

A unique tool for metabolite research

Q Exactive GC coupled with IRMS (QE GC-IRMS)

Authors

Dieter Juchelka and Jens Griep-Raming, Thermo Fisher Scientific, Bremen, Germany

Thomas Piper and Mario Thevis, German Sport University Cologne, Center for Preventive Doping Research – Institute of Biochemistry, Cologne, Germany

Keywords

Metabolite research, Q Exactive GC, Delta V, GC-IRMS, GC IsoLink II, Trace 1310 GC, ConFlo IV, Isotope Research

Goal

Isotopically labeled metabolites and their corresponding structural information can simultaneously be determined to facilitate and accelerate the discovery of metabolites using a robust workflow. Combining the Thermo Scientific™ Delta V™ IRMS or the Thermo Scientific™ 253 Plus™ IRMS system with Thermo Scientific™ Q Exactive™ GC connected to a single Thermo Scientific™ Trace™ 1310 GC provides a new approach to metabolite research.



Introduction

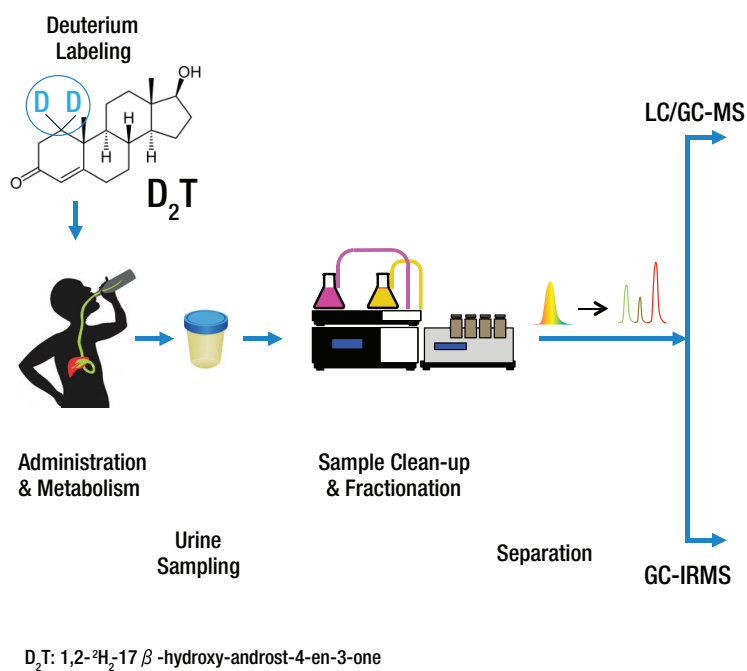
Comprehensive studies on the metabolic fate of drug candidates require the identification and structural elucidation of metabolites. Structural information on metabolites can serve to accelerate the drug discovery and method development process. In sports drug testing, studies on the metabolism of therapeutic agents with potential to misuse as well as designer compounds and drug candidates are necessary to identify metabolites that provide utmost retrospectivity and specificity.

Gas chromatography coupled with mass spectrometry (GC-MS) is routinely used for metabolism studies due to its inherent advantages, such as chromatographic resolution, reproducibility, peak capacity, and convenient spectral libraries. GC provides an excellent chromatographic separation capability for metabolite discovery but has previously been limited by the lack of high-end mass spectrometry techniques providing the dynamic range, accurate mass, and scan rate sufficient to analyze very complex samples. The Q Exactive GC enables ultra-high mass resolution, sub-ppm mass accuracy and a large dynamic range for untargeted and targeted metabolite research.

However, the identification of metabolites in complex matrices can be time consuming and challenging due to the requirement to both identify and quantify hundreds of different compounds with limited a priori knowledge of the metabolites.

Compound-specific stable isotope analysis has been used to provide insights into the metabolism of many compounds in plants, animals and humans to the inherent fractionation of isotopes in cycling reactions. Small natural variations of the stable isotopes of carbon, nitrogen, oxygen and hydrogen can be measured at natural abundances.

