

Derivatization Procedure and Negative Chemical Ionization GC/MS/MS Conditions for the Analysis of Steroidal Analogs

Application Note

Clinical Chemistry

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Abstract

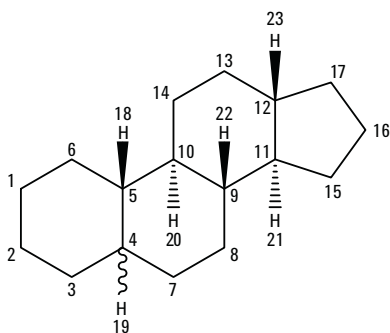
This application note describes a chemical derivatization procedure required to facilitate measurement of steroidal analogs in electron capture negative chemical ionization mass spectrometry and the GC/MS/MS conditions for highly sensitive and selective analysis.

Introduction

Steroids comprise a broad chemical class inherent to animals, plants, and fungi and can be divided into at least seven subclassifications such as estrogens, androgens, pregnanes, cholanes, cholestanes, corticosteroids, and mineralocorticoids. In biological systems, steroids perform hormonal and neuromodulating activities including anabolism, regulation of metabolism, immunomodulation, sexual development, and pregnancy control. In humans, the biosynthesis of steroids begins through NADPH reduction of two farnesyl pyrophosphate molecules to form squalene, then a two-step enzymatic cyclization to form the protosteroid lanosterol. Of the hundreds of known steroids, all are analogs constructed around the basic perhydrocyclopentaphenanthrene scaffold. Steroids have a number of medically approved uses including birth control, growth stimulation, and hormone replacement therapies. They can also be monitored as biomarkers of disease states such as the relationship of estrogen dysregulation in breast cancer and hypertension (Macherone, *et al* 2010). However, steroids are more commonly known for their abuse in popular sports and doping control. It is because of their natural importance in biology and their proper use and improper abuse that there exists great interest in the analysis of steroidal analogs. These interests range from clinical and forensic chemistry to emerging contaminants and their effect on the environment, flora, and fauna. In response to this broad interest across diverse research areas, this application note describes a chemical derivatization procedure and the instrument conditions required to monitor several estrogens and androgens through negative chemical ionization gas chromatography triple quadrupole mass spectrometry.



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(8*S*,9*R*,10*S*,13*S*,14*R*)-hexadecahydro-1*H*-cyclopenta[*a*]phenanthrene

Figure 1. The perhydrocyclopentaphenanthrene scaffold of steroids.

Experimental

Background

Estrogens and androgens differ from one another primarily through functional group substitution at carbons 2, 16, and 17 of the steroidal scaffold and by the number of carbon atoms inherent to each chemical subclass. For the purposes of derivatization, one must consider the analytical methodology. For example, will the analyst develop a gas chromatographic method using electron impact or chemical ionization mass

spectrometry to measure the analytes and which mode of mass spectrometry will be implemented: single stage such as SIM or SIS on a single quadrupole or ion trap, respectively or SRM on a triple quadrupole? This application note describes analysis through negative chemical ionization gas chromatography triple quadrupole mass spectrometry (NCI-GC/MS/MS) and therefore, must synthesize derivatization products that are amenable to the primary mechanism of negative chemical ionization, that is, electron capture. This can be achieved by a selective procedure that adds electronegative atoms such as fluorine to the basic chemical structure of the analyte.

Estrogens are comprised of 18 carbon atoms and a phenolic hydroxyl moiety at carbon 2 while carbons 16 and 17 are either hydroxyl, keto, or multiply substituted. Androgens are comprised of 19 carbon atoms with a ketone moiety at carbon 2 and either a hydroxyl or keto at carbon 17. From a synthetic chemistry point of view, alcohols and phenols can undergo esterification while ketones readily form stable oximes. Commercially available reagents for adding fluorine to a molecule through esterification or oximation include pentafluorobenzoyl chloride (PFBCl) and pentafluorobenzoyl hydroxylamine hydrochloride (PFBHA), respectively. A select representation of the derivation products using these reagents is given in Figure 2.

