade (Burdick & Jackson, Muskegon, MI, USA). Dichloromethane (CH_2Cl_2) and formic acid were of reagent grade. Methyltestosterone (MT), acetobromo-α-glucuronic acid, and silver carbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oasis MAX strong anion exchange (3 mL, 60 mg) solid-phase extraction (SPE) columns were obtained from Waters Corp. (Milford, MA, USA).

Standard Solutions. *Primary Stock Solution (88 μg/mL):* MT-Glu (2.2 mg) was weighed into a 25 mL volumetric flask. MeOH was added to dissolve the solid.

Working Stock Solutions. Working MT-glu standard solutions at 1.0, 2.0, 4.0, 6.0, and 10.0 ng/μL were prepared by diluting the MT-glu primary stock solutions with MeOH/water (60:40 v/v). All solutions were stored at −10 °C or below.

Synthesis of MT-glu. A mixture of methyltestosterone (270 mg, 0.89 mmol), bromopyranose (660 mg, 1.66 mmol), and silver carbonate (1.0 g, 3.63 mmol) in 5 mL of CH_2Cl_2 was stirred at room temperature for 48 h. (Figure 1). The mixture was filtered and concentrated. The resulting residue was treated with 10% NaOH (10 mL) and stirred at room temperature for 18 h. The reaction mixture was diluted with water and washed with EtOAc (3 × 10 mL). The aqueous layer was acidified with 2 M HCl (pH ∼2) and extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with brine and concentrated. The crude product was purified by preparative HPLC to produce 15 mg (3.5% yield) of the product as a slightly yellow solid.

^{1}H NMR (CD_3OD, 600 MHz): δ 5.71 (s, 1H, H-4), 4.47 (d, J = 8.0 Hz, 1H, H-1′), 3.67 (d, J = 9.6 Hz, 1H, H-5′), 3.52 (dd, J = 9.6, 9.2 Hz, 1H, H-4′), 3.37 (dd, J = 9.2, 8.9 Hz, 1H, H-3′), 3.18 (dd, J = 8.9, 8.0 Hz, 1H, H-2′), 2.48 (dt, J = 14.8 and 5.0 Hz, 2H), 2.37 (t, J = 12.9 Hz, 1H), 2.27−2.32 (m, 2H), 2.10 (ddd, J = 13.4, 5.0, 3.1 Hz, 1H), 1.88−1.90 (m, 1H), 1.54−1.73 (m, 6H), 1.47−1.52 (m, 1H), 1.32−1.42 (m, 2H), 1.20−1.80 (m, 1H), 1.25 (s, 3H), 1.24 (s, 3H), 0.99−1.06 (m, 1H), 0.98 (s, 3H), 0.92−0.96 (m, 1H). ^{13} C NMR (CD_3OD, 150 MHz): δ 202.51, 175.42, 173.33, 124.26, 100.53, 89.35, 77.89, 76.55, 75.24, 73.41, 55.54, 51.14, 47.66, 40.19, 37.63, 36.95, 36.34, 34.85, 34.11, 33.24, 33.22, 24.57, 23.24, 21.93, 17.87, 15.03.

Fish Dosing. Dosing of fish was performed at the Center for Veterinary Medicine aquaculture facility. Tilapia (*Oreochromis* species, not sex reversed) were obtained from Aquasafra Inc. (Bradenton, FL, USA) as 1 g fry. Fish were raised in freshwater recirculating systems consisting of two 500 gal tanks at a temperature range of 20−30 °C until they reached a suitable size for dosing (400−700 g). Tilapia were given a single oral dose of 60 mg/kg body weight MT and sacrificed at 1, 2, 3, 7, and 14 days after the dose. Dosed fish were maintained in separate tanks. One fish was used for each time point. Filets and bile samples were removed and stored at −80 °C until analysis.

Analysis of MT-glu from Bile. *Sample Preparation.* Fish bile (∼100 mg) was weighed into a 15 mL polypropylene centrifuge tube. Prefortification of samples was done at this stage. To each sample was added 1 mL of 5% aqueous ammonium hydroxide solution, and the mixture was sonicated for 5 min.

