Using GC-MS/MS for Superior Sensitivity, Specificity and Precision in Free Testosterone Analysis

By Rohan A. Thakur, Clark Williard and Agnita Rajasekaran Taylor Technology - a subsidiary of PharmaNet Development Group, Princeton, NJ

This article discusses biologically important steroid measurements which have, in the past, seen a lack of analytical sensitivity. Examples include determination of testosterone levels in women and children and estrogen levels in post-menopausal women. The article gives insight into the analysis of free testosterone at physiologically relevant levels in plasma ultra-filtrate, concentrating on the sensitivity, specificity, precision and accuracy advantages offered by GC-MS/MS.

Introduction

Until 1955, mass spectrometry (MS) was most commonly used for the direct analysis of volatiles. Gas chromatography (GC) was coupled to MS for the first time at Dow Chemical Company with the aim to expand the analytical capabilities of MS to cover complex mixtures of unknowns [1]. Subsequent technological developments involved the introduction of hybrid mass analyzers such as the triple stage quadrupole (TSQ) mass spectrometers and the use of tandem MS (MS/MS or SRM) as a high specificity technique for routine quantitative analysis of complex mixtures. GC-MS/MS was quickly established as the technology of choice for bioanalytical applications.

Although highly efficient for the analysis of complex mixtures, GC-MS/MS could not address the requirement of the industry for the analysis of larger non-volatile molecules. To overcome this new challenge, high performance liquid chromatography (HPLC) was coupled to MS/MS (LC-MS/MS) leading to the development of the atmospheric pressure based ionization (API) technique of electrospray (ESI). This new method soon was soon adopted as the preferred technology for bioanalysis [2].

In addition, LC-MS/MS was seen as offering a number of advantages that allowed for easy, uncomplicated bioanalysis of small molecules. Compared to GC-MS/MS, the technique requires very little sample preparation while delivering good detection limits and is also capable of analyzing a wider range of compound classes and molecular weights. Latest advances have seen the introduction of TurboFlow, on-line solid phase extraction (SPE) and other chromatographic techniques that facilitate direct injection of biological fluids into LC-MS/MS systems, eliminating sample preparation [3]. These methods are particularly beneficial for high-throughput laboratories, where rapid analysis of thousands of samples is needed. However, in applications where assay precision and accuracy are of utmost importance, LC-MS/MS may not always deliver the required results. Indeed for the highly sensitive and specific detection of steroids for example, it has been suggested that GC-MS/MS might actually be the preferred technique.

Estrogens (estradiol and estrone) and androgens (testosterone and androstenedione) are a class of biologically important endogenous compounds that routinely require reproducible results at very low detection limits (attogram level). Endogenous and exogenous estrogens are associated with the pathogenesis of breast cancer [4] whereas testosterone affects major biological functions and is used as a therapeutic agent for the treatment of hypogonadism, the stimulation of erythropoiesis and hereditary angioneurotic edema [5]. As a consequence, measurements of estrogens and androgens is of high biological importance, determining for example testosterone levels in women and children and estrogen levels in post-menopausal women [6,7]. Analytical sensitivity and specificity play major roles in the interpretation of steroid measurements. Sample preparation is critical for the sensitivity, precision and accuracy of results. Ion suppression can be directly related to inadequate sample preparation and is a major problem with ESI based LC-MS/MS techniques [8].

The limitations of LC-MS/MS related to accuracy, precision, specificity and sensitivity have been significant enough to initiate an inter-laboratory comparison study of serum total testosterone measurements performed by mass spectrometric methods [6]. Recent data indicates that GC-MS/MS offers three key advantages over LC-MS/MS when it comes to this type of analysis. The derivatization technique improves sensitivity (Neg Ion CI dramatically reduces chemical noise), the high number of theoretical plates of a GC column results in highly focused chromatographic peaks (approximately 3 sec or less), and the entire column eluent enters the ion source (unlike LC-MS/MS ion sources which sample 5% of the LC eluent) . As a

result, GC-MS/MS provides the specificity, sensitivity and precision required to reliably interpret physiologically relevant levels of free testosterone in plasma.