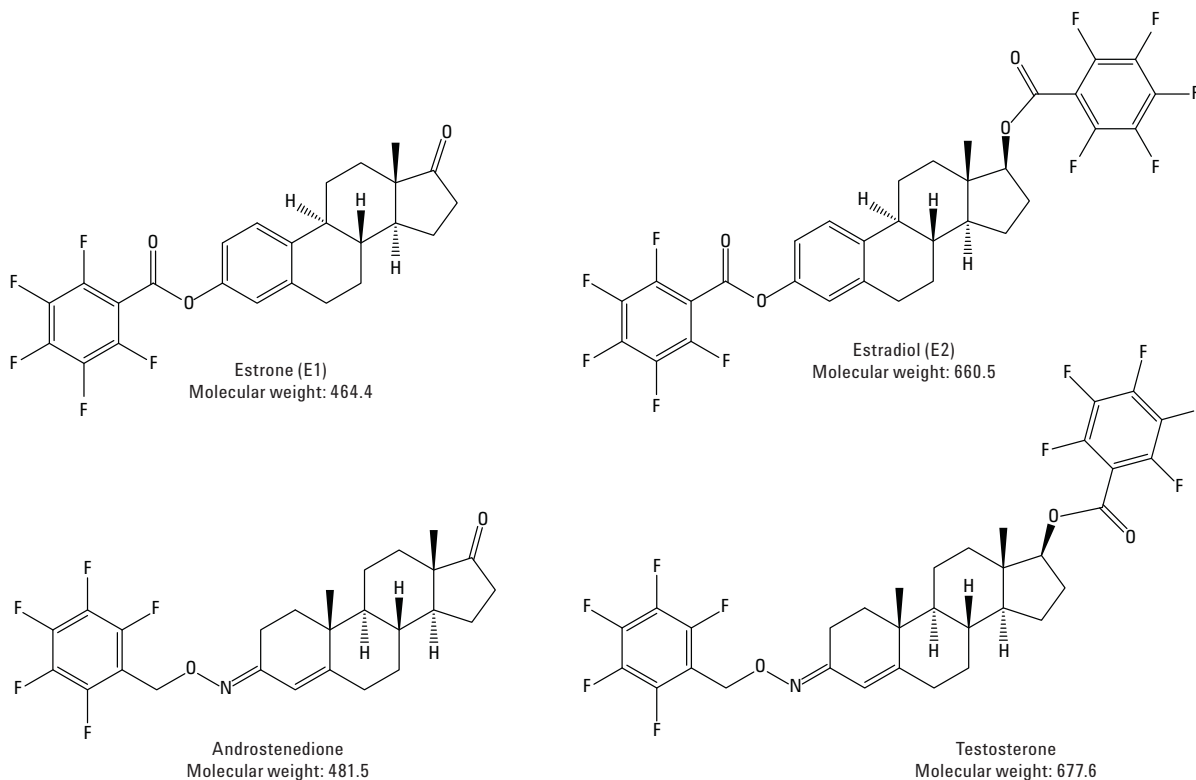


Figure 2. Representative derivatization products.

Derivatization Procedure

Esterification of phenols and alcohols with acid chlorides occurs through the nucleophilic acyl substitution reaction mechanism. Two considerations for reaction efficacy are: the solvent properties and the basicity of the leaving group. Acid chlorides are susceptible to the presence of water. Therefore the use of an anhydrous, polar, aprotic solvent is recommended in conjunction with a tertiary amine base to scavenge the chloride ion and pull the reaction equilibrium towards the derivatized product.

Oximation of ketones proceeds through a more complex imine-type mechanism with a reaction rate that can be optimized as a function of pH. The general mechanism begins through nucleophilic addition of the nitrogen on the pentafluorobenzyl hydroxylamine to the ketone followed by protonation and dehydration resulting in the oxime. Polar, aprotic solvents stabilize the charged intermediates, or a tertiary amine base can be used in its concentrated form to assure complete dissolution of PFBHA.

With the above considerations in mind, the following derivatization procedure was developed:

1. To dried extract, add 1.0 mL of pentafluorobenzoylchloride/anhydrous ethyl acetate (1.0%; v/v).
2. Add 100 μ L of anhydrous pyridine
3. Incubate at 60 °C for 30 minutes.
4. Add 0.5 mL of 0.5 M NaHCO₃ solution, and vortex.
5. Centrifuge for 5 minutes.
6. Transfer the organic phase into an Agilent high recovery auto sampler vial (p/n 5183-2073).
7. Evaporate under nitrogen at 50 °C.
8. To dried partially derivatized sample, add 100 μ L 0.1% (wt/v) pentafluorobenzyl hydroxylamine HCl in pyridine.
9. Incubate at 60 °C for 30 minutes.
10. Evaporate under nitrogen at 50 °C.
11. Reconstitute each tube with 50 μ L of dodecane or isooctane and vortex 30 seconds.

