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GC-C-IRMS Detection of Steroid Metabolites - Some Hardware Considerations.

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Introduction

The $^{13}C/^{12}C$ ratio in natural compounds such as steroids is determined by the metabolic pathway by which they were produced. The two main biosynthetic groups of plants, namely C-3 and C-4, produce compounds with measurably different carbon isotope ratios. The starting material in the production of synthetic testosterone is derived from soy, a C-3 plant and therefore the synthesis yields a steroid depleted in carbon-13. Steroids produced by the body strongly reflect an individuals dietary intake and are generally a little more enriched in carbon-13 by comparison (1). These small but measurable differences in isotope ratio between synthetic and naturally produced steroids are the basis for recently developed testing procedures which provide another approach to identifying cases of misuse of synthetic versions of endogenous steroids among athletes (2-5).

These small differences in carbon isotope ratios are routinely measured using Carbon Isotope Ratio Mass Spectrometry. This is a well known technique and has been in use in the geological field for many years. Results are generally expressed as the difference between the $^{13}C/^{12}C$ ratio in the sample and a known standard (PDB for carbon) and are therefore expressed using delta notation (δ) in parts per thousand (per mil, $^{o}/_{oo}$). The combination of IRMS with gas chromatography (GC) is a more recent development (6) which allows isotope analysis at a molecular level and has extended the capability and specificity, and hence the available applications of the technique (7).

IRMS requires conversion of compounds of interest to simple gases. In the case of carbon isotope analysis, carbon dioxide is the preferred analyte gas. Its use in conjunction with gas chromatography therefore requires a combustion interface (C) where the compounds eluting from the GC column are oxidised to CO_2 prior to entering the source of the mass spectrometer. Masses 44, 45 and 46 only are then monitored by Faraday cup detectors and the $^{13}C/^{12}C$ ratio is determined from the m/z 45 and 44 abundances. Corrections are made for

small amounts of oxygen-17 contributing to the 45 ion abundance by using the ratio of abundances of the m/z 46 and 44 ions (8,9). Unlike GC-MS, GC-C-IRMS does not give any structural information regarding the analytes and also requires stringent chromatographic conditions in order to accurately measure individual isotope ratios (10).

Water is a by-product of the oxidation process in the combustion interface. Its presence in the ion source can lead to the formation of HCO_2^+ species. $^{12}C^{16}O_2$ is the most abundant of the isotopomers of carbon dioxide and has a mass to charge ratio of 44. $^1H^{12}C^{16}O_2^+$ has a mass to charge ratio of 45. It is therefore important that the water of combustion be efficiently removed prior to the carrier gas entering the source or the isotope ratio measurements will be artificially high (11,12).

Aims

A Finnigan-MAT 252 IRMS equipped with a Varian 3400 GC and a prototype combustion interface and also dual and multiport inlets was made available for this study. Many problems had been experienced with this interface and the system had not been used successfully in GC mode. Because the system was a prototype, instruction manuals were not available.

Our aim in this project is to use the system for GC-C-IRMS measurements. As such the system needs to be made to function and the isotope ratio reproducibility optimised. Therefore three of the interface components were targeted: the GC column, the combustion furnace and the water removal system.

Materials and Methods

GC-MSD analyses were undertaken on an HP-5890/5970B system fitted with a 30m SGE BPX50 column of 0.25mm i.d. and 0.25μm film thickness. Helium carrier gas and splitless injection were used.

GC-C-IRMS analyses were performed on a Finnigan-MAT 252 IRMS system with a Varian 3400 GC and prototype interface. The GC was equipped with a Varian 1077 injector operated in splitless mode and an SGE BPX50 column identical to that used in the GC-MS analyses.

Testosterone and testosterone acetate were donated by the National Analytical Reference Laboratory, Pymble, Australia. 17-Methyltestosterone was obtained from the

Sigma Chemical Company. Solutions were made to a nominal 10 or 20 μg/mL in either chloroform or methanol (HPLC grade).