An experiment was developed to demonstrate the high sensitivity, precision and accuracy offered by GC-MS/MS. A bioanalytical method was used for the analysis of free testosterone at physiologically relevant levels in plasma ultra-filtrate (UF) from post-menopausal women.

Experimental

EDTA (ethylenediaminetetraacetic acid) plasma from post-menopausal women was stored at -20°C and used to form quality control samples (QCs). The plasma was also stripped with charcoal to prepare calibration standards (STDs). Plasma UF was prepared using a centrifugal filter device (Centricon YM-30, Millipore Corporation, Billerica, MA). Sample preparation was completed in three steps. First, a penta deuterated testosterone (d5- testosterone) solution was added to 0.5 mL of the plasma UF, followed by liquid:liquid hexane/ethyl acetate extraction. Oxime of the steroids was then prepared by evaporating a volatile extraction solvent with N2 and derivatizing with O-(2,3,4,5,6 pentafluorobenzyl)-hydroxylamine HCl. Silylation of the OH group was enabled using N-Methyl-N-trifluoroacetamide (MSTFA).

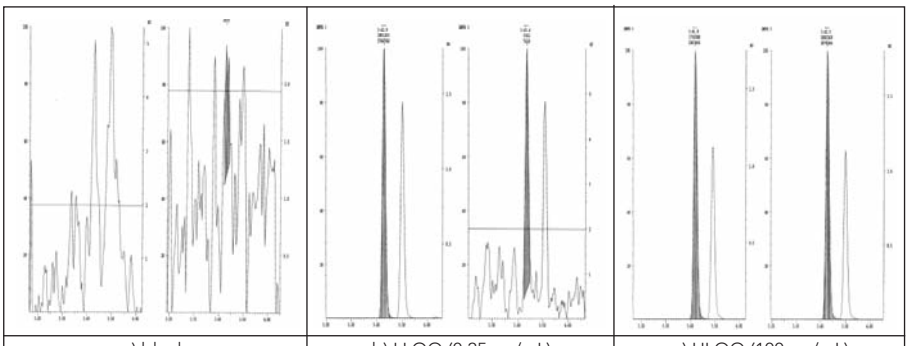
A gas chromatograph (Varian 3400, Varian, Inc., Walnut Creek, CA) was implemented together with a 15 m x 0.250 mm DB-1 fused silica capillary column (J&W Scientific, Folsom, CA). The split flow rate was 50:1 and the temperature program spanned from 210°C to 303°C. A triple stage mass spectrometer (Finnigan TSQ-7000, Thermo Fisher Scientific, San Jose, CA) was also used, operated in negative ion chemical ionization (NICI) mode.

Results and Discussion

The assay range of the GC-MS/MS method was determined to be between 0.250 and approximately 100 pg/mL. The calibration standard curves yielded consistent regression slopes and all acceptance criteria were met. Table 1 demonstrates the intrarun and interrun precision and accuracy results whereas Figure 1 shows the chromatograms that were derived over the course of the experiment. Recovery was 78-79%. UF samples were stable after three freeze/thaw cycles, after 30.5 hours at ambient temperature, after 42 days at -20°C and after 46 days at -70°C. Extracts were stable in the autosampler after 239 hours. Testosterone levels were stable in human plasma stored at -70°C for at least 62 days and -20°C for at least 42 days.

