Investigation of HPLC–MS Using a Monolithic Column to Separate a Diverse Suite of Steroids

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Abstract

A rapid method for profiling steroids with a wide range of polarity has been developed using high-performance liquid chromatography–mass spectrometry with a monolithic LC column. Steroids are detected using tandem mass spectrometry (MS^n) with a quadrupole ion trap and quantified using testosterone-d_3 as the internal standard. The method is compared to two similar methods using a traditional particulate column in terms of number of steroids eluted, peak area reproducibility, limits of detection, and overall analysis time. The monolithic method elutes the steroids in a 20-min analysis time, whereas the particulate methods elute the steroids in 30 and 45 min, respectively. The monolithic column also allows for improved reproducibility (relative standard deviations from 5–23%, as opposed to 14–42% for the shorter particulate method) and lower limits of detection (typically 2–5 times lower) when compared to the particulate column. Finally, the method is evaluated with unextracted, spiked alligator plasma, giving responses within 80–90% of those expected for standards for all steroids tested (except androstenedione).

Introduction

Steroids are messengers responsible for a wide array of functions in the endocrine system, including sexual development (1–4), reproduction (3,4), metabolic and immune system control (3,4), and general muscle growth and development (3,4). Endogenous steroid profiles can change as a result of drug intake, use of anabolic enhancements, and exposure to endocrine disrupting compounds (EDCs) and other pollutants (5–7). Steroid levels can be further altered by changes in physical and mental stress and by many endocrine diseases and disorders, and can even vary across differing races and backgrounds (8–10). Such changes and differences in the levels of steroidal hormones can have a significant effect on the growth, lifespan, and reproductive success of an individual (6,7). Therefore, it is important to develop rapid and reliable methods to simultaneously analyze a wide range of endogenous steroids. Moreover, it is of particular interest that these be quantitative chromatographic methods, in order to allow for the analysis of changes in the levels of such a large suite (due to exposure to endocrine disruptors or other environmental stresses) within a single analysis.

Primarily due to limitations in separation capabilities of particulate columns, existing steroid-monitoring methods tend to concentrate on the analysis of only a small range of steroids at one time, usually within a specific class. Attempting to extend these methods to separate steroids across several classes would become prohibitively long, and would significantly broaden the peaks of those steroids that eluted last (3,11,12). Many circumstances and stimuli are capable of simultaneously affecting multiple steroid pathways and concentrations (5,8); furthermore, the exact endocrine responses are often unknown (5,6,10), which makes the extension to broader analysis techniques more appealing. A method to quickly profile multiple subclasses of steroids in a single analysis would provide a more complete interpretation of steroid pathways and function.

The standard method among biologists to monitor steroid levels is radioimmunoassay (RIA). RIA, however, suffers from cross-reactivity and are only capable of monitoring single steroids per assay (3,13,14). Furthermore, the availability of an RIA test for a given steroid is dependent on the availability of the corresponding antibody, which makes unknown analysis extremely difficult. Therefore, immunoassay methods are not suitable for the comprehensive analysis of steroids. While gas chromatography–mass spectrometry (GC–MS) offers the high sensitivity, specificity, and resolution necessary for profiling, many steroids are not GC-amenable and require a derivatization step prior to analysis. Derivatization procedures are often time-consuming and laborious, taking upwards of 30 min in some cases (15–18). Furthermore, sample loss due to incomplete derivatization and the formation of artifacts can reduce overall sensitivity (16–18). In contrast, high-performance liquid chromatography–mass spectrometry (LC–MS) methods using traditional particulate columns require no derivatization, but often have inadequate resolving power for the comprehensive analysis of steroids. Existing LC–MS methods tend to concentrate on only a small number of steroids, usually within a single sub-class (11,12). Previous work in our lab has shown that LC–MS methods designed to separate a diverse range of steroids often result in poor peak shapes and poor limits of detection. In the search for ways to improve LC–MS profiles, monolithic LC columns were evaluated.

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Monolithic LC columns can be considered a bridge between existing GC–MS and particulate LC–MS methods. For steroid analysis, monolithic columns require no derivatization, and compared to a particulate LC column, the monolithic column's macropores and mesopores allow for an increase in interactions of the steroids with the stationary and mobile phases (19,20). This enhanced interaction can result in improved separation of the steroids along the same length of column. Simultaneously, the porous, open nature of the monolithic stationary phase allows the mobile phase, along with analyte, to flow more efficiently along a more direct path compared to traditional particle-filled columns, which reduces the multipath peak-spreading and results in sharper, taller chromatographic peaks (19,20). Furthermore, because of their solid column interior, monolithic columns require less sample preparation than traditional columns, often reducing or eliminating the need for extraction prior to injection because excess sample matrix and impurities cannot easily clog the column (19).