Results and Discussion

1. Chromatography

Choice of GC column is important in this application. GC-C-IRMS inherently requires a larger sample than GC-MS for example and the column must therefore support sample loadings large enough for analysis while maintaining good chromatography, baseline separation of components and low bleed (13).

Derivatisation of steroids prior to GC analysis on non-polar methyl-silicone phases is common analytical practice. However, the extra carbon atoms introduced during the derivatisation must be accounted for before reporting the isotope ratios (14). A more straightforward approach is to select a GC column with a somewhat more polar phase suited to separation of underivatised steroids.

In the past, polar chromatographic phases have been unstable at temperatures greater than approximately 240 °C and were prone to high bleed. Recent developments in column phase technology include production of phases with benzene molecules substituted into the phase backbone. This is found to reduce the effects of chemical and temperature degradation resulting in phases with high maximum operating temperatures and low bleed.

SGE's BPX50 is one such phase and a 30m 0.25 mm i.d. column with 25μm film of this phase was selected to evaluate its suitability for GC-CIRMS analyses. This column has an operating temperature range of 40 to 360 °C and is sold as a high temperature, low bleed, moderately polar column tested for GC-MS.

This column was installed into a GC-MSD for testing. Acetylated and free testosterone were introduced onto the column via splitless injector at 250 °C. The column temperature was initially 70 °C and after one minute, it was increased at 30 °C /min to 250 °C then at 3 °C/min to 280 °C and then at 10 °C/min to 300 °C which was held for 5 minutes prior to cooling. The peak shapes and baseline were examined (fig. 1).

Acetylated testosterone was found to have longer retention times than its underivatised counterpart. In both cases the peaks were sharp with minimal or no tailing and the baseline remained sufficiently flat for the duration of the run.

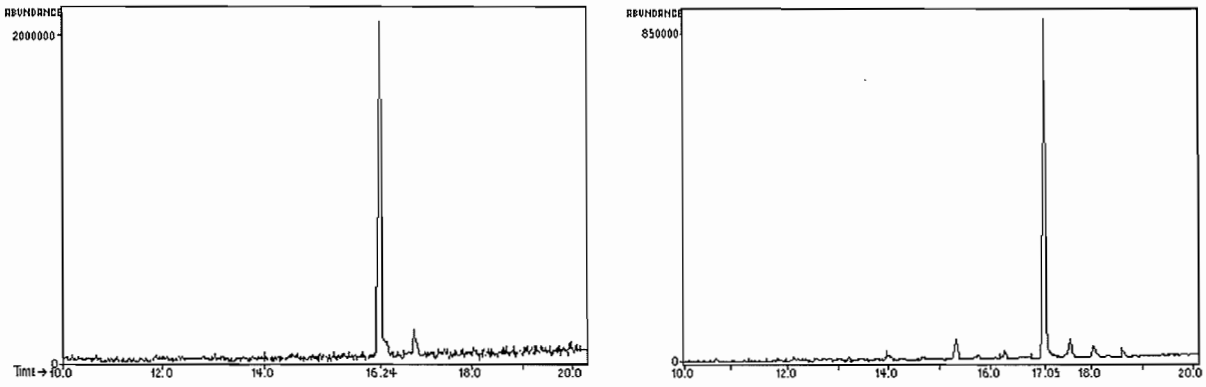

Figure 1: Total Ion Chromatograms of Free (left) and Acetylated (right) Testosterone analysed on the BPX50 column

These analyses suggest this column provides low bleed and good chromatography for both free and acetylated testosterone. Further analyses show that associated free steroids and their metabolites are also resolved on this phase under appropriate temperature conditions. The 0.25 mm i.d. column with 0.25 μm film thickness gives a good balance between column loading, resolution and bleed.

2. Combustion Furnace

The existing prototype combustion interface is represented diagrammatically in figure 2.

