Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (9)

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Sport und Buch Strauß, Köln, 2001

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In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (9). Sport und Buch Strauß, Köln, (2001) 115-123

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Enhanced Steroid Screening achieved by fast GC/MS with Hydrogen Carrier Gas

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Abstract

Electronic pressure control and the fast ovens currently available on mass production gas chromatographs permit the use of rapid temperature programs. Improvements in benchtop mass spectrometers make it possible to integrate narrower peaks. Substitution of hydrogen for helium as a carrier gas allows for higher flow rates without compromise of separation efficiency, thus allowing the analyst to take full advantage of the technological improvements offered by today's benchtop GC/MS instruments. Modifications to the standard steroid GC/MS screening, consistent with the capabilities of an Agilent Technologies 6890/5973 instrument and using hydrogen as carrier gas, have reduced analysis time to ten minutes, while preserving separation efficiency and enhancing sensitivity. An unanticipated result of the change in carrier gas and consequent analysis conditions has been increased longevity of the chromatographic column.

Introduction

Historically, analytical strategies in chromatography have been adjusted to move analysis in the direction of greater separation efficiency. A profound example of just such an adjustment was the shift from packed column to capillary chromatography. Typical run time for complex mixtures such as urinary steroids in doping control is about 30 minutes. As testing volume increases, fast more productive chromatography becomes an important practical consideration. Based on theory of chromatography, speed may be increased by use of hydrogen as a carrier gas, narrow-bore capillary columns, fast chromatographic ovens, and constant flow controllers. EI mass spectrometer, as a relatively slow chromatographic detector, imposes some limitations to the speed of analysis: too fast peaks cannot be properly integrated, and hydrogen carrier gas, more reactive than helium, causes rapid contamination of the ion source. The latter discouraged earlier attempts to use hydrogen in routine steroid analysis in doping control. The newer

instrumentation, as shown below, resolves most of the problems, allowing 10 minutes routine steroid analysis without sacrifice in separation.

Instrumentation

The established GC/MS steroid screening procedure in use in our laboratory is performed on a Hewlett Packard 5890/5970 GC/MS system. Table 1 contrasts this system with a newer system, capable, in principle, of faster analysis. The 5890 GC works at constant pressure and, consequently, variable flow. The 6890 is equipped with electronic pressure control, which offers the possibility of constant flow analysis. In addition, the 6890 is equipped with a fast oven, allowing for steeper temperature ramps. The 5970 MS has a maximum scan speed of 1500 amu/s, contrasted with 5200 amu/s for the 5973. Both systems use turbo molecular pumps to maintain high vacuum, but the new system will accommodate recommended flows of up to 4 mL/min, while the older system is limited to 1 mL/min. For both systems, the capillary column is a HP1 fused silica, crosslinked methylsilicon, 16.5 m, 0.2 mm i.d., 0.11 micron film thickness. The carrier gas for the established unit is helium, delivered through a centralized distribution system using refillable helium tanks. The high pumping capacity of the "performance turbo pump" in the new unit, in conjunction with the rapid scanning rate of its mass spectrometer, allow for routine operation at higher flows. In this higher flow domain, helium is no longer the optimal carrier gas. Because of its greater efficiency for flows exceeding 1 mL/min, hydrogen becomes preferable. For our new system, hydrogen is introduced as the carrier gas; it is provided by a benchtop Whatman 75-32-V452 hydrogen generator.

Methodology

The established screen for anabolic steroids, as performed routinely in the laboratory, follows the protocols of the familiar IOC Procedure 4 for conjugated steroids. Sample preparation involves enzymatic hydrolysis by β–glucuronidase from *Helix pomatia*, followed by C_{18} solid phase extraction and subsequent derivatization of the dry residue with 75μL of MSTFA/NH_4I-Dithioerythritol; 1μL of the final extract is injected into the GC/MS. Analysis on the established instrumentation proceeds in single ion monitoring mode, under constant pressure conditions, using helium as the carrier gas at an initial flow rate of 0.65 mL/min and the parameters indicated in table 2.