In this experiment, the novel use of a monolithic liquid chromatography column for the separation of a diverse suite of steroids within a single chromatographic run was examined. Because of its enhanced resolving power, the monolithic column employed here was able to sufficiently separate a suite of steroids without overly broadening the chromatographic peaks. The monolithic column was compared to a traditional particulate column in terms of overall analysis time, number of steroids separated, peak area reproducibility, and limits of detection. The developed method was then applied to American alligator (*alligator mississippiensis*) plasma spiked with endogenous steroids.

Experimental

Materials

Standards and gases

Standards of testosterone (T), androstenedione (AE), 17-methyltestosterone (MT), 5-β-dihydrotestosterone (DHT), 17-β-estradiol (E2), estrone (E1), diethylstilbestrol (DES), progesterone (P), pregnenolone (PREG), corticosterone (CORT), cholesterol (CHOL), and testosterone-d_3 (T-d_3, internal standard) were obtained from Sigma-Aldrich (St. Louis, MO). All HPLC-grade solvents (water, methanol, acetonitrile) were obtained from Fisher Scientific (Fairlawn, NJ). Ultra-high-purity (99.999%) helium and nitrogen were used.

Standard solutions of steroids at 100 mg/L were prepared by dissolving 2.00 mg of each steroid in 0.020 L methanol. A 1000 µg/L mixture of all standards used for subsequent dilutions was prepared by mixing 20.0 µL of each standard solution in a 4-mL vial, evaporating under nitrogen, and redissolving in 2.00 mL 50:50 acetonitrile–water.

Instrumentation

LC was performed using a Paradigm MS4 mulitdimensional separations module (Michrom BioResources, Auburn, CA) coupled to an LCQ Deca MS with atmospheric pressure chemical ionization (APCI) (Thermo Finnigan, San Jose, CA). Data analysis was performed using Xcalibur software (Thermo Finnigan). Compounds were separated using either a packed-particle Luna C-18 LC column (250 × 4.6 mm i.d., 5-µm particle size) with a SecurityGuard HPLC guard column or using a monolithic Onyx C-18 LC column (100 × 4.6 mm i.d.) (Phenomenex, Torrance, CA).

LC

Sample loading

Sample loading onto each column was performed using the Paradigm MS4's Magic autosampler, employing the microliter pickup function. The operational settings used a 20-µL injection volume with a methanol rinse.

Mobile phase

Three different mobile phase methods were examined: one for the monolithic column and two for the particulate column. The monolithic column method (MM) used a steady mobile phase flow rate of 500 µL/min and a mobile phase gradient beginning at 70:30 water–acetonitrile (H_2O–ACN), holding at 70:30 H_2O–ACN for 5 min, and then ramping the ratio to 20:80 H_2O–ACN over 18 min, followed by a return to 70:30 H_2O–ACN over the last 5 min for re-equilibration. The first particulate method (PM-1) attempted to elute the steroids in a similar analysis time to the monolithic method by use of a faster flow rate. PM-1 used a steady mobile phase flow rate of 1.00 mL/min and a mobile phase gradient beginning at 70:30 H_2O–ACN, holding at 70:30 H_2O–ACN for 7.5 min, and then ramping the ratio to 25:75 H_2O–ACN over 25 min, followed by a return to 70:30 H_2O–ACN over the last 5 min for re-equilibration. The second particulate method (PM-2) attempted to elute the steroids with similar chromatographic quality to the monolithic column. PM-2 used a mobile phase flow rate of 500 µL/min (similar to the monolithic method) and had a mobile phase gradient beginning at 70:30 H_2O–ACN, holding at 70:30 H_2O–ACN for 10 min, and then ramping the ratio to 17:83 H_2O–ACN over the next 38 min, followed by a return to 70:30 H_2O–ACN over the last 5 min for re-equilibration.