GC/MS/MS Analysis

All analyses were performed on an Agilent 7890A Gas Chromatograph with an Agilent 7000 Triple Quadrupole GC/MS System. The Agilent 7890A GC was equipped with a 1 m Agilent J&W DB-17ht column connected to a 15 m Agilent J&W DB-17ht column with a pressure controlled tee (PCT) known as the Purged Ultimate Union (Agilent Technologies, Inc., Santa Clara, CA). This column configuration allowed intrarun backflush of the 1-m precolumn beginning at 9 minutes into the analysis for the following benefits:

- Efficient removal of heavy matrix through the reversal of column flow
- Significantly improved retention times and LOD precision
- Increased column life and a clean source for an extended period

The Agilent 7000 Triple Quadrupole GC/MS was operated in negative chemical ionization (NCI) mode using methane as the reagent gas in selected reaction monitoring (SRM) for all analytes. SRM transitions were developed empirically from reference standards to identify optimal precursor/ product ion pairs for the analysis. The collision cell energy was optimized for each unique SRM transition to achieve the maximum ion intensity. The GC/MS/MS conditions are given in Table 1 and the retention times of the derivatized analytes under these conditions are given in Table 2. The optimized SRM transitions for each analyte measured are given in Table 3.

Table 1. GC/MS/MS Conditions

Conditions	
GC run conditions	
Columns	Column 1: Agilent J&W DB-17ht 0.18 mm \times 1 m, 0.1 μ m Column 2: Agilent J&W DB-17ht 0.18 mm \times 15 m, 0.1 μ m CFT device: Purged Ultimate Union
Injection mode	Pulsed splitless. 40 psi, 0.9 min
Inlet temperature program	280 °C
Injection volume	2 μ L
Carrier gas	Column 1: Helium, constant flow mode, 1.0 mL/min, 9 min then 99 mL/min to -15.05 mL/min (2 psi at 295 °C oven) Column 2: Helium, constant flow mode, 1.2 mL/min
Oven program	205 °C for 1 min then 10 °C/min to 295 °C for 0 min then 2.5 °C/min to 305 °C for 0 min
Transfer line temperature	290 °C
MS conditions	
Tune	Autotune
Gain factor	100
Chemical reagent gas	Methane, 40% (2.0 mL/min)
Acquisition parameters	Negative Chemical Ionization, Selected Reaction Monitoring (SRM)
Collision gas	Nitrogen, 1.5 mL/min; Helium quench gas 2.25 mL/min
MS temperatures	Source 150 °C, Quadrupoles 150 °C

Results and Discussion

In vivo, estradiol (E2) is present at very low concentrations especially in postmenopausal women and limits of quantification are typically as low as 1 pg/mL or 1 fg/ μ L of injected sample. As such, E2 requires the most analytical sensitivity and can be used as a probe for the overall sensitivity of the method. To this end, eight replicate injections were made of a 10-pg/mL E2 standard and the average signal-to-noise (S/N) was determined to be 441 using a root mean square algorithm based on signal height and at least 0.2 minutes of noise in the estradiol data collection channel. To this end, eight replicate injections were made of a 10 pg/mL E2 standard and a percent relative standard deviation (RSD) of 3.7% of the absolute area counts was determined. Additionally, a percent relative standard deviation (RSD) of 3.0% of the area counts was determined. The instrument detection limit (IDL) can therefore be determined by the equation: [3]

Equation 1. IDL

$$IDL = (t_{\alpha})(\%RSD)(\text{amount of standard})/100$$

t_{α} : Statistical confidence factor found in the Student t- distribution table.

With 99% confidence, the t_{α} value for 8–1 degrees of freedom is 2.998. Substituting this into Equation 1 gives:
 $(2.998)(3.7\%)(10 \text{ pg/mL})/100 = 1.1 \text{ pg/mL}$.

Table 4 illustrates the raw data for the IDL determination.

Figure 3 shows the absolute area counts for the eight replicate injections and RMS signal-to-noise (S/N) with noise region of 10.85 minutes through 11.35. Figure 4 illustrates the calibration curve from 0.5 pg/mL through 250 pg/ML with correlation coefficient of 0.998.