The new "fast" analysis modifies the established procedure only in the GC/MS stage; sample preparation, extraction and derivatization are unchanged. Again, injecting 1μL of extract, with an identical column, proceeding in single ion monitoring mode, but now under constant flow conditions using hydrogen as the carrier gas and with the additional temperature modifications listed in table 2.

Results and Discussion

Figure 1 displays total ion current chromatograms of SIM screening runs for the same steroid standard mixture analyzed on the established system using helium (upper chromatogram) and the fast system using hydrogen (lower chromatogram) as carrier gases. For comparative purposes, we can bracket the time display with relatively early and late eluting anabolic steroids. Early, we see that 19-nor-androsterone appears in the upper chromatogram at 10.0 minutes and on the lower "fast" chromatogram at 4.7 minutes. Near the end of both chromatograms, 3'-OH-stanozolol appears. For the upper chromatogram, although not visible, it elutes at 20.23 minutes. On the lower chromatogram it is barely visible at 9.15 minutes. Examining androsterone and etiocholanolone, we see that they elute near the middle of both chromatograms, just prior to 12 minutes in the helium regime, compared to 5.5 minutes when hydrogen is used. The difference between the two chromatograms is simply compression in time, but that difference is significant. Using hydrogen and the modified analytical parameters, each individual compound is eluting faster by a factor of two, when compared with the established, helium utilizing, analysis. Elution order and proportional time evolution remain the same, so that the twofold increase in speed extends to total run time (table 2). The interinjection time, which is additionally influenced by the response and equilibration rates of the GC heating and sampling systems, reflects a similar increase in speed, due to improvements in instrument performance.

Resolution is retained despite the time compression of the chromatogram. As is evident in figure 1, androsterone and etiocholanolone display baseline separation in both chromatograms. Separation of the 5α and 5β isomers of 17α-methyl-3α,17β-androstandiol, critical to distinctions between metabolites of methyltestosterone, methandrostanolone and oxymetholone is achieved in the hydrogen regime (figure 2), as is epitestosterone from potential endogenous interference.

Sensitivity is enhanced using the fast analysis achievable with hydrogen and the improved GC/MS workstation. Figure 3 gives a sample of the results obtained when a steroid

standard mixture is diluted 1:10 with deionized water, taken through full hydrolysis and solid phase extraction, in order to produce realistic interferences and recoveries, then taken through routine screening analysis using the fast hydrogen system. At 5 ng/mL, 19-nor-androsterone (fig. 3,A) and testosterone and epitestosterone (fig. 3,B), display good signal to noise. At 10 ng/mL, methenolone (fig. 3,C), is strong; and although at 3 ng/mL, 3'-OH-stanozolol (fig. 3,D), appears perhaps weak and baseline challenged to the ungenerous eye, its very appearance in a broad spectrum screen represents an improvement in sensitivity over the established method. As a final, albeit somewhat artificial example of the sensitivity of the new screening method, figure 4 displays a SIM chromatogram of 3'-OH-stanozolol at 1 ng/mL after direct derivatization of a standard solution.

An unexpected, but most welcome, consequence of the change to hydrogen carrier gas and higher flow rates has been increased longevity of the capillary column. Previously, average column life was 300 to 350 screening injections between replacements, with severe degradation of some analytes, notably 3'-OH-stanozolol, during the latter portion of this interval. The new high flow hydrogen system yields unimpaired column performance for 2000 injections, with a less rapid degradation thereafter. Offsetting this advantage to a limited extent, is the more frequent source cleaning required with the hydrogen method; but this inconvenience is partially allayed by the comparative simplicity of this procedure for the Agilent 5973.

Table 1. INSTRUMENTATION

	Established System	Fast System
GC	HP 5890	Agilent 6890
Flow Control	Constant Pressure	Electronic Pressure Control
MS	HP 5970	Agilent 5973
Vacuum System	Turbo Pump	High Performance Turbo
Injector	HP 7673	Agilent 7683
Operating System	UNIX	Windows NT

Table 2. INSTRUMENT PARAMETERS

	Established System	Fast System
Column	HP1	HP1
Carrier Gas	Helium	Hydrogen
Flow	0.65 mL/min. (variable)	1.8 mL/min. (constant)
Injection Temp.	270°C	270°C
Purge	OFF/ON 0.15 min.	OFF/ON 0.3 min.
Detector Temp.	280°C	280°C
Oven Ramp	180°C (0.3 min. hold) 3°C/min. to 231°C 30°C/min. to 310°C	160°C (0.4 min. hold) 35°C/min. to 180°C 5.8°C/min. to 215°C 32°C/min. to 310°C
Run Time	20.6 min.	10.0 min.
Interinjection Time	24.15 min.	12.03 min.