Table I. The Steroids Tested Along with the *m/z* Values used for Tandem Mass Spectrometry and Quantification

Steroid	Abbreviation	MS^2 or MS^3 *m/z*	Ion Type(s)	Quantification
Testosterone	T	289	$[M+H]^+$	97, 109
Androstenedione	AE	287	$[M+H]^+$	97, 109
17-Methyltestosterone	MT	303	$[M+H]^+$	97, 109
5β-Dihydrotestosterone	DHT	291	$[M+H]^+$	97, 109
17β-Estradiol	E2	255	$[M–H_2O+H]^+$	133, 159
Estrone	E1	271→253	$[M+H]^+$→$[M–H_2O+H]^+$	158, 199
Diethylstilbestrol	DES	269	$[M+H]^+$	135, 199
Pregnenolone	PREG	299→281	$[M–H_2O+H]^+$	172, 212
Progesterone	P	315	$[M+H]^+$	97, 109
Corticosterone	CORT	347	$[M+H]^+$	121, 269
Cholesterol	CHOL	369	$[M–H_2O+H]^+$	177, 296
Testosterone-d_3	T-d_3	292	[M+H]	97, 109

MS

APCI

Analytes were ionized using APCI as they eluted off of the column. APCI parameters were as follows: capillary temperature, 150°C; APCI temperature, 450°C; sheath gas flow, 50; auxiliary gas flow, 10; and corona needle discharge current, 4.00 µA.

Tandem MS

Analyte ions were detected using the quadrupole ion trap MS. The MS and MS^n parameters were as follows for all ions: scan range, *m/z* 75–400; isolation width, 1.5; normalized collision energy, 30%; activation q, 0.250; and activation time, 30 ms. Steroids were quantified using tandem MS (MS–MS) by isolating the precursor ion, either $[M+H]^+$ or $[M-H_2O+H]^+$ depending on the steroid, and generating ion chromatograms using the expected product ions. For estrone and pregnenolone, a third level of tandem mass spectrometry (MS^3) was necessary, selecting the product ions $[M+H]^+ \rightarrow [M-H_2O+H]^+$, and $[M-H_2O+H]^+ \rightarrow [M-2H_2O+H]^+$, respectively, from MS–MS for MS^3. This information and the actual *m/z* values used are summarized in Table I.

Table II. Relative Standard Deviations (*n* = 3) for Each of the Steroids of Interest in the Three Methods Used*

Steroid	MM (%)	PM-I (%)	PM-II (%)
T	5.4	17	13
AE	12	14	ND
MT	12	14	5.9
DHT	9.6	ND	ND
E2	7.7	16	7.7
E1	4.7	19	4.5
DES	20	ND	30
PREG	5.9	33	21
P	8.4	17	9.5
CORT	18	23	19
CHOL	23	42	29

* ND indicates that the steroid was not detected; MM indicates the monolithic method; PM-I indicates the first particulate method, optimized for rapid elution; and PM-II indicates the second particulate method, optimized for separation and quantification

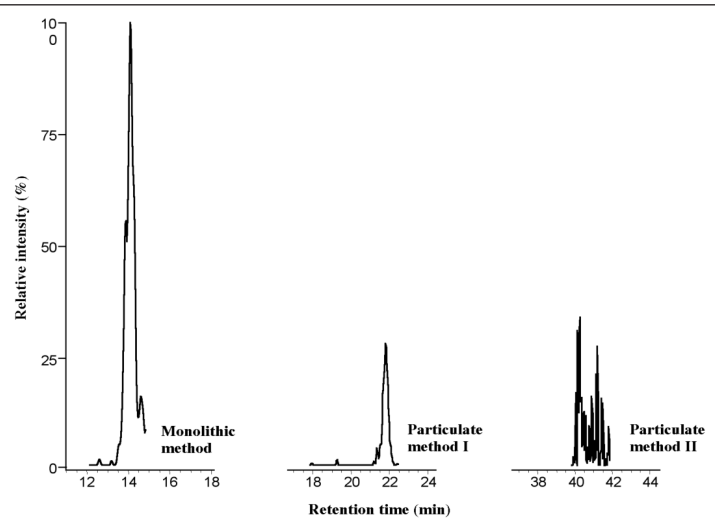

Figure 1. Comparison of peak shapes and normalized heights for testosterone in each of the three methods. Peaks are normalized to MM.

Comparisons of chromatograms

To determine the precision of the method, a standard mixture of all steroids and the internal standard, each at 1000 µg/L, was analyzed three times, and Xcalibur software was used to integrate the peak areas. The steroid peak areas were then divided by the peak area of the internal standard, testosterone-d_3, to give a relative area for each peak. This corrected for instrumental variability and demonstrated the precision (%RSD) of the methods using both column types at 1000 µg/L.