SPE Processing. Processing was done using a RapidTrace (Charlotte, NC, USA) SPE workstation. Waters Oasis MAX SPE cartridges (60 mg, 3 mL) were preconditioned with 2.5 mL of MeOH (42 mL/min) followed by 2.5 mL of water (42 mL/min). After the samples (1 mL/min) had been loaded, the cartridges were rinsed successively with 2 mL of 0.5 M NH_4OH in 5% MeOH (42 mL/min) followed by 2 mL of 20% MeOH (42 mL/min). Cartridges were dried by passing 6 mL of air (42 mL/min). Glucuronide was eluted with 2 mL of freshly prepared 2% formic acid in MeOH (1 mL/min). Formic acid fractions were evaporated by blowing N_2 at 40 °C. Postfortification was done at this stage. Samples were reconstituted to 200 μL with MeOH/water (60:40 v/v).

Fortification. Pre- and postfortifications were carried out at five levels by adding 50 μL of each of the working standard solutions (1.0, 2.0, 4, 6, and 10 ng/μL) to bile before and after extraction. Solvent standards were prepared by mixing 50 μL of working standard solutions with 150 μL of MeOH/water (60:40 v/v).

Liquid Chromatographic Conditions. The LC system consisted of an Agilent series 1100 pump (Palo Alto, CA, USA). Extracts were analyzed for MT-glu using the following gradient LC conditions: mobile phase, ACN/0.1% aqueous formic acid; linear gradient of 30−90% ACN in 10 min, hold at 90% ACN for 5 min, linear gradient of 90−30% ACN in 1 min, followed by equilibration at 30% ACN for 9 min before the next injection. The flow rate was 0.2 mL/min. The column was a C18 (2) Luna, 150 × 2.0 mm, 3 μm column with a guard column of the same packing. The injection volume was 10 μL. The column oven was held at 40 °C, and the autosampler tray was maintained at 10 °C. The LC flow was diverted away from the mass spectrometer after 12 min of each run. An HPLC system was connected to the Agilent 6530 Q-TOF mass spectrometer. A typical injection sequence was as follows: solvent blank, standards, sample set, solvent blank, and standards again to bracket the samples.

The purification of synthesized MT-glu was performed on a Shimadzu preparative HPLC system (pump, LC-10ATvP; PDA, M10AvP; autosampler, SIL-HTa; system controller, SCL-10AvP). The pure MT-glu eluted ∼7.5 min from the semipreparative column (C18, 10 × 250 mm, 5 μm, Phenomenex, Torrance, CA, USA) running a linear gradient of 40−80% ACN in 15 min, at a 4 mL/min flow rate.

Mass Spectrometry. An Agilent 6530 Q-TOF mass spectrometer with an electrospray (ESI) interface with Agilent Jet Stream Technology in the positive ion mode was used. The instrument was calibrated in the high-resolution (4 GHz, High Res Mode), lower mass range (<m/z 1700) in the positive ion mode. Data were collected in both centroid and profile formats. Reference masses at m/z 121.0508 and 922.0098 were continually introduced along with the LC stream for accurate mass calibration. The drying gas (nitrogen) temperature was set at 325 °C, drying gas flow at 12 L/min, nebulizer pressure at 50 psi, and capillary voltage (Vcap) at 4000 V. Fragmentor voltage was set at 165 V. Both centroid and profile data within mass range m/z 100−1000 were acquired at a 1 spectra/s rate with Mass Hunter workstation (Agilent). The $[MT\text{-}glu + H]^+$ ion at m/z 479.2639 was extracted from the TIC and was selected as the precursor ion for collision-induced dissociation (CID). For targeted MS/MS analysis the product ion scan range was 50−500 Da. A retention time window of ±0.5 min and a medium (m/z 4) precursor ion isolation width were used with a scan rate of 1 spectra/s. High-purity nitrogen was introduced into collision cell as fragmentation gas.

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In the targeted MS/MS experiment, the collision energy was optimized at 30 V to obtain maximum intensity for fragments 285.2200, 267.2090, and 109.0644 and ~10% intensity for the precursor ion 479.2639 compared to the most intense fragment ion 285.2200. For confirmation, the ion ratio between m/z 285.2200 and 267.2090 was determined for both the synthetic and the MT-glu in the incurred tilapia bile. For quantitation, peak areas were computed of the MT-glu, m/z 479.2639, in the full-scan MS spectra. A 10 mDa window was used for extraction.