Deuterium labeling of compounds has long been recognized as a valuable means to identify metabolites (Figure 1). Hence, it has been used for decades to investigate human metabolism. Dosages up to more than 5% deuterium are considered safe, clinical approval may be given during pregnancy and childhood. Gas chromatography coupled with isotope ratio mass spectrometry (GC-IRMS) enables the compound-specific hydrogen isotope analysis at natural abundance and deuterium enrichment. Small natural variations of the $^2\text{H}/^1\text{H}$ can be measured with precision in the range of 1-3 per mille. Compounds eluting from the GC column are online converted to hydrogen which is admitted to the isotope ratio mass spectrometer (Figure 2). A quantitative reaction (high temperature conversion) can be accomplished using temperatures above 1400 °C, but consequently, all structural information is lost.



Structural Elucidation

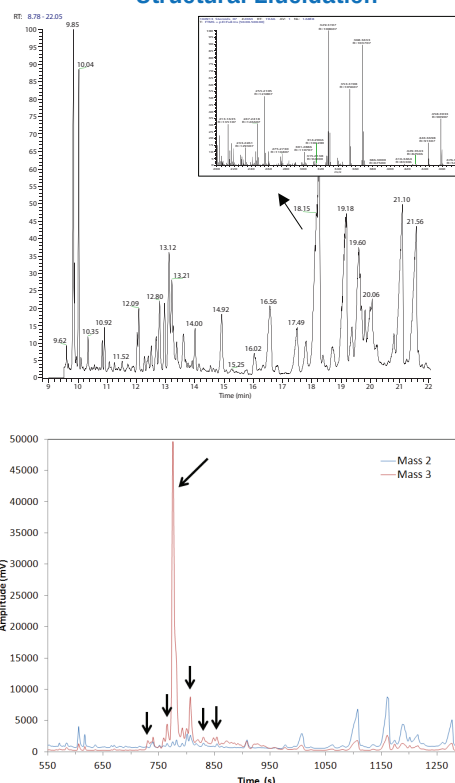


Figure 1. Metabolite research: excretion study.

Metabolite Detection

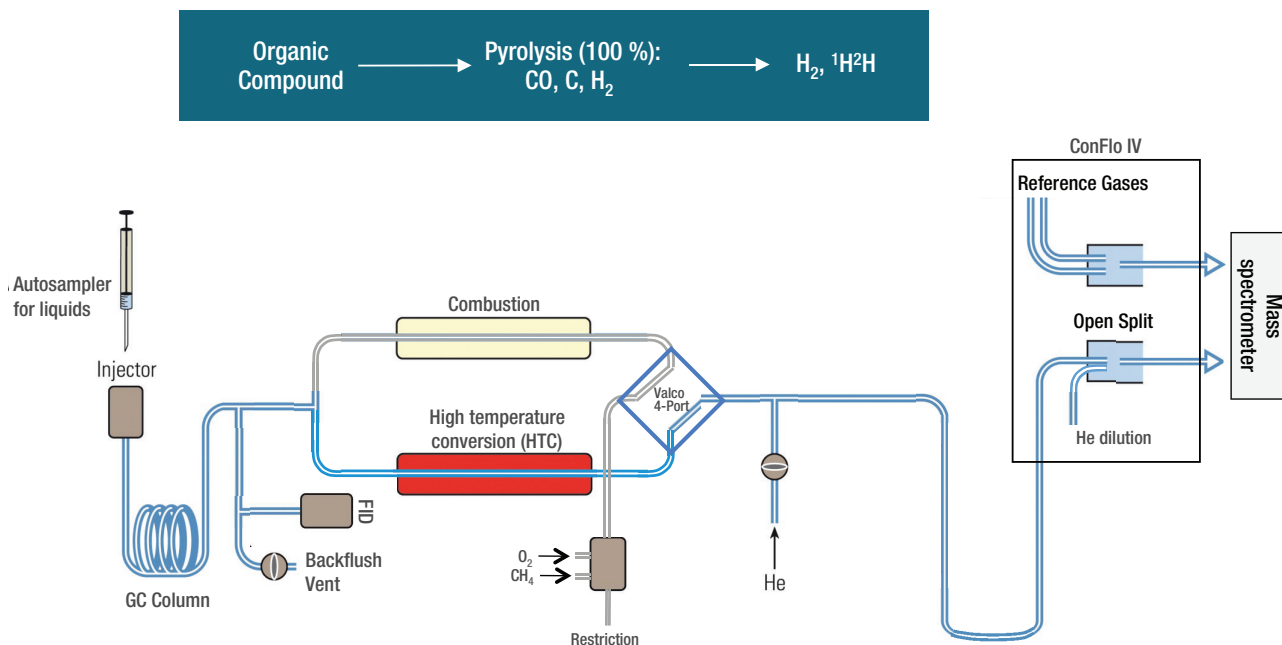


Figure 2. ²H/¹H analysis by high temperature conversion.