Estimated limits of detection

Standard mixtures were prepared at 1000, 100, 50, 10, and 1 µg/L, and each was run in triplicate using each of the three methods. The limit of detection (LOD) (defined as the lowest level standard that yielded a signal-to-noise ratio of at least 3) was estimated independently for each steroid in standard solution.

Alligator plasma

Plasma samples were obtained from several captive-raised alligators and were pooled together into one large sample. Steroids were spiked into the plasma at a concentration of 500 µg/L. For each 1 mL sample, 3 mL of acetonitrile was used to precipitate the proteins, and the supernatant (approximately 1 mL) was transferred to an autosampler vial for injection. No extraction step was performed beyond protein precipitation.

Results and Discussion

Profile parameters

The MM was developed to elute the diverse suite of steroids as quickly as possible while still allowing for quantification. The particulate column used was of the same phase (C-18), but was 2.5 times as long as the monolithic column, to account for the reduced resolving power. The first particulate method (PM-I) was chosen to elute the steroids in a similar time frame to MM without regard for quantification or peak shape; it does so by doubling the flow rate to correct for the added length of the column. The second particulate method (PM-II) was chosen to give similar separation and quantification parameters to MM, albeit with a longer analysis time, which was accomplished at the same flow rate as the monolithic method. The steroid profile used was chosen to emulate the wide polarity range of steroids, which could possibly appear in an endogenous sample. Steroids were separated in each method in order of polarity, with the most polar steroids eluting first and the least polar eluting last. Several steroids (androstenedione, dihydrotestosterone, and the steroid-like diethylstil-

bestrol) were not detected in the particulate methods because of the higher limits of detection.

Comparison of peak areas

The relative standard deviations for the relative peak area of each of the steroids in all three methods are presented in Table II. Note that whereas the MM and the slow particulate method (PM-II) have comparable precision for many steroids, the fast particulate method (PM-I) has a much poorer precision in every case. The increased relative standard deviation in PM-I makes quantification difficult because of the large variability in the chromatographic peak areas. For several of the analytes, including PREG and DES, the monolithic method's precision is superior to both particulate column methods. This is most likely due to the superior peak shapes and greater peak height obtained using the monolithic method, as shown using testosterone in Figure 1.

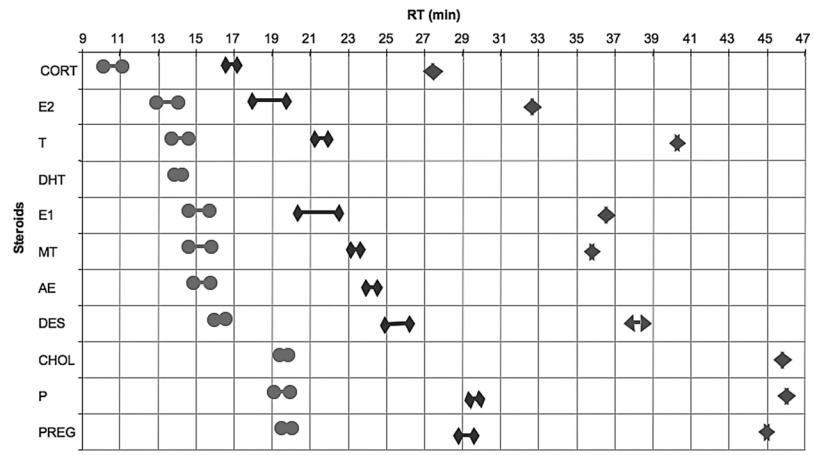

Figure 2. Comparison of the retention times and peak widths of the monolithic method (●—●), the fast particulate method (◆—◆), and the slow particulate method (◆—◆).