Table 2. Analytes and Retention Times

Name	Retention time
Estrone	8.960
Androstenedione	9.179
DHT isomer 1	11.353
DHT isomer 2	11.493
Estradiol	11.831
Testosterone	11.978

Table 3. Time Segments, SRM Transitions, Dwell Times and Collision Energies

Time segment (TS)	TS start time	Name	Precursor	Product	Dwell	CE
1	8.62	Estrone	464	400	250	10
2	9.08	Androstenedione	461	431	250	10
3	11.07	DHT isomer 1	679	659	125	10
		DHT isomer 2	679	629	125	10
4	11.45	Estradiol	660	596	250	10
5	11.90	Testosterone	677	657	125	8
		Testosterone	677	627	125	7

Table 4. Raw E2 Data for IDL Determination

Estradiol	
Injection no.	Area
1	22263
2	22194
3	21101
4	20603
5	22590
6	22988
7	21420
8	21634
Average	21849
St dev	800
% RSD	3.7

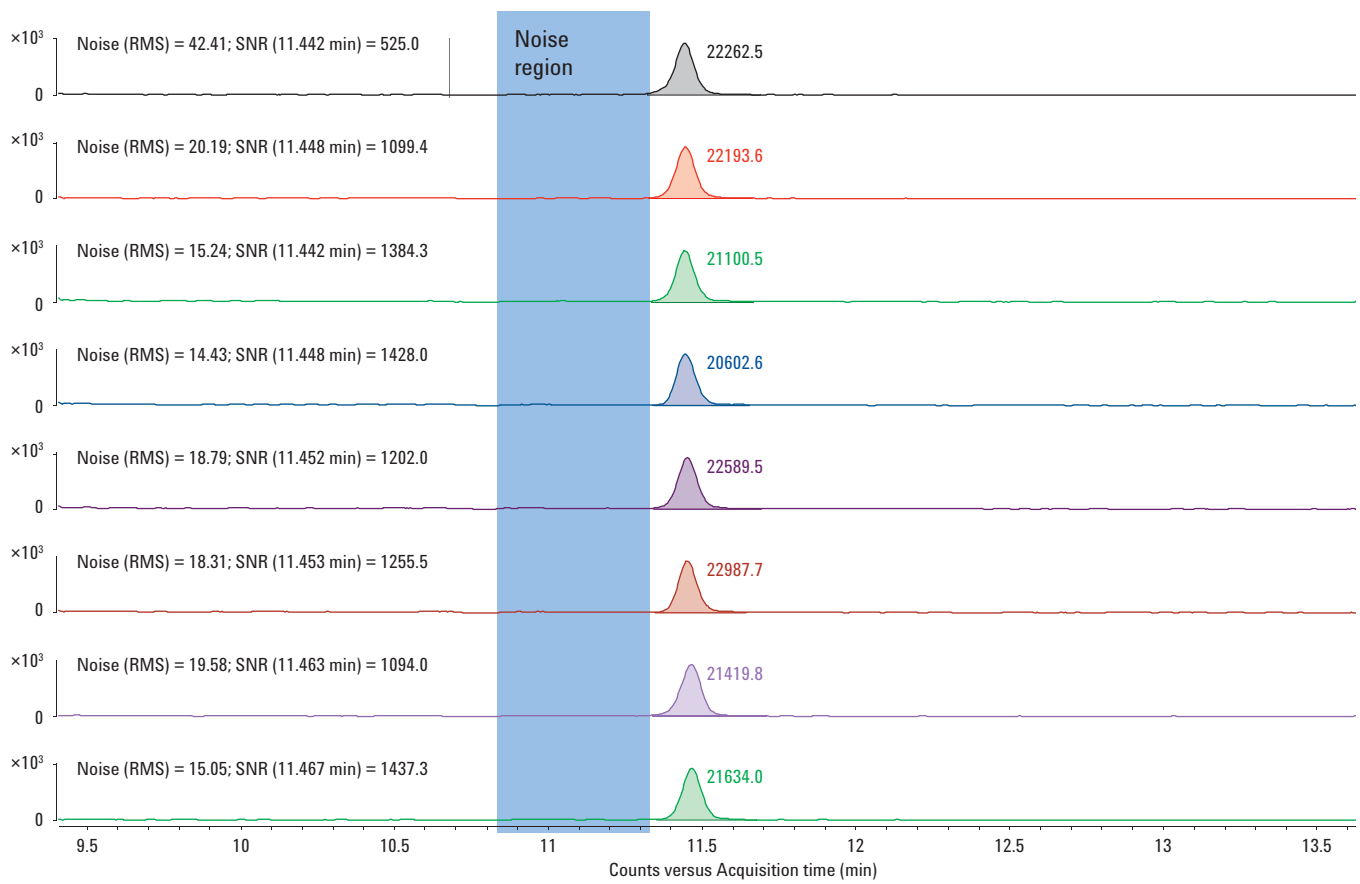


Figure 3. Chromatographs of eight replicate injections.