NMR Spectrometry. A Bruker (Billerica, MA) Avance 600 MHz NMR spectrometer was used.

RESULTS AND DISCUSSION

We used NMR and MS to confirm the structure of the synthesized glucuronide of MT as MT-glu. As MT contains only one possible site for glucuronidation, there was no ambiguity about the position of the glycosidic linkage. The carbon chemical shifts of the synthesized glucuronide were identical to the values published in the literature.[12] The appreciable shifts of the vicinal carbons in the ^{13}C NMR spectrum of carbons 17, 16, and 13 of MT-glu compared to those of MT also offered additional evidence of glucuronidation at C17 (Table 1).

Table 1. Chemical Shifts (Parts per Million) of Selected Carbons (CD_3OD at 25 °C)

carbon no.	MT	MT-glu
17	82.21	89.36
16	34.87	36.34
13	46.80	47.66

Integration of the ^1H NMR signals of the glucuronide/aglycone ratio confirmed the formation of the monoglucuronide. The MS spectra produced a molecular ion at m/z 479.2656 substantiating the formula $C_{26}H_{38}O_8$. The MS/MS spectra of the synthesized glucuronide exhibited strong fragment ions at m/z 97.0639 and 109.0644 consistent with the presence of the intact 4-ene-3-keto moiety in the glucuronide.[13] The MS/MS spectra of the synthesized MT-glu and the glucuronide in the incurred bile extract showed identical fragmentation patterns with close relative intensities (Figure 2). The comparable accurate mass values of the fragments also provided strong additional evidence that the glucuronide in the incurred bile was indeed MT-glu.

Even though MT-glu is stable as a solid, it decomposes readily in methanol/water solutions during freeze–thaw cycles. Therefore, stock solutions were stored in the freezer at −10 °C or below, and the sample tray in the LC system was maintained at 10 °C.

Bile is a very dirty matrix that consists of dark green pigments and bile acids. An anion exchange mixed mode SPE was the best sorbent for extraction of glucuronides from the bile. It was selective for acidic compounds and removed the polar and nonpolar neutral and basic components before the elution of glucuronides. When we first started using our optimized method of SPE, to our surprise we discovered large variations of recovery of the glucuronide from prefortified matrix extracts. After performing a controlled troubleshooting exercise, we discovered that the variability of the recovery from the SPE step is attributed to the instability of our eluting solvent, 2% formic acid in methanol. The instability of formic acid in methanol is documented in the literature.[14] Therefore, a fresh elution solvent must be prepared each day for elution of the glucuronide from the anion exchange resin.

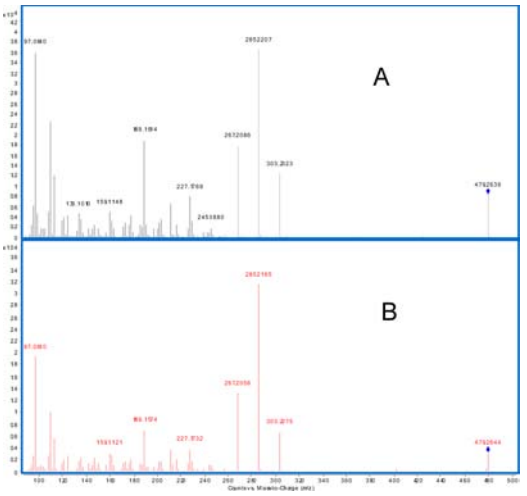

Figure 2. MS/MS spectra of the synthesized MT-glu standard in bile (A) and glucuronide in incurred tilapia bile extract (B).

The evaluation of matrix effects on the quantitative analysis of drugs in biological fluids is an important and sometimes overlooked aspect of the assay validation. FDA guidelines on bioanalytical analysis explicitly require the evaluation of matrix effects to ensure that precision, selectivity, and sensitivity will not be compromised.[15] Unfortunately, most of the LC-MS/MS methods published in the literature do not address the matrix effect issue, even though it is critical to the development of reliable methods. Correlation coefficients for all three (solvent, postfortified, and prefortified) five-point calibration curves of the MS response (area counts) against the amount of the MT-glu (ng) were >0.99. We calculated a matrix effect (suppression) of 17% by comparing the slope of the MS response of the analyte MT-glu spiked postfortified at five different concentration levels to the slope of the MS response of the analyte at same concentration levels in the "neat" mobile phase according to eq 1.[16] We determined a recovery of 90%, according to eq 2, a "true" recovery value that is not affected by the matrix.