In this approach the IRMS system has been used to effectively detect the deuterium label of metabolites deduced from the deuteration of the administered drug. Coupling GC-IRMS with the Q Exactive GC provides simultaneously the structural information of the metabolites at the same time as located into the IRMS chromatogram.

An elimination study with an oral administration of doubly- and triply deuterated testosterone (16,16,17-²H₃-17 β -hydroxy-androst-4-en-3-one (D₃T) and 1,2-²H₂-17 β -hydroxy-androst-4-en-3-one (D₂T)) has been performed. Urine samples were collected for 8 days and the specimens were processed according to established

sample preparation procedures.² In order to allow for utmost coverage of potential metabolites, glucuronidated, sulfoconjugated and unconjugated steroids were measured by the Q Exactive GC-IRMS system.

Instrument and method setup

Sample preparation

Steroids are excreted as glucuronidated and sulfated species. Sample clean-up includes the hydrolysis of conjugated steroids using β -glucuronidase and acidic solvolysis. A slightly modified sample preparation method for routine doping control analyses was used in this study.^{1,2} In order to further purify the steroidal extracts, fractionation was performed on a HPLC system. Table 1 shows the fraction collection intervals.

Table 1. Fraction collection times for HPLC cleanup and expected analytes using established methods.^{1,2}

Fraction	Time (min)	Steroids (exemplarily)
I	2.5-6.0	unknown
II	6.0-9.0	4-androstene-7 α , 17 β -diol-3-one
III	9.0-12.0	5 β -androstane-3 α , 6 α -diol-17-one, 6 α - and 6 β -OH-T
IV	12.0-14.0	4-androstene-3,6,17-trione
V	14.0-16.7	T, 5b-diol, DHEA
VI	16.7-22.0	ETIO, AND, 5a-diol, PD
VII	22.0-30.0	16EN

Analytes: T – testosterone, 5b-diol – 5 β -androstane-3 α , 17 β -diol, DHEA – dehydroepiandrosterone, ETIO – 5 β -androstane-3 α -ol-17-one, AND – 5 α -androstane-3 α -ol-17-one, 5a-diol – 5 α -androstane-3 α , 17 β -diol, PD – 5 β -pregnane-3 α , 20 α -diol, 16EN – 5 α -androst-16-en-3 α -ol.

Sample derivatization

Collected fractions were evaporated under a stream of nitrogen and trimethylsilylated by adding 100 μL of a solution containing MSTFA in ethyl acetate (20/80, v/v) for 45 min at 70 °C. The samples were dried, reconstituted with ethyl acetate and transferred to autosampler vials.

Q Exactive GC-IRMS analysis

All experiments used a single Thermo Scientific™ Trace™ 1310 GC with a Thermo Scientific™ Q Exactive™ GC hybrid quadrupole-Orbitrap™ mass spectrometer coupled with a GC-IRMS system consisting of a Thermo Scientific™ GC IsoLink™ II preparation unit, Thermo Scientific™ ConFlo™ IV interface and a Thermo Scientific™ Delta™ V Plus mass spectrometer. The GC oven incorporates a micro channel device (MCD) for splitting the effluent of the GC column. The split of the GC effluent is set to 5:1 (IRMS/ Q Exactive GC) and was adjusted by the length of transfer fused silica capillary from the MCD to the ion source of the Q Exactive GC.

Sample injection was performed using a Thermo Scientific™ TriPlus™ RSH autosampler, and chromatographic separation was obtained using a Thermo Scientific™ TraceGold™ TG-5MS 30 m \times 0.25 mm I.D. \times 0.25 μm film capillary column (P/N: 26098-1420). Additional details of instrument parameters are shown in Tables 2a and 2b.

Results and discussion

81 samples were collected before and after the administration of differently deuterated testosterone. After sample preparation, HPLC fractionation and derivatization, the corresponding aliquots were measured by gas chromatography / thermal conversion / isotope ratio mass spectrometry (GC/TC/IRMS) connected with high resolution / accurate mass (HR/AM) spectrometry. Compounds eluting from the GC column were split with ratio (5:1 IRMS / Q Exactive) and directed to both mass spectrometers enabling simultaneous analysis of samples.