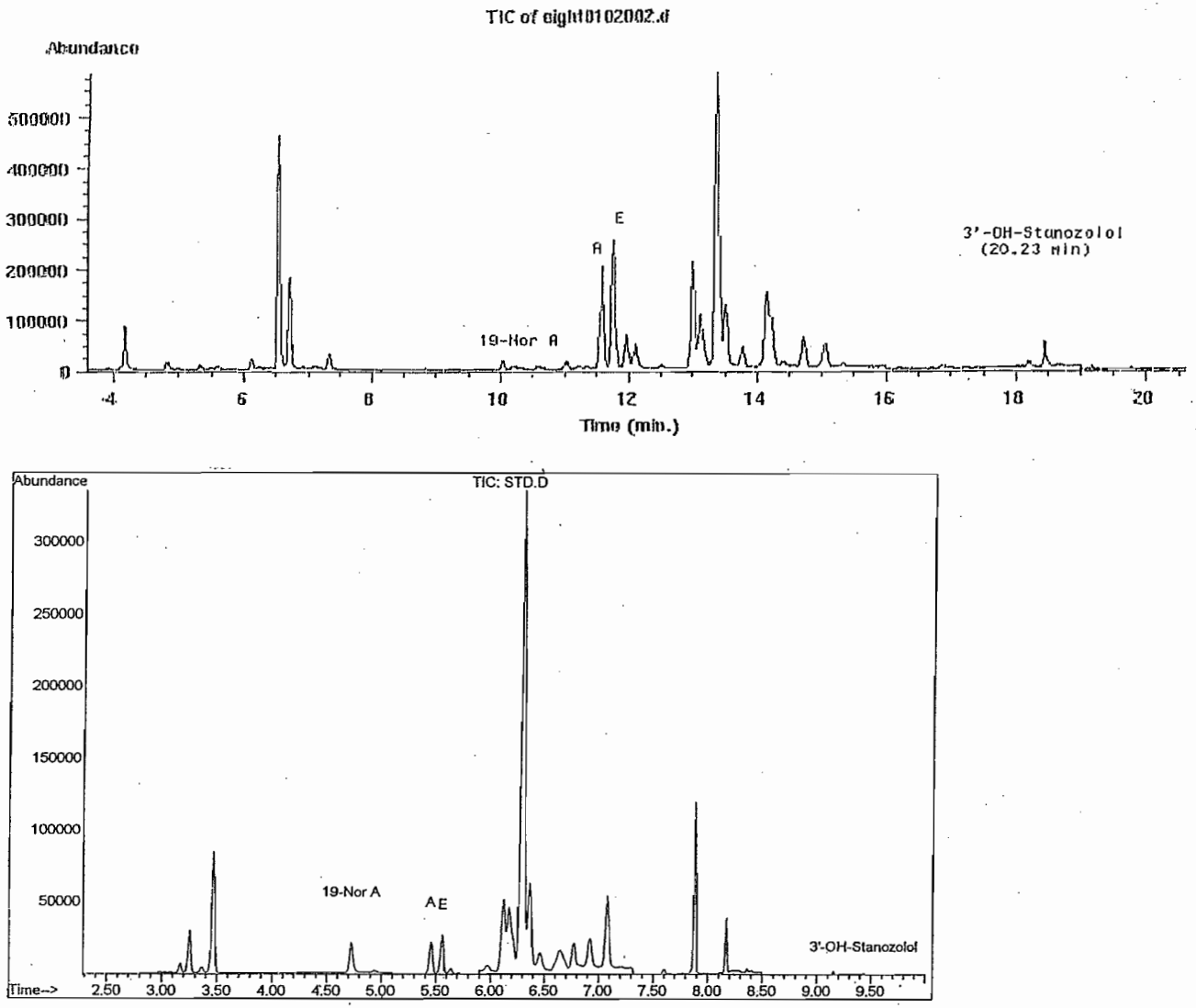

Figure 1. TIC of a steroid standard mixture acquired in SIM screen using helium (upper chromatogram) and hydrogen (lower chromatogram) as carrier gases.