$$\begin{aligned}\text{matrix effect} &= [\text{slope(postfortified)} \\ &\quad - \text{slope(solvent standard)}] \\ &\quad /\text{slope(solvent standard)} \\ &= -17\% \end{aligned} \tag{1}$$

$$\begin{aligned}\text{recovery} &= \text{slope(prefortified)}/\text{Slope(postfortified)} \\ &= 90\% \end{aligned} \tag{2}$$

Our primary goal in this study was to develop a fast screening and confirmation method for MT-glu. Because MS data indicated a linear response and good recovery for MT-glu, we used our data to obtain a semiquantitative analysis with our limited samples. Typical extracted ion chromatograms of control, fortified control, and bile of fish dosed over a 7 day period are shown in Figure 3. A sample was confirmed positive if the mass accuracy of the parent ion was within 5 ppm of the calculated, the retention time agrees within 2%, and the ion ratios between fragments produced in the targeted MS/MS agree arithmetically within 10% compared to MT-glu in postfortified bile. The molecular formula of fragments agreed

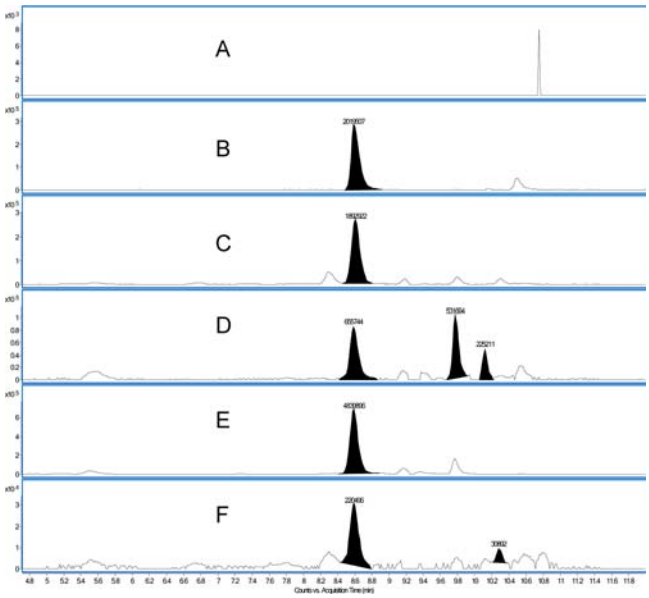

Figure 3. Extracted ion chromatograms of control bile (A), MT-glu spiked bile (B), and incurred bile 1 day (C), 2 days (D), 3 days (E), and 7 days (F) postdose. Note that the *y* scales for each extracted ion chromatogram are different.

with the postulated fragmentation mechanism of MT-glu (Figure 2). All fortified and incurred samples were confirmed positive with these criteria, whereas the control was confirmed negative.

The glucuronide levels found in bile of dosed fish over 7 days are reported in Table 2.

Table 2. Levels of MT-glu Found in Dosed Tilapia

days after dosing	amount of MT-glu (ng/mg)
1	1.8
2	0.8
3	4.2
7	0.5

The highest concentration of MT-glu (4.2 ng/mg) was detected 3 days after dosing. Compared to the low levels (ppb) of the parent compound MT detected in the muscle of tilapia by our previous study, we detected a much higher concentration (ppm) of the glucuronide metabolite MT-glu in the bile up to 7 days. The glucuronide was not detected in the bile at 14 days. The low concentration of MT-glu observed on day 2 could be due to fish-to-fish variation.

We also analyzed the muscle of tilapia for MT-glu using an extraction method we developed using Oasis HLB SPE, which provided 50−60% recovery of MT-glu from prefortified muscle extracts. We did not detect any MT-glu in the dosed fish muscle even at a lower parts per billion concentration at any time point of our study (unpublished data). Therefore, it is possible that the glucuronide does not reside in the muscle but is excreted via the bile.