Table 2a. GC conditions.

Trace 1310 GC inlet parameters	
Injection volume	1.0 μL
Liner	Single taper (P/N453A1345)
Injector	280 °C
Injector module and mode	SSL, splitless
Splitless time and purge	1 min, 100 mL/min
Carrier gas	Helium, 1.5 mL/min
Oven temperature program	
Initial temperature	100 °C
Hold time	2 min
Rate 1	40 °C/min
Temperature 1	260 °C
Hold time 1	0 min
Rate 2	2 °C/min
Temperature 2	285 °C
Hold time 2	0 min
Rate 3	40 °C/min
Temperature 3	300 °C
Hold time 3	5 min

Table 2b. Mass spectrometer conditions.

GC-IRMS parameters	
IRMS	Delta V Plus
Amplifications	m/z 2: 10^9 m/z 3: 10^{12}
Conversion unit	GC IsoLink II
Mode	High temperature conversion
Reactor temperature	1420 °C
Interface	ConFlo IV
Q Exactive GC-MS parameters	
Transfer line	280 °C
Ionization type	EI
Ion source	250 °C
Electron energy	70 eV
Acquisition mode	Full scan
Mass range (m/z)	50-500
AGC target	1e6
Mass resolution (FWHM at m/z 200)	120,000

Table 2c. Application softwares.

Softwares	
IRMS	Isodat 3.0.94
Q Exactive GC-MS	Xcalibur 4.0 (Foundation 3.1 SP3, Trace Finder 4.0)

With hardware and software synchronization.

Metabolite identification by IRMS

The IRMS system was used for detection of deuterium-labeled metabolites. At present, the required amount of an analyte for the determination of natural hydrogen isotope abundance is approximately 50 ng 'on-column'. The use of deuterated compounds for metabolism studies substantially increases the signal intensities of analytes bearing approximately 10% of deuterium atoms. Concentrations in the sub-ng/mL range result in a detectable signal above the natural deuterium abundance in IRMS.^{1,3} Labeled metabolites of D₃T and D₂T are readily detected in the *m/z* 3 ion trace of the IRMS as demonstrated in Figure 3.

While the majority of urinary steroids is conjugated, a minor fraction is found unconjugated due to hydrolysis events or elimination without undergoing conjugation reactions. In an appropriately stored urine sample, the amounts of unconjugated steroids are commonly below 5% of the conjugated fraction and mostly even below 1%. For the steroids highlighted in Figure 3, the amount ranges from 10 to 20 ng/mL for AND and ETIO and falls below 1 ng/mL for T demonstrating the sensitivity of the IRMS approach. More than 70 different T-metabolites could be identified.⁴

One new metabolite with unexpected structure (T_METH at 730 s in Figure 3) will be discussed in detail.

Structural elucidation of metabolites by HR/AM Q Exactive GC-MS

The Q Exactive GC system was operated in full GC/MS scan mode in order to obtain high-resolution / high-accuracy mass data on all urinary metabolites previously identified by IRMS during the initial experiments. In order to obtain MS/MS data of relevant metabolites for further structural elucidation the Q Exactive GC system was operated in the parallel reaction monitoring mode.

Metabolic methylation of testosterone

In the fraction of unconjugated metabolites especially the above mentioned unknown metabolite T_METH was of particular interest as the respective mass spectrum did not fit any prediction (Figure 4). The measured accurate mass of *m/z* 304.2371 was not the anticipated oxygenation (calculated mass of 304.2007 Da) but obviously the addition of a methylene unit resulting in a molecular formula of C₂₀H₂₈D₂O₂ and, thus, a metabolically methylated T metabolite. While the methylation of steroid receptors is described in the literature, there is no publication available that reports on the methylation of androgens in humans so far.

