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Mass Spectrometry & Spectroscopy

Is there any such thing as a free hormone?

Opportunities for online TFC-LC-MS/MS analysis of serum free steroids

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The majority of steroid and other small molecule hormones are extensively bound to carrier proteins in the blood stream. Whilst there is debate regarding the function of these carrier proteins there is a considerable body of evidence to suggest that the concentration of unbound or 'free' hormone is the most biologically and clinically relevant measure of hormone activity. This is commonly cited as the 'free hormone hypothesis' [1], the underlying assumptions being that: i) the hormone receptor is only accessible by the unbound hormone and that: ii) the hormone:protein complex is unable to extravasate the capillary bed.

Unfortunately, the measurement of free hormone presents both analytical and theoretical challenges. Many hormones have the capacity to bind to several plasma proteins, and both the capacity and binding constants for these different proteins can vary over at least four orders of magnitude. As many specific hormone binding proteins have sub-nanomolar dissociation constants, the concentration of free hormone can be in the picomolar range and the bound to free ratio stacked heavily against the free hormone. Theoretically, free hormone measurement presents a problem similar to the uncertainly principle [2] - that is, any attempt to measure the concentration of free hormone will itself perturb the binding equilibrium. Despite these concerns there is no doubt that current methods that estimate the free fraction of certain hormones are of clinical relevance. For example, UK clinical laboratories use analogue immunoassay measurements to measure free thyroxine (FT4) in the diagnosis of thyroid disease (though this methodology is still not universally accepted in the USA). In contrast, nearly all laboratories estimate total rather than free steroid hormone concentrations, despite considerable literature showing the shortfalls of these methods [3]. This is largely due to the absence of a simple, reliable method to measure free steroid hormones. Unlike with FT4, analogue immunoassay methods for steroids have been largely discredited [4,5].

Clinical laboratories are increasingly drawn towards LC-MS/MS instrumentation for the quantitation of steroids and other small molecule hormones, due to superior analytical selectivity when compared to the immunoassay methods currently in use. An attractive solution to the problem of routine free hormone analysis in clinical laboratories would be to combine the selectivity of MS/MS detection with an on-line method that has the capacity to resolve free from bound hormones. Contemporary methods such as selective binding protein precipitation methods, equilibrium dialysis or ultra filtration [6], suffer technical and practical limitations and none are particularly amenable to on-line MS/MS methods, particularly for routine, high-throughput analysis.

In this article we describe our investigations, using serum testosterone as an example, with an application of a simple on-line, column based extraction system for the separation of bound and free hormones prior to analytical HPLC separation and quantitation by MS/MS.

Serum Testosterone

Total serum testosterone concentration is routinely analysed in clinical laboratories for the investigation of infertility or hirsuitismin women and sexual hypofunction in men. There has been much recent interest in testosterone analysis to either prove or refute the existence of the male menopause [7].

In vivo, testosterone is extensively and tightly bound to the carrier protein sex hormone binding globulin (SHBG), a dimeric glycoprotein of molecular weight 90 kDa (*Figure 1*).

Testosterone also binds to serum albumin, though with a much lower affinity (30,000 fold). However, the very high concentration of albumin (40-50 g/L) in serum gives a distribution of testosterone of approximately 40% SHBG bound, 58% albumin bound and 2% free in a typical male serum sample (*Figure 1*).

It is important to consider, when interpreting total testosterone results, that the relevant concentration of these binding proteins influences the distribution of testosterone significantly. For instance the concentration of SHBG can be quite variable [3] – it is affected by very common conditions such as oral contraceptive use, obesity, hyperthyroidism and insulin resistance. It is also regulated by sex hormone status itself. As such, many authors agree that a calculated value based on measurement of total testosterone, SHBG and albumin concentration correlates best with the clinical androgenic status [6].

It is also important to consider the kinetics of the hormone binding protein interactions as well as the binding equilibria when considering potential analytical methods. Whilst these have not been extensively studied, a reasonable estimate of the half-life of the off-rate of testosterone from SHBG are 12 s, compared to <0.35 s for albumin [8] relative to a blood capillary transit time of <0.1 s.