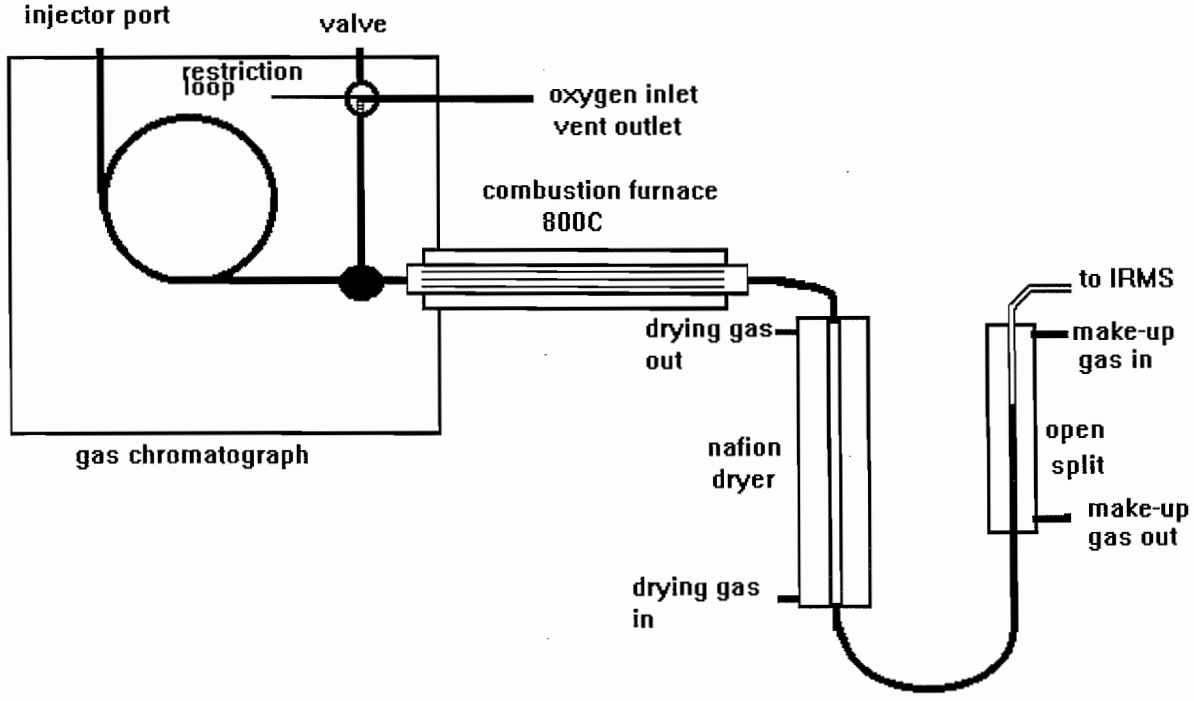

Figure 2: Finnigan prototype combustion interface

The oxidation furnace is an alumina ceramic tube containing two copper and one platinum wires. This tube is connected to the column and the deactivated fused silica tubing, which forms the interface flow path, using Valco unions with a 1/32" connection to the column and a 1/16" connection to the furnace. Polyimide type ferrules were used in both connections. At the operating temperature of 750-800 °C and with the oven temperature cycling, it is difficult to maintain leaktight seals at these connections.

This problem is not unique to this particular instrument. One report suggests a viable alternative is to bypass these connections by constructing an oxidation reactor of deactivated silica tubing which forms a continuous flow path from the end of the column to the open split (15). This system also employs a liquid nitrogen trap for water removal, which requires periodic "defrosting".

The existing interface in the GC-C-IRMS system utilises Nafion with a countercurrent dry helium flow for water removal. Nafion is a polymer material consisting of a teflon backbone with alkyl sidechains bearing terminal sulphite groups attached to it (16). It has an. exceptionally high affinity for water and a very low affinity for carbon dioxide. For these reasons the existing Nafion dryer, which utilises a length of Nafion of approximately 10 cm, was retained.

The first model of the capillary interface is represented in figure 3.