		LLOQ	Low	Medium	ULOQ
		0.250 pg/mL	1.29 pg/mL	9.56 pg/mL	96.1 pg/mL
		Intrarun			
Run 1	Mean	0.242	1.37	9.36	94.9
	CV (%)	14.8	6.69	7.18	4.58
	Bias (%)	-3.2	6.20	-2.09	-1.25
	N	5	6	6	6
Run 2	Mean	0.222	1.25	9.61	96.1
	CV (%)	10.4	3.26	2.26	6.50
	Bias (%)	-11.2	-3.10	0.523	0.00
	N	6	6	6	6
Run 3	Mean	0.207	1.25	9.73	97.3
	CV (%)	22.5	7.00	0.741	2.70
	Bias (%)	-17.2	-3.10	1.78	1.25
	N	5	5	6	6
		Interrun			
	Mean	0.224	1.29	9.56	96.1
	CV (%)	16.1	7.16	4.36	4.66
	Bias (%)	-10.4	0.00	0.00	0.00
	N	16	17	18	18

Table 1. Intrarun/Interrun Precision and Accuracy. LLOQ is the the lower limit of quantitation and ULOQ is the upper limit of quantitation.



a) blank | b) LLOQ (0.25 pg/mL) | c) ULOQ (100 pg/mL)

Note: in each panel, the first chromatogram is a trace of the IS ion channel; the second is a trace of the ion channel for testosterone. The second peak in the LLOQ and ULOQ chromatograms is due to the testosterone isomer formed during derivatization.
Figure 1. Chromatograms of Extracted Standards that are: a) blank, b) at the LLOQ, and c) at the ULOQ

GC-MS/MS was able to routinely and reproducibly deliver results at a LOQ of 3 femtograms on column, considerably lower than most LC-MS/MS systems can routinely deliver. The liquid-liquid extraction technique used for this assay is proven to reduce matrix effects, resulting in the analytical column always being exposed to the cleanest possible biological samples. As a result, build-up of problematic matrix components such as phospholipids, polar metabolites and other polar interferences was reduced. (8) In addition, the derivatization step required to make compounds volatile for injection into the GC offered some degree of specificity.

The GC column provided increased efficiency of chromatographic separation, generating highly focused peaks. Since the majority of the analyte was chromatographically concentrated in a narrow peak (3 seconds or less at base), separation from background noise became easier, resulting in clear delineation as to when the signal started and ended. Consequently, precision was improved since peak inflection and deflection points were easier to assess through automated peak integration algorithms.

The entire column effluent from the GC column entered the MS ion source making it far more efficient than atmospheric pressure ionization (API) techniques employed by LC-MS/MS. API sources (ESI/APCI) are usually 1-10% efficient in transferring the ions from the LC eluent into the MS. This means while close to 100% of the column eluent enters

the GC-MS/MS ion source, exactly the opposite occurs in an LC-MS/MS API ion source. Until this sampling rate is improved, it appears that GC-MS/MS will be more efficient and this has a direct impact on sensitivity.

Conclusion

Choosing whether to implement LC-MS/MS or GC-MS/MS for bioanalysis depends on the specific application requirements. LC-MS/MS is the preferred technique for simple bioanalysis of an extended range of compound classes and molecular weights from small to large non-volatile molecules. However, the method does not offer the accuracy, precision, specificity and sensitivity required for specific applications including the analysis of steroids (estrogens and androgens). A highly sensitive GC-MS/MS bioanalytical assay for free testosterone in UF plasma from post-menopausal women has been successfully implemented to help elucidate the pharmacokinetic parameters of testosterone-related therapy and the monitoring of endogenous testosterone as a pharmacodynamic biomarker.

References

1. *Gohlke, R.S. and McLafferty, F. W. J. Amer Soc. Mass Spectrom. (1993), 4, 367*
2. *Yamashita, M., and Fenn, J.B. J. Phys. Chem. (1984) 88, 4671.*
3. *Herman J. L. Rapid Commun.Mass Spectrom. (2002) 16, 421.*
4. *Wang, S., Paris, F., Sultan, C. S., Song, R., Demers, L.N., Sudaram, B., Settlege, J., Ohorodnik, S., and Santen, R. JCEM (2005) 3, 1407.*
5. *Santen, R., Demers, L., Ohorodnik, S., Settlage, J., Langecker, P., Blanchett, D., Goss, P. E., and Wang S. Steroids (2007) 72, 656.*
6. *Vesper, H.W., Bhasin, S., Wang, C., Tai. S., Dodge, L.A., Singh, R. J., Nelson, J., Ohorodnik, S., Clarke, N. J., Salameh, W. A., Parker, R. C. Jr., Razdan, R., Monsell, E. A. and Myers, G. L. Steroids (2009) 74, 498.*
7. *Singh. R. J. AACC (2008) 34, 12.*
8. *Bonfiglio, R., King, R.C., Olah, T. V., Merkle, K. Rapid Commun. Mass Spectrom. (1999) 13, 1175.*