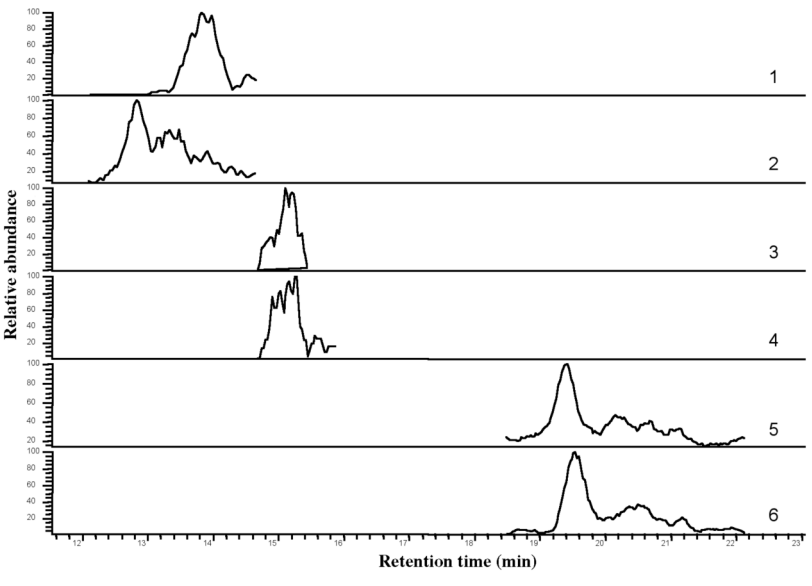

Figure 3. Spiked plasma chromatogram. Each steroid is simultaneously analyzed using its own tandem mass spectrometry method. 1, Testosterone; 2, 17β-estradiol; 3, 17-Methyltestosterone; 4, Androstenedione; 5, Progesterone; 6, Pregnenolone.

Elution times

The average peak widths at 10% peak height and retention times for each steroid are illustrated in Figure 2. Although the separation of PM-II is similar to that of MM, the final few steroids did not elute until approximately 45 min after the injection, over twice as long as in the monolithic method, which required only 20 min. The increased mobile phase flow rate of PM-I allows for the separation of the steroids in a time frame similar to the monolithic method, albeit with poorer resolution; however, PM-I still required nearly 10 min more than the monolithic method.

Limits of detection and quantitation

The LOD of each steroid were estimated using the three methods; the results are summarized in Table III. The first particulate method, although rapid, suffered from poor LODs in exchange for speed, whereas the second particulate method had improved LODs compared to the first particulate method, but was still inferior to the monolithic method for many of the steroids.

Alligator plasma injection

The unextracted, protein-crashed, spiked plasma was injected and analyzed for steroid concentrations using the monolithic column method. Each of the endogenous steroids was detected at this concentration (500 µg/L) despite the fact that no extraction was performed. No retention time shifting was observed compared to the standard samples, likely because the supernatant (acetonitrile) was similar enough to the starting mobile phase that the separation was unaffected. The responses were between 80% and 90% of the expected peak areas for each of the steroids, with the notable exception of androstenedione at 45%.

Conclusion

The monolithic column method was found to be an overall improvement over both of the particulate column methods tested. Whereas the particulate column forces the user to choose between a short run time and accurate quantification, the monolithic column simultaneously achieves both of these goals. The monolithic column produced significantly better precision when compared with both particulate methods, while profiling the steroids in less time. The estimated LODs of the monolithic method also proved to be equal or superior to the particulate methods for each of the steroids tested. Furthermore, the monolithic column holds the potential to eliminate

Table III. Estimated Limits of Detection for Each Steroid Standard Using the Three Methods*

Steroid	MM LOD (µg/L)	PM-I LOD (µg/L)	PM-II LOD (µg/L)
T	15	40	20
AE	20	60	ND
MT	20	150	60
DHT	200	ND	ND
E_2	15	50	20
E_1	20	60	40
DES	20	ND	30
PREG	20	80	60
P	20	60	30
CORT	500	500	500
CHOL	150	300	200

* ND indicates that the steroid was not detected; MM indicates the monolithic method; PM-I indicates the first particulate method, optimized for rapid elution; and PM-II indicates the second particulate method, optimized for separation and quantification.

the need for solid-phase extraction due to the solid nature of the mobile phase; plasma may be run without an extraction step beforehand, depending on the concentration of the analytes. Although it is still susceptible to certain limitations common to most HPLC–MS analysis (the use of a solvent gradient requires re-equilibration of the column, the need to reconstitute samples in the starting mobile phase to prevent shifting of retention times), the MM can allow for higher sample throughput when quantitatively profiling samples, facilitating comparison studies of steroid concentrations in multiple samples, and aiding comparison studies of steroid levels across many differing variables.