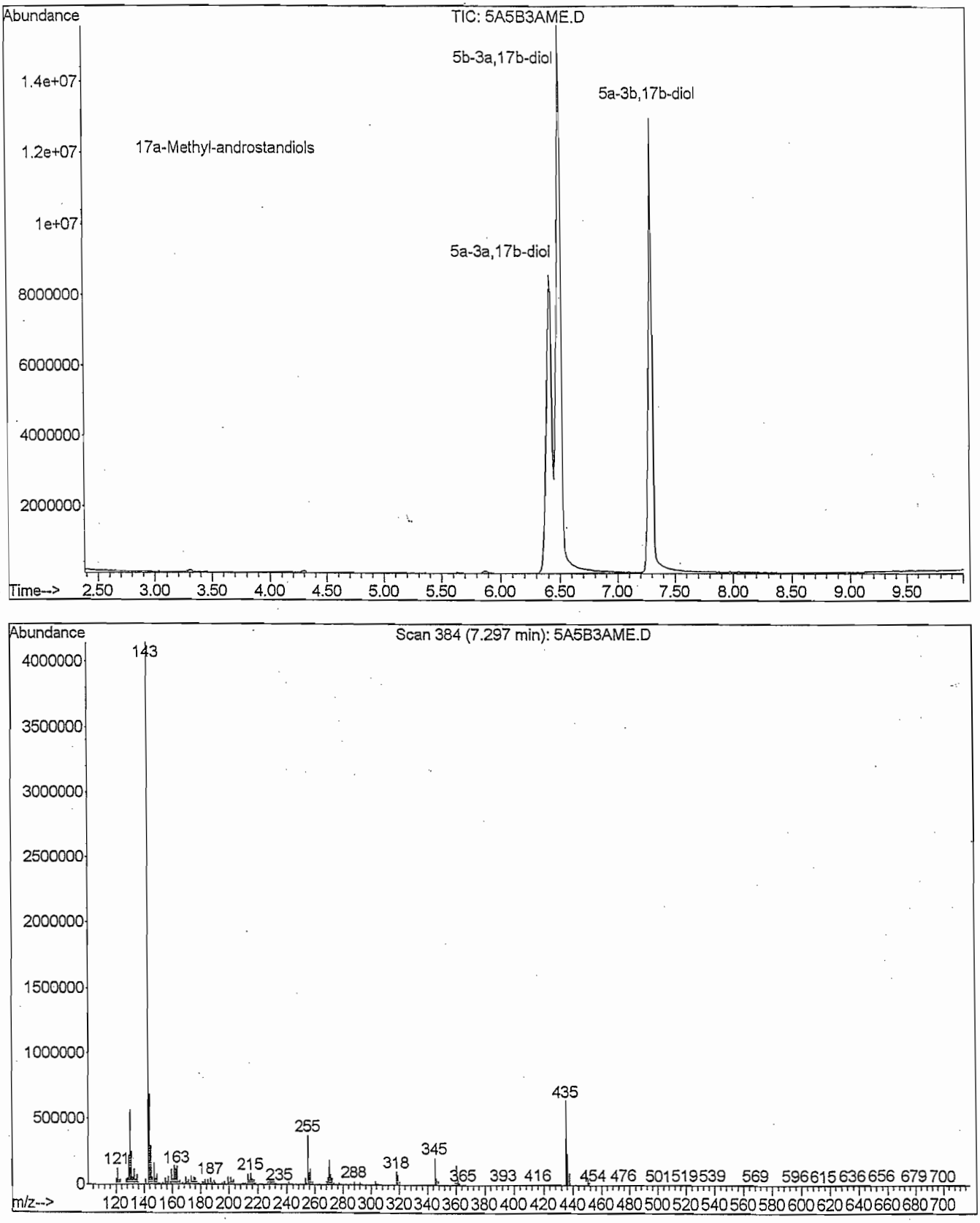

Figure 2. Separation of isomeric methyltestosterone metabolites.