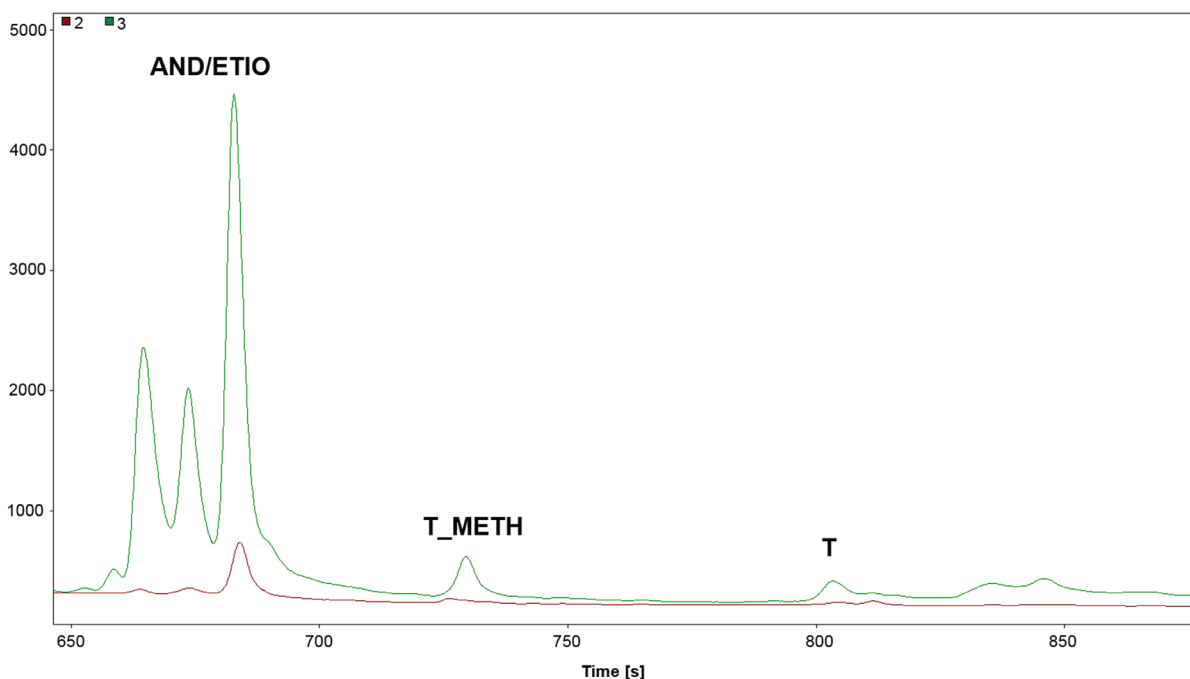


Figure 3. IRMS chromatogram of a post-administration urine (unconjugated fraction) after the administration of 40 mg of deuterated testosterone.