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References

1. A.T. Kicman. Pharmacology of anabolic steroids. *Br. J. Pharmacol.* **154:** 502–21 (2008).
2. M.B. Renfree, J.D. Wilson, and G. Shaw. The hormonal control of sexual development, Genetics and biology of sex determination, Vol. 244, Novartic Foundation Symposium, Chichester, New York, pp. 136–56.
3. O. Nozaki. Steroid analysis for medical diagnosis. *J. Chromatgr. A* **935:** 267–78 (2001).
4. H. Noppe, B. Le Bizec, K. Verheyden, and H.F. De Brabander. Novel analytical methods for the determination of steroid hormones in edible matrices. *Anal. Chim. Acta* **611:** 1–16 (2008).
5. S.A. Hall, S.T. Page, T.G. Travison, R.B. Montgomery, C.L. Link, and J.B. McKinlay. Do statins affect androgen levels in men? Results from the Boston area community health survey. *Cancer Epidemol. Biomarkers Prev.* **16:** 1587–94 (2007).
6. J.S. Fisher. Are all EDC effects mediated via steroid hormone receptors? *Toxicology* **205:** 33–41 (2004).
7. M.A. Ottinger, M. Abdelnabi, M. Quinn, N. Golden, J. Wu, and N. Thompson. Reproductive consequences of EDCs in birds: What do laboratory effects mean in field species? *Neurotoxicol. Teratol.* **24:** 17–28 (2002).
8. D.I. Spratt, J.R. Morton, R.S. Kramer, S.W. Mayo, C. Longcope, and C.P.H. Vary. Increases in serum estrogen levels during major illness are caused by increased peripheral aromatization. *Am. J. Physiol. Endocrinol. Metab.* **291:** E631–38 (2006).
9. C.H. Kroenke, S.E. Hankinson, E.S. Schernhammer, G.A. Colditz, I. Kawachi, and M.D. Holmes. Caregiving stress, endogenous sex steroid hormone levels, and breast cancer incidence. *Am. J. Epidemiol.* **159:** 1019–27 (2004).
10. A.H. Wu, A.S. Whittemore, L.N. Kolonel, E.M. John, R.P. Gallagher, D.W. West, J. Hankin, C.Z. Teh, D.M. Dreon, and R.S. Paffenbarger. Serum androgens and sex hormone-binding globulins in relation to life-style factors in older African-American, white, and Asian men in the United-States and Canada. *Cancer Epidemol. Biomarkers Prev.* **4:** 735–41 (1995).
11. K. Deventer, O.J. Pozot, P. Van Eenoo, and F.T. Delbeke. Detection of doping agents by LC-MC and LC-MC-MC. *LC/GC N A* **26:** 376 (2008).
12. K. Yamashita, R. Nakagawa, M. Okuyama, S. Honma, M. Takahashi, and M. Numazawa. Simultaneous determination of tetrahydrocortisol, allotetrahydrocortisol and tetrahydrocortisone in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids* **73:** 727–37 (2008).
13. M. Hill, R. Hampl, D. Lukác, O. Lapcík, V. Pouzar, and J. Sulcová. Elimination of cross-reactivity by addition of an excess of cross-reactant for radioimmunoassay of 17[alpha]-hydroxypregnenolone. *Steroids* **64:** 341–55 (1999).
14. T. Wong, C.H.L. Shackleton, T.R. Covey, and G. Ellis. Identification of the steroids in neonatal plasma that interfere with 17-alpha-hydroxyprogesterone radioimmunoassays. *Clin. Chem.* **38:** 1830–37 (1992).
15. H. Budzinski, M.H. Devier, P. Labadie, and A. Togola. Analysis of hormonal steroids in fish plasma and bile by coupling solid-phase extraction to GC/MS. *Anal. Bioanal. Chem.* **386:** 1429–39 (2006).
16. M. Axelson. Exchange of oxime functions—useful reaction in GC-MS analysis of steroids. *Anal. Biochem.* **86:** 133–41 (1978).
17. W.H. Kwok, D.K.K. Leung, G.N.W. Leung, F.P.W. Tang, T.S.M. Wan, C.H.F. Wong, and J.K.Y. Wong. Unusual observations during steroid analysis. *Rapid Commun. Mass Spectrom.* **22:** 682–86 (2008).
18. D.H. van de Kerkhof, R.D. van Ooijen, D. de Boer, R.H. Fokkens, N.M.M. Nibbering, J.W. Zwikker, J.H.H. Thijssen, and R.A.A. Maes. Artifact formation due to ethyl thio-incorporation into silylated steroid structures as determined in doping analysis. *J. Chromatogr. A* **954:** 199–206 (2002).
19. K. Nakanishi, H. Minakuchi, N. Soga, and N. Tanaka. Double pore silica gel monolith applied to liquid chromatography. *J. Sol-Gel Sci. Technol.* **8:** 547–52 (1997).
20. Q.C. Wang, F. Svec, and J.M.J. Frechet. Macroporous polymeric stationary-phase rod as continuous separation medium for reversed-phase chromatography. *Anal. Chem.* **65:** 2243–48 (1993).

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