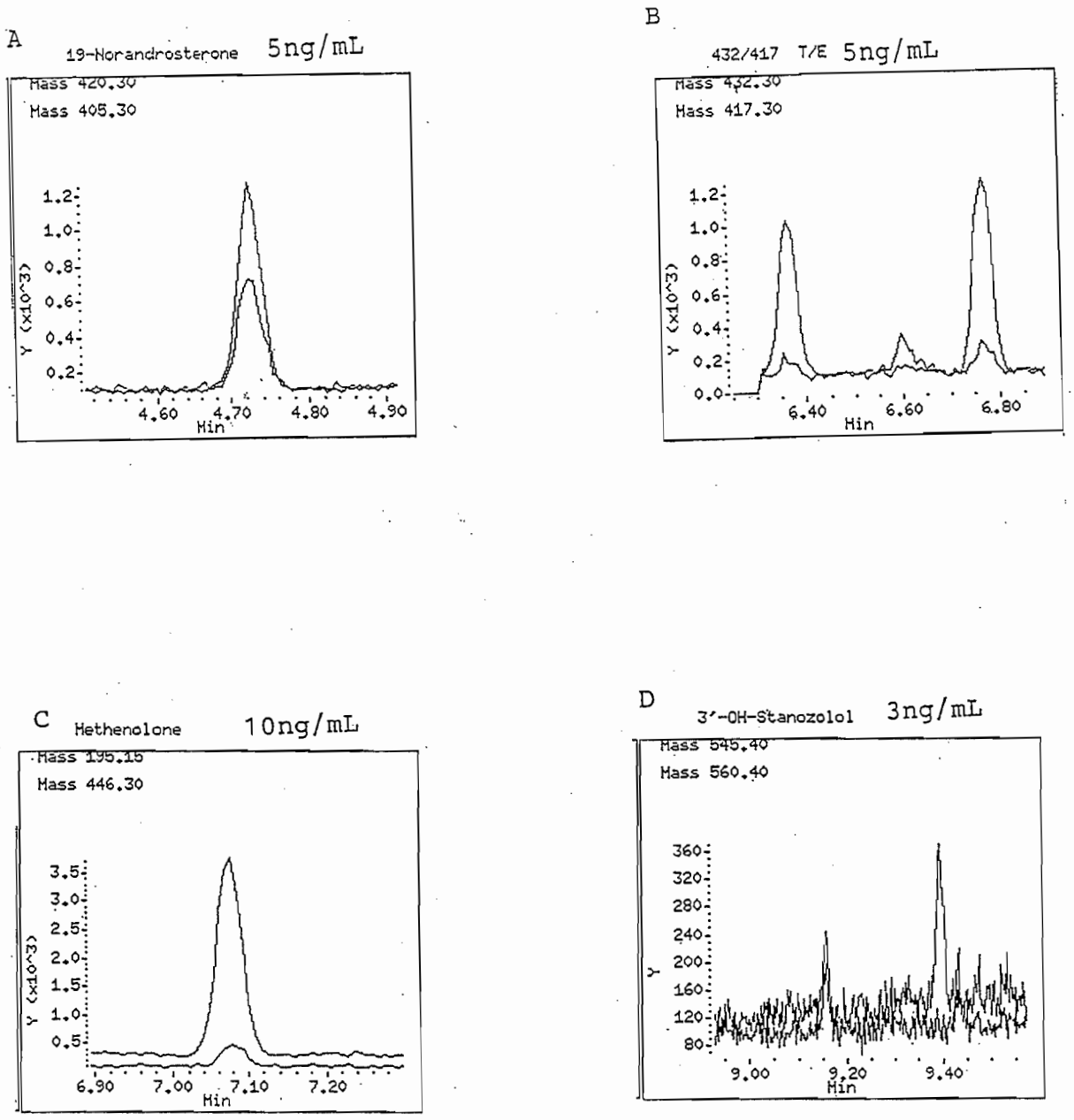

Figure 3. SIM screen extracted ion chromatograms for steroid standard mixture diluted 1:10.