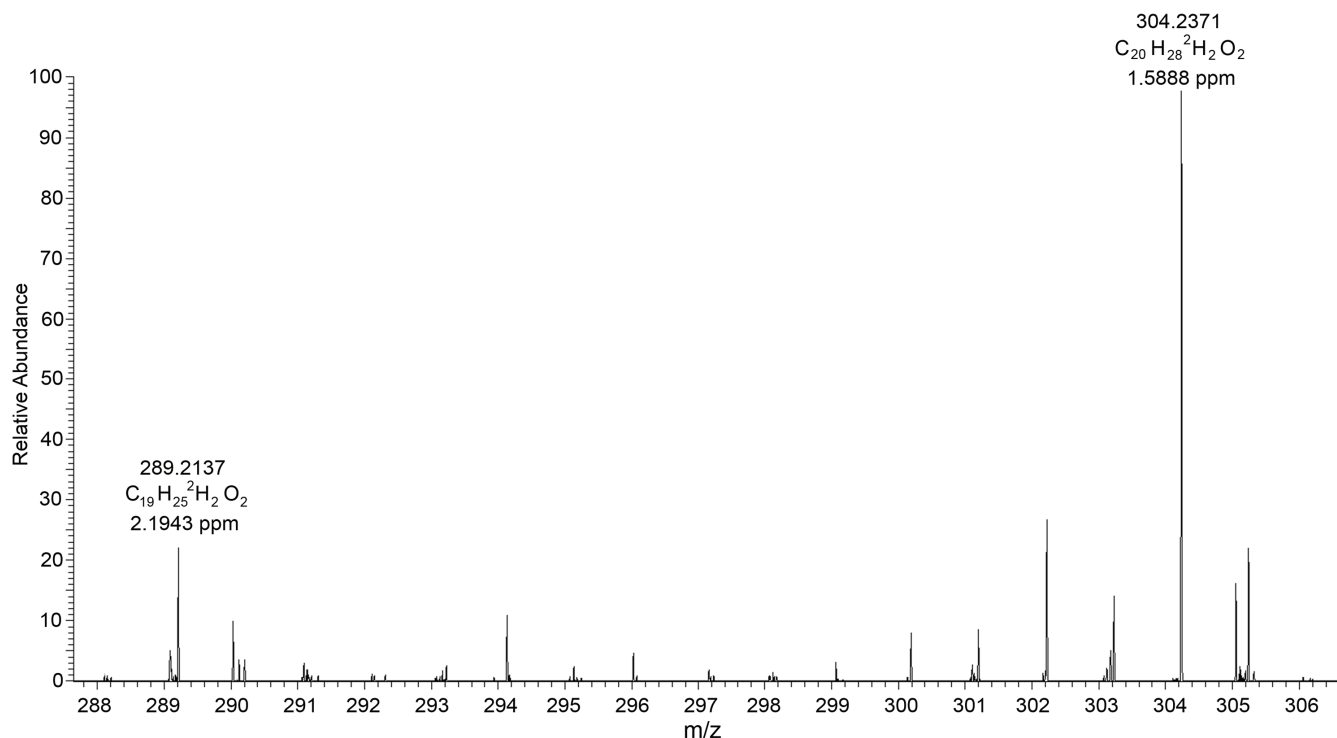


Figure 4. High resolution GC-Orbitrap mass spectrum in scan mode of the newly discovered testosterone metabolite.

The loss of a methyl radical (-15 Da) from the molecular ion was a preferred fragmentation pathway of this metabolite (Figure 4) under EI conditions. Taking also into account that the derivatization with MSTFA was hindered, the conclusion that this metabolite is methylated at a hydroxyl function appears plausible. Chemical methylation of T with methyl iodide yielded one mono-methylated and two isomeric di-methylated T derivatives. None of the mass spectra was identical to the one of T_METH.

In order to generate additional information on this yet unreported metabolite, excretion studies conducted in 2 separated trials were performed, using either the doubly A-ring-deuterated T or the triply D-ring-deuterated T. Urine samples were analyzed by means of the Q Exactive GC providing the required sensitivity and mass resolution to further characterize the detected compound. In both excretion studies the target metabolite was identified at m/z 304.2731, indicating that both the doubly- and triply deuterated T yield a doubly labeled metabolite, pointing

towards the formation of a 17-oxo-function. Further conclusions regarding the structure could be drawn by comparing the MS/MS spectra of both metabolites as depicted in Figure 5.

While both species show fragments at m/z 289.2137 ($C_{19}H_{25}D_2O_2$, attributed to the loss of a methyl group) and 257.1869 ($C_{18}H_{21}D_2O$, suggested to result from the loss of CH_3 and CH_3OH) the main difference is the presence or absence of ions at m/z 273.1853 and 275.1973 with molecular formulae of $C_{18}H_{25}O_2$ and $C_{18}H_{23}D_2O_2$, respectively. The loss of both deuterium atoms in the A-ring-deuterated testosterone during the fragmentation process suggests a methylation at the oxygen at C3. This is corroborated by abundant ions at m/z 139.1087 ($C_9H_{11}D_2O$) for the A-ring-deuterated analogue and m/z 137.0961 ($C_9H_{13}O$) for the D-ring deuterated testosterone. These fragments correspond to methylated species of the commonly observed A-ring fragment of testosterone at m/z 124.0883 ($C_8H_{12}O$), but proof for this hypothesis by chemical synthesis is yet required.