We would like to acknowledge Robert White, Randy Weintraub, Bhaskar Sundaram, James Settlage, and Susan Ohorodnik.

Impressive Speaker Line Up Focuses on Efficiency Improvements

The full line-up of speakers has now been announced for the 2010 **Anatune** conference, entitled 'Automation in the Chromatography Laboratory'. The aim of the conference, which will be held at Down Hall Country House Hotel in Hatfield Heath, UK, on 5-6 October 2010, is to help delegates find innovative ways of improving efficiency in their laboratories. The conference promises to be packed full of inspiration and interest for owners or managers of chromatography laboratories and offers plenty of opportunity to interact with the speakers and network with other delegates. Throughout the two day conference the focus will be on lowering overheads whilst improving data quality.

Speakers for this year's conference come from the UK, Europe, the Far East and the US; all have a wide and impressive background in analytical technology: Dr Jaroslav Slobodnik, Director of Environmental Institute in Slovakia; Dr Jim Jones, Managing Director of Jones Environmental, Chester, UK; Nobuo Ochiai, Technical Director, GERSTEL Japan; Mr Ray Perkins, Managing Director, Anatune Limited, Cambridge, UK; Mr Bob Green, Technical Director, Anatune Limited, Cambridge, UK; Mr Pierre Alves, Export Technical Sales Manager, OI Analytical, Texas, USA; Mr Angus Macmaster, Analytical Team Leader, Givaudan UK Ltd, Kent, UK; Mr Don Harris, President, ASAP Analytical, Kentucky, USA; and Mr Kevin MacNamara, Head of Technical Development, Irish Distillers Co. Cork, Ireland. Down Hall Country House Hotel, the venue for the conference, is set in 110 acres of beautiful parkland and is easily accessible, being just 15 minutes from the M11 and Stansted airport.

For more information, visit www.anatune.co.uk

New Standalone Ultrahigh-Pressure Pump for Proteomics Workflows Using Liquid Chromatography

Dionex announce the expansion of its family of industry-leading Rapid Separation LC (RSLC) systems with the new UltiMate® 3000 NCP-3200RS nano/capillary LC pump module. This new standalone ultrahigh-pressure pump features an extensive flow-pressure footprint, delivering continuous, direct flow rates from 20 nL/min to 50 L/min at backpressures of up to 800 bar (11,600 psi) for nano, capillary, and micro LC analyses.

The pump can be used to configure an entry-level RSLCnano system for reversed-phase separation of peptides in LC-MS proteomics workflows. Alternatively, the pump can be used as an add-on module to our industry-leading RSLCnano system for advanced applications, such as automated off-line 2D-LC or tandem nano LC. Tandem nano LC can be an attractive technique for customers who want to improve their mass spectrometry use and increase sample throughput. Dedicated application kits have been introduced for the RSLCnano system, such as automated off-line RP × RP of peptides and tandem nano LC, to facilitate instrument installation and get effective data from the first experiments.

In addition, Dionex has also released new biocompatible switching valves, featuring a maximum pressure specification of 345 bar (5,000 psi), for sample injection and column switching. These values are intended for use with the RSLCnano system, to provide a biocompatible sample flow path that can be important to customers dealing with the analysis of phosphopeptides.

For more information, visit www.dionex.com.