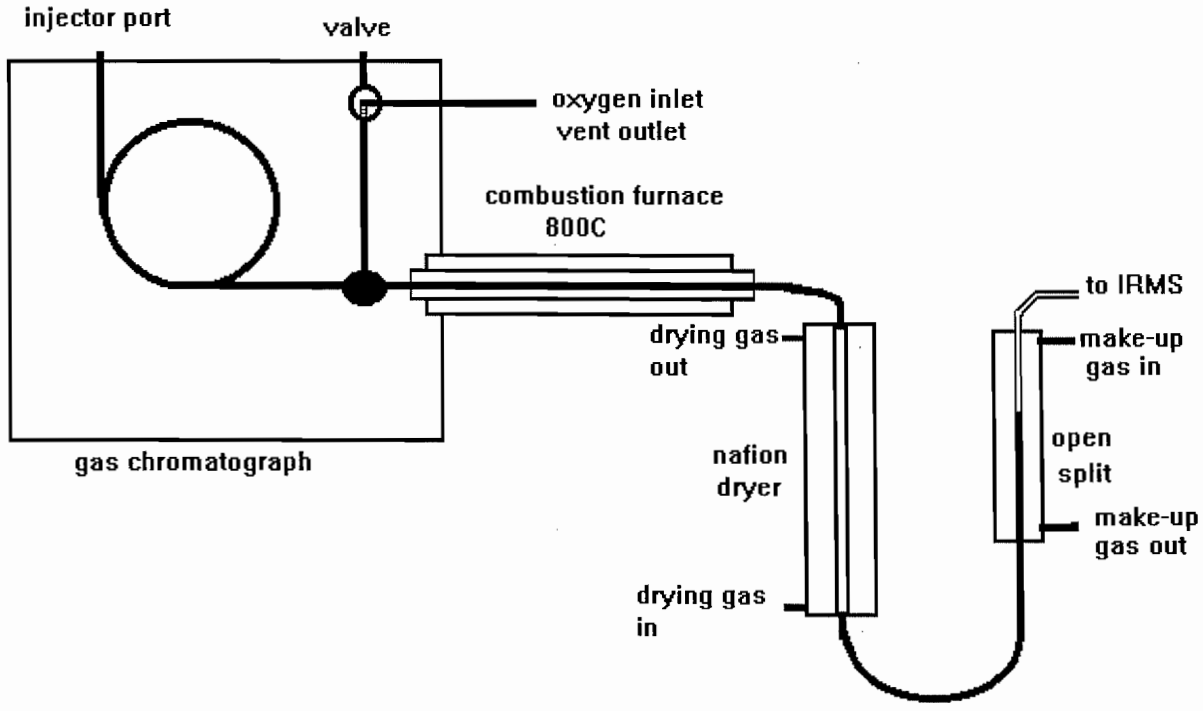

Figure 3. ASDTL Capillary Interface.

The interface capillary connects to the Valco T union (1/32" fittings) at the end of the column and the Nafion is swollen in solvent and slipped over the other end of the interface capillary, forming a leak-tight seal either end of the furnace. The interface is constructed of 0.32 mm i.d. fused silica deactivated tubing throughout, with two copper wires inserted a known distance from the column end of the furnace. The section containing the wires is contained within one of the old furnace tubes and positioned in the centre of the furnace housing, to enable oxidation in the hottest part of the furnace.

This interface was found to function well after the restriction loop was removed from the valve assembly inside the oven and the port sealed. The background argon level was maintained at or below 1V on cup 4 and water background at or below 2.5 V on cup 6, criteria specified in an in house instruction manual. The furnace in this system was oxidised overnight, at a temperature of 500 °C. The furnace temperature was increased to 750 °C prior to commencement of analysis.

The system, in conjunction with the BPX50 column, gave good chromatograms and isotope ratio traces (fig. 4). The isotope swings were even, showing good combustion efficiency, the peaks sharp and the baseline flat.