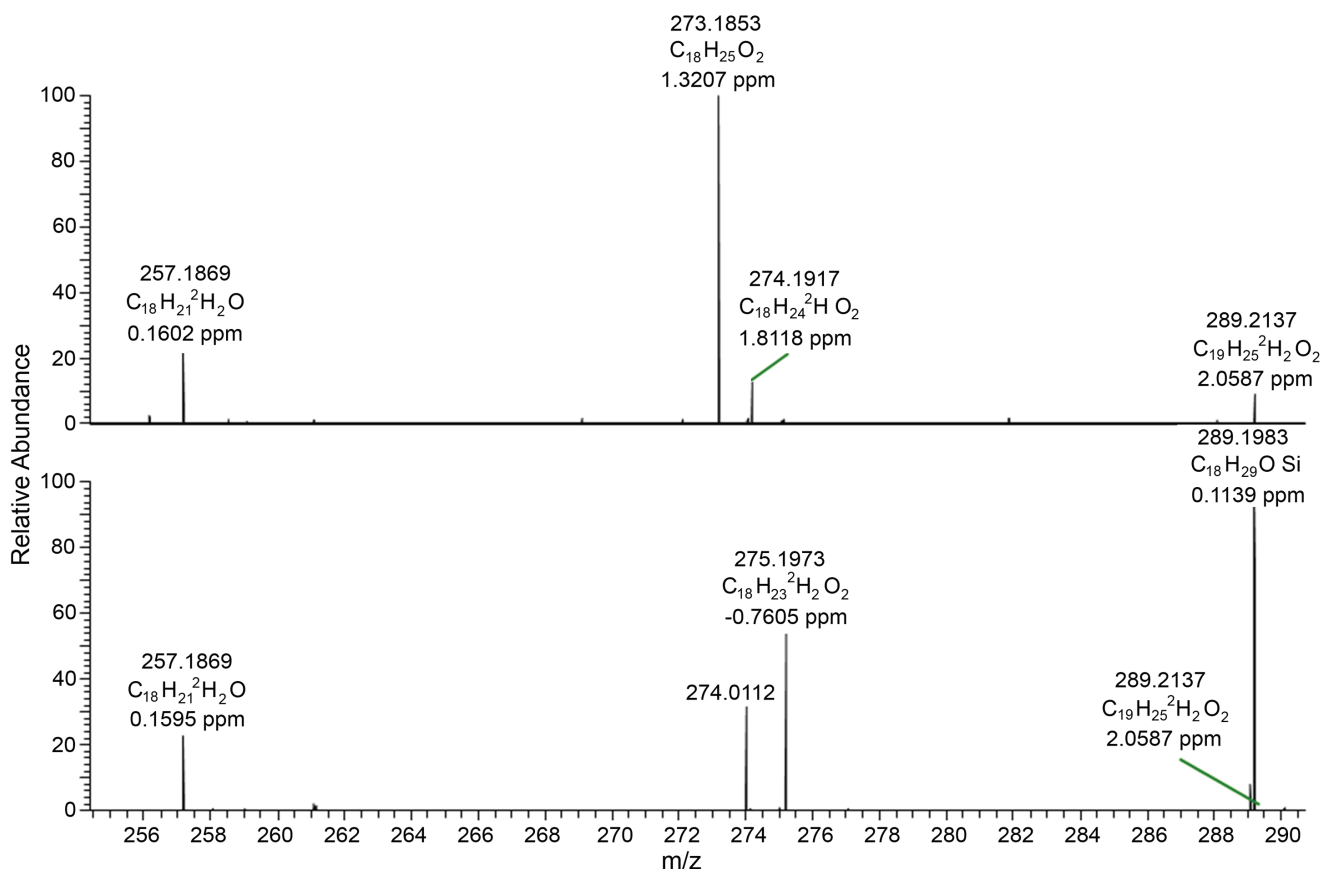


Figure 5. High resolution GC-Orbitrap mass spectrum of the newly discovered testosterone metabolite after administration of 1,2-²H₂-T (upper spectrum) and 16,16,17-²H₃-T (lower spectrum). Formulae were calculated with as mass error < 2 ppm.

Conclusion

Comprehensive studies on the metabolism of therapeutic agents and drugs with potential to misuse are vital for comprehensive doping controls. Isotope ratio MS can be used as an invaluable tracer detecting metabolites with outstanding sensitivity of < 0.5 ng/mL on column. The full GC/MS scan capabilities and accurate mass determination of the Q Exactive GC Orbitrap system can be applied in compound discovery and structural elucidation of metabolites with highest confidence. This makes the QE-GC-IRMS system amenable for unambiguous metabolite identification providing invaluable information for metabolism research.

Furthermore, detection and structural elucidation of metabolites can contribute and accelerate the drug discovery and development process.

The concept is also applicable to other areas of drug research. This allows omitting the preparation and administration of radioactively labeled substances (³H or ¹⁴C) and scintillation counters to detect the compounds of interest. The sensitivity of the method might further increase by increasing the relative amount of deuterated substance in the experiment.