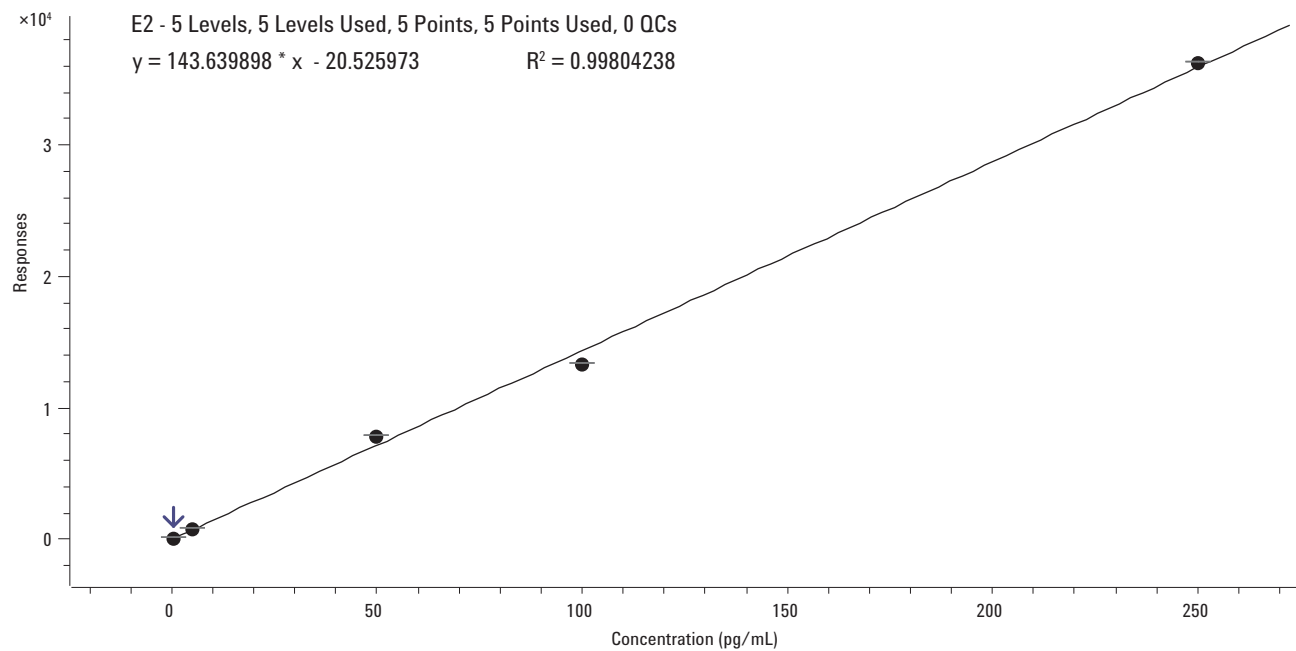


Figure 4. Estradiol calibration curve.

Summary

The accurate and sensitive measurement of steroidal analogs in a variety of matrices is an important requirement for clinicians to understand these compounds as biomarkers for disease and monitor their fate and transport *in vivo* and *ex vivo*. This application note outlines a chemical derivatization procedure that modifies steroidal analogs so they are amenable to electron capture negative chemical ionization mechanisms. The procedure also provides GC/MS/MS conditions required for a highly sensitive and robust analytical method with the IDL on the order of 1 pg/mL or less. Moreover, the derivatization procedure described is amenable to matrices other than serum or plasma and can be extended to environmental analyses on waste water effluent, soil, and bio-solids for *in natura* studies (Churley *et al*, 2011). The GC/MS/MS analytical method can be applied to any of these regardless of the source of the analytes or discipline of the researcher.

References

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2. A. Macherone, M. Churley, and R. White, "Ultra-low Detection of Estrogenic Compounds by GC-NCI/MS/MS", (2010) LC/GC Special Issues, Advanstar Communications, Inc.
3. G. Wells and H. Prest, "Why use signal-to-noise as a measure of MS performance when its often meaningless", (2011) Agilent Application Note 5990-8341EN, Agilent Technologies, Santa Clara, CA.

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