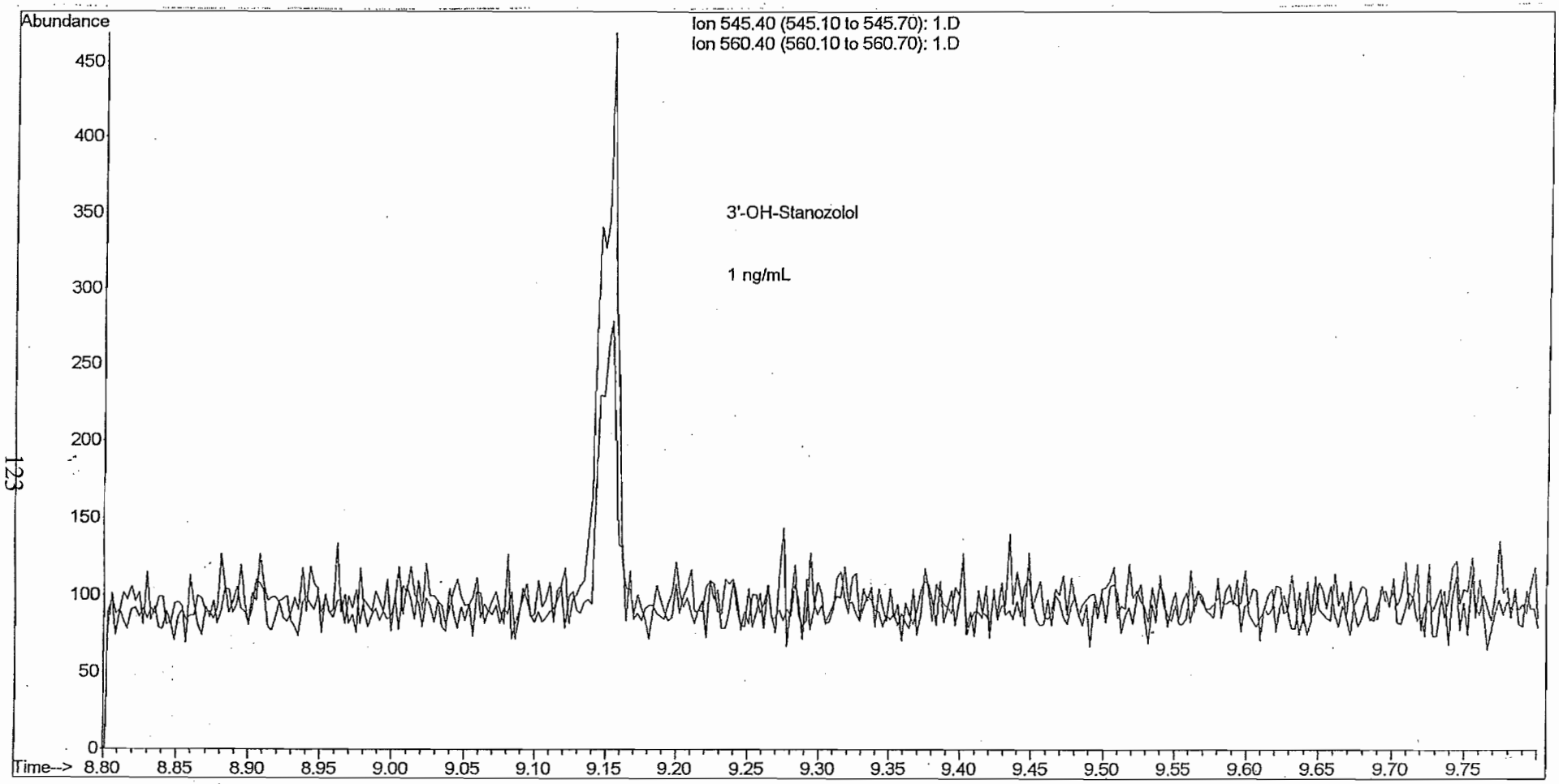

Figure 4. SIM screen of 3'-OH-stanozolol (1 ng/mL standard solution, unextracted)