Thermo Scientific isotope ratio analyzers

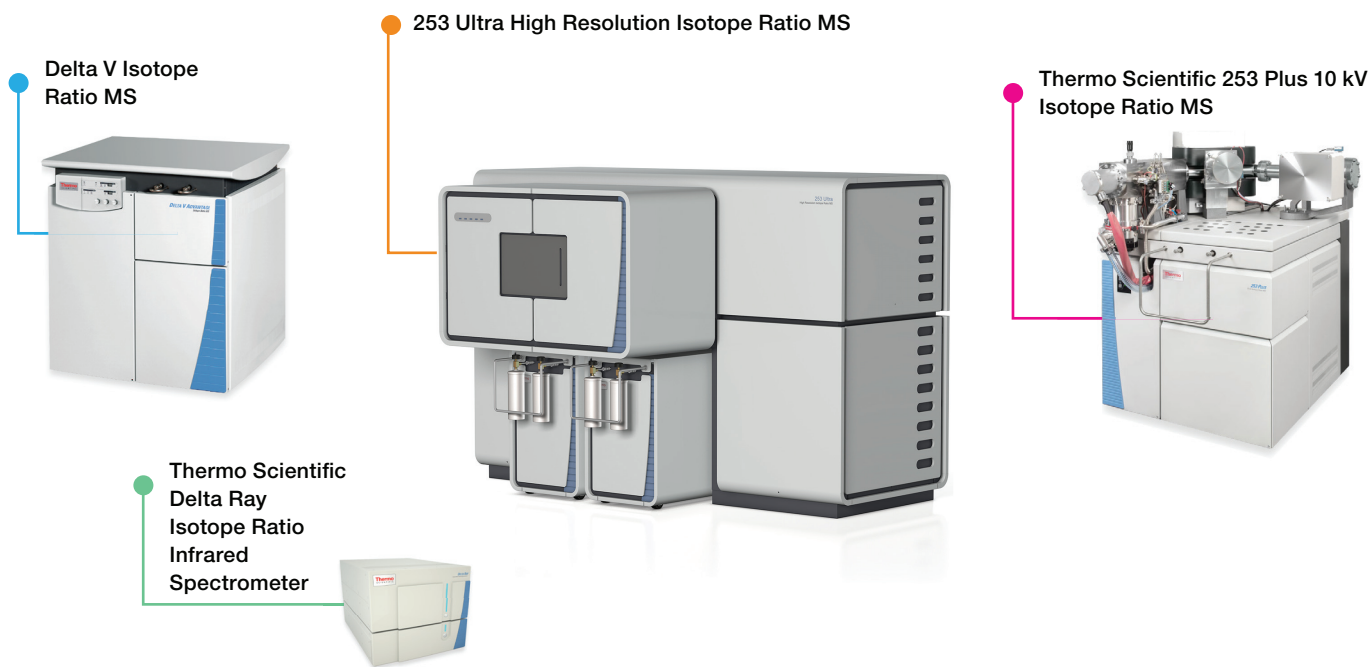
For over 60 years Thermo Fisher Scientific has been the leading supplier of isotope ratio instrumentation to scientists across the world.

Our GC-IRMS systems have enabled some of the most exciting pioneering discoveries in the study of metabolic pathways and the history and origin of all kind of samples.

Our foundations are based on strong collaborative partnerships that have allowed us to become the leading provider of gas chromatography.

References

1. M. Thevis, T. Piper, S. Horning, D. Juchelka and W. Schänzer: Hydrogen isotope ratio mass spectrometry and high-resolution/ high-accuracy mass spectrometry in metabolite identification studies: Detecting target compounds for sports drug testing. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 1904–1912.
2. T. Piper, U. Mareck, H. Geyer, U. Flenker, M. Thevis, P. Platen, W. Schänzer. Determination of $^{13}\text{C}/^{12}\text{C}$ ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2161-2175.
3. T. Piper, W. Schänzer, M. Thevis. Revisiting the metabolism of 19-nortestosterone using isotope ratio and high resolution/high accuracy mass spectrometry. *Journal of Steroid Biochemistry & Molecular Biology* **2016**, *162*, 80–91
4. T. Piper, W. Schänzer, M. Thevis. Genotype-dependent metabolism of exogenous testosterone – new biomarkers result in prolonged detectability. *Drug Test. Analysis* **2016**, *8*, 1163-1173.



Find out more at thermofisher.com/IRMS

ThermoFisher
SCIENTIFIC