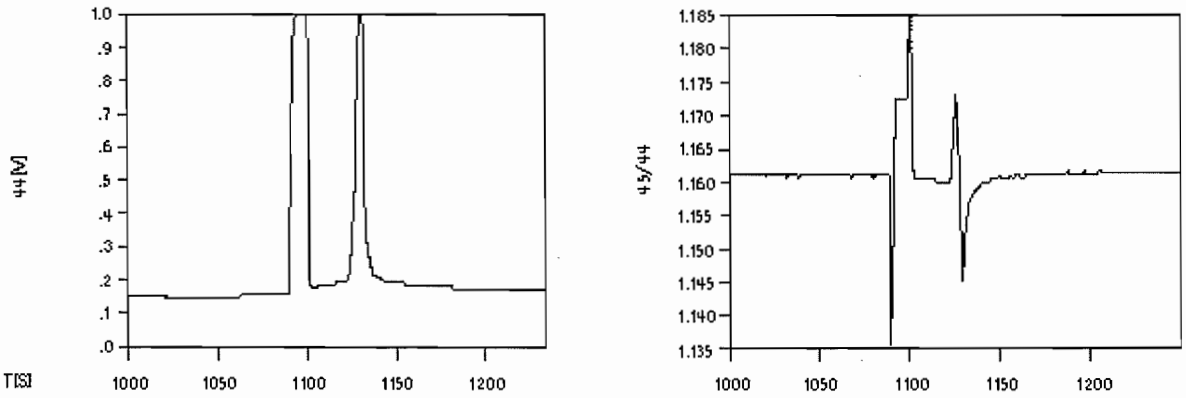

Figure 4. Isotope Ratio Chromatogram and Ratio Trace of Testosterone Acetate obtained using the ASDTL Capillary Interface

The main problem with the ASDTL Capillary Interface is that the section of silica tubing inside the furnace housing lasts a little over a week before fracturing and the whole interface then needs to be replaced. This a cumbersome task and requires a quantity of silica tubing, ferrules and Nafion each week.

A second model was therefore developed reversing the order in the flow path of the Nafion dryer and the open split (fig. 5). This utilises the natural disconnection of the flow path which occurs in the open split so that only the furnace section of the interface and none of the

Nafion need be replaced each time the furnace is rebuilt, without introducing any new connections. The length of Nafion in the dryer and the purge gas flow rate were also optimised according to published criteria (16).

This system is much less cumbersome to construct than the Capillary Interface and because only two connections are required for each furnace installation, those being at the T-unions at the end of the column and at the top of the open split, it is also much easier to maintain. No loss of resolution, peak broadening nor altered isotope ratios were observed.