In this paper we describe an efficient screening method to detect and confirm MT use in aquaculture of tilapia, which can be extended to other fish species. Because MT is not approved for use in aquaculture in the United States, this method can be developed for monitoring its use and for surveillance purposes.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Gale, W. L.; Fitzpatrick, M. S.; Lucero, M.; Contreras-Sanchez, W. M.; Schreck, C. B. Masculinization of Nile tilapia (*Oreochromis niloticus*) by immersion in androgens. *Aquaculture* **1999**, *178* (3−4), 349−357.

(2) Kuwaye, T. T.; Okimoto, D. K.; Shimoda, S. K.; Howerton, R. D.; Lin, H. R.; Pang, P. T. K.; Grau, E. G. Effect of 17α-methyltestosterone on the growth of the euryhaline tilapia, *Oreochromis mossambicus*, in fresh water and in sea water. *Aquaculture* **1993**, *113* (1−2), 137−152.

(3) Pandian, T. J.; Sheela, S. G. Hormonal induction of sex reversal in fish. *Aquaculture* **1995**, *138* (1−4), 1−22.

(4) Chu, P. S.; Lopez, M.; Serfling, S.; Gieseker, C.; Reimschuessel, R. Determination of 17α-methyltestosterone in muscle tissues of tilapia, rainbow trout, and salmon using liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* **2006**, *54* (9), 3193−3198.

(5) Clarke, D. J.; George, S. G.; Burchell, B. Glucuronidation in fish. *Aquat. Toxicol.* **1991**, *20* (1−2), 35−56.

(6) Cravedi, J. P.; Delous, G.; Debrauwer, L.; Prome, D. Biotransformation and branchial excretion of 17-α-methyltestosterone in trout. *Drug Metab. Dispos.* **1993**, *21* (2), 377−385.

(7) Hintikka, L.; Kuuranne, T.; Leinonen, A.; Thevis, M.; Schanzer, W.; Halket, J.; Cowan, D.; Grosse, J.; Hemmersbach, P.; Nielen, M. W. F.; Kostiainen, R. Liquid chromatographic-mass spectrometric analysis of glucuronide-conjugated anabolic steroid metabolites: method validation and interlaboratory comparison. *J. Mass Spectrom.* **2008**, *43* (7), 965−973.

(8) Curtis, L.; Diren, F.; Hurley, M.; Seim, W.; Tubb, R. Disposition and Elimination of 17 α-methyltestosterone in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **1991**, *99* (1−2), 193−201.

(9) Goudie, C. A. Extraction of a synthetic androgen from fish muscle and quantitation by high performance liquid chromatography. *Steroids* **1984**, *44* (3), 241−252.

(10) Kuuranne, T.; Vahermo, M.; Leinonen, A.; Kostianen, R. Electrospray and atmospheric pressure chemical ionization tandem mass spectrometric behavior of eight anabolic steroid glucuronides. *J. Am. Soc. Mass Spectrom.* **2000**, *11* (8), 722−730.

(11) Maurer, H. Liquid chromatography mass spectrometry in forensic and clinical toxicology. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **1998**, *713* (1), 3−25.

(12) Thevis, M.; Opfermann, G.; Schmickler, H.; Schanzer, W. Mass spectrometry of steroid glucuronide conjugates. II. Electron impact fragmentation of 3-keto-4-en- and 3-keto-5α-steroid-17-*O*-β glucuronides and 5α-steroid-3α,17β-diol 3- and 17-glucuronides. *J. Mass Spectrom.* **2001**, *36* (9), 998−1012.

(13) Pozo, O. J.; Van, E.; Deventer, K.; Grimalt, S.; Sancho, J. V.; Hernandez, F.; Delbeke, F. T. Collision-induced dissociation of 3-keto anabolic steroids and related compounds after electrospray ionization. Considerations for structural elucidation. *Rapid Commun. Mass Spectrom.* **2008**, *22* (24), 4009−4024.

(14) Snoble, K. A. J.; Fox, N. M.; Lorenz, S. M.; Kemperman, A. R.; Przybytek, J. T. Stability of formic acid in methanol solutions and the implications for use in LC-MS gradient elution analysis. *LCGC N. Am.* **2008**, *26* (9), 946−950.

(15) Department of Health and Human Services, Food and Drug Administration. Guidance for Industry on Bioanalytical Validation. *Fed. Regist.* **2001**, *66* (100), 28526 (docket 98D-1195. 2001)..

(16) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **2003**, *75* (13), 3019−3030.