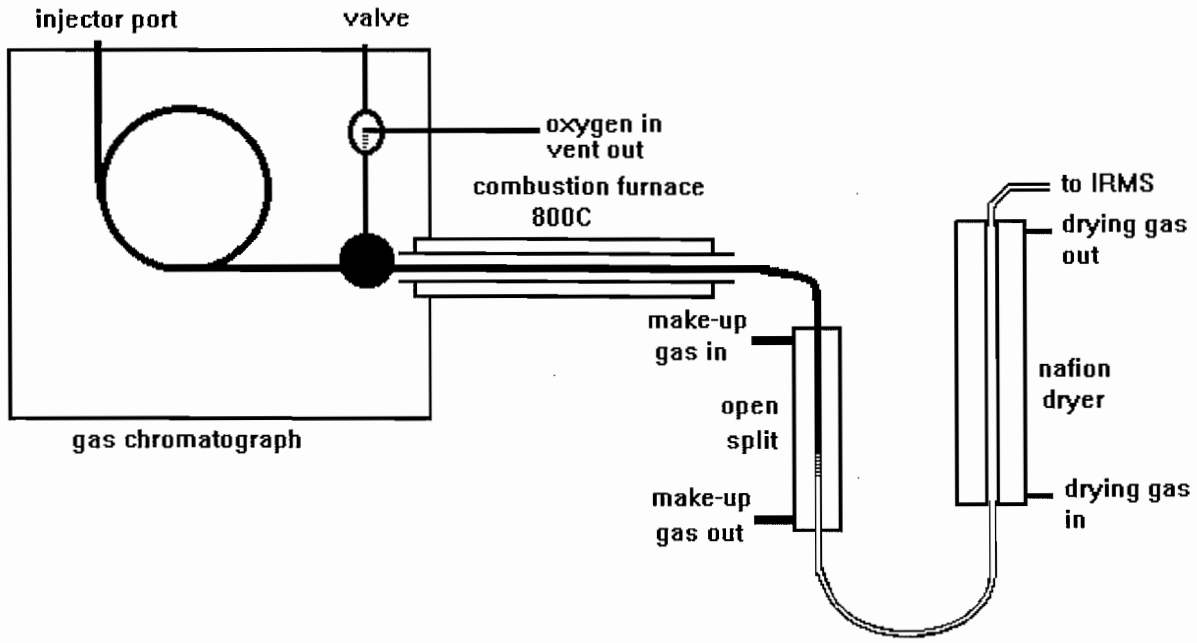

Figure 5. ASDTL Capillary Furnace

Each Capillary Furnace requires only 70-80 cm of deactivated fused silica tubing, compared with more than twice that amount for the Capillary Interface. The argon background was consistently 0.5 V (cup 4) or less and the water background 1 V or less. After some practice a new furnace could be constructed and installed in less than half an hour, compared with half a day for the capillary interface.

The disadvantages of this system is that it has a lifetime of a maximum of ten days and is also very fragile to errant solvent peaks. It also requires daily reoxidation. However, the relative ease of installation and minimal leakage associated with this system are good compensations for these inconveniences.

3. Reproducibility

Repeated analyses of testosterone acetate were performed to assess the accuracy and precision of the system. The average value obtained was -30.4 $^{o}/_{oo}$ with a standard deviation of 0.1 $^{o}/_{oo}$ over six analyses. Repeating these analyses on a new furnace gave -29.9 $^{o}/_{oo}$ with a standard deviation of 0.4 $^{o}/_{oo}$ over 3 analyses.

These results compare reasonably well with the values obtained from combustion (-29.1 ± 0.2 $^{o}/_{oo}$), and from other instruments (-30.5 $^{o}/_{oo}$, std. dev. 0.2 $^{o}/_{oo}$, Finnigan, Germany, and -30.4 $^{o}/_{oo}$, std. dev. 0.2 $^{o}/_{oo}$, Micromass, England). However, some discrepancy is apparent from furnace to furnace; the main source of reproducibility error was found to be the oxidation condition of the furnace. This was improved by increasing the carrier flow rate during oxidation to improve the flow of oxygen over the copper wires while protecting the capillary column.

Multiple analyses of 17-methyltestosterone were then performed to test and assess the uncertainty in the isotope ratio measurement (table 1). The confidence limits (95 %) were found to be ±0.5 $^{o}/_{oo}$ over seven days. This value was obtained from 41 analyses performed over two weeks including a number of furnaces and reoxidations.

Table 1: Reproducibility of the ASDTL Capillary Furnace Using 17-Methyltestosterone.

Date of Analyses	Average for day	Number of replicates	Standard Deviation for day's analyses
7-Jan	-29.7	5	1.3
11-Jan	-30.6	7	1.8
12-Jan	-30.3	10	0.5
13-Jan	-29.9	3	0.7
19-Jan	-31.4	4	2.0
20-Jan	-29.5	10	0.3
21-Jan	-30.4	2	1.1
Average	-30.3		
Number of days	7		
Day to Day Standard Deviation	0.6		
Confidence limits*	**0.5**		

* at 95% confidence

Conclusions

The BPX50 capillary column was found to be stable at the temperatures required and resolved both free and acetylated sterols well. It provides the advantage of the ability to analyse underivatised compounds and hence avoids the problems of accounting for the isotope contribution from the extra carbon atoms in the derivatising agent and of isotopic effects during the derivatisation reaction.

The ASDTL Capillary Furnace shows promise as a combustion interface. Although not ideal, it does allow the instrument to be utilised in conjunction with gas chromatography, a facility not available with the existing configuration. The current configuration of the furnace allows rapid and simple replacement of furnaces and the system is easy to maintain.

The accuracy and precision were found to improve by modifying the oxidation conditions. Further improvements may also follow after testing oxidation temperature, furnace diameter and the number of copper wires inserted into the tubing.

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