As the albumin bound fraction is so labile some authors consider that the free testosterone concentration plus the albumin bound fraction, which is called the 'bio-available' fraction by its proponents, may be a better marker of testosterone status. In terms of analysis, 'bio-available' testosterone is the easiest fraction to measure due to its relative concentration along with the long half-life of the SHBG dissociation reaction. Also, in most situations, 'bio-available' testosterone will be highly correlated with 'free'.

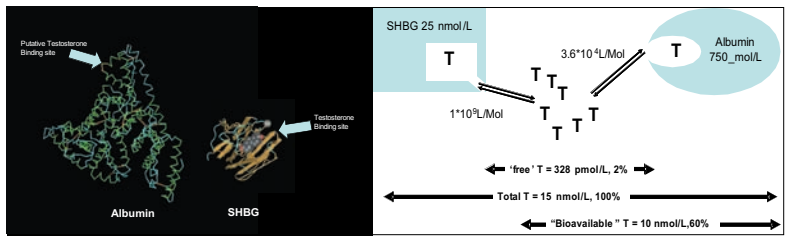

Figure 1. Albumin and SHBG binding sites for testosterone and the distribution of testosterone in serum.

Analytical considerations

If we are to use an online solid-phase extraction (SPE) system to separate bound from free testosterone it must fulfill several criteria:

1) The SPE partition must reflect the true equilibrium between bound and free.

2) It must be possible to load neat serum onto the cartridge, as any pre-treatment may alter the testosterone binding equilibria.

3) The SPE eluate must be of sufficient purity and concentration to pass directly onto an LC-MS/MS system without extensive off-column preparation.

For these reasons we investigated the TurboFlow™ technology from ThermoFisher Scientific as a likely candidate.

TurboFlow™ technology

Turbulent flow chromatography (TFC – *Figure 2*) is based on the direct injection of neat biological samples onto a column packed with relatively large particles at high flow-rates (1.5-5.0 mL/min). In the resulting turbulent flow, analytes move in and out of the pores by mass transfer, small analytes are retained whilst proteinaceous material, which does not have sufficient time to diffuse into the pores flows to waste. Once the compounds of interest are extracted from the biological matrix onto the TurboFlow column they are eluted onto the analytical column and subject to chromatographic separation prior to MS/MS detection.

We have investigated the theory that by injecting neat serum directly onto the column only the non-bound testosterone is retained whilst protein-bound

testosterone is quickly eluted the waste. The high flow-rate coupled with the low volume of the TurboFlow column (~7 µL - 50 x 0.5mm internal diameter) means that the non-retained molecules have an on-column retention time many fold less than the half-life of the testosterone-SHBG dissociation reaction. As such, we assumed that SHBG-bound testosterone would not have time to dissociate within the TurboFlow column whilst the albumin-bound testosterone would re-equilibrate with the column interstices during transit such that the retained fraction would represent bio-available testosterone.

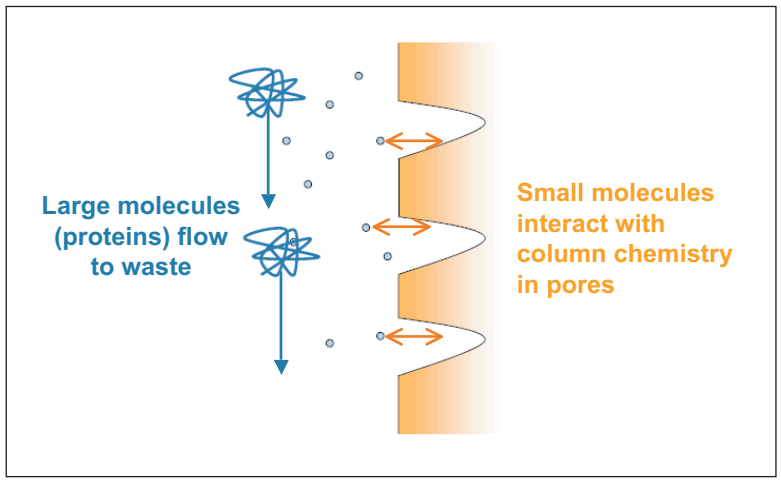

Figure 2. Principles of TurboFlow. Small molecules can move into the particle pores and bind to the column chemistry whilst larger molecules (such as proteins) flow to waste. Target molecules can then be eluted onto an analytical column followed by quantification by MS/MS.

Figure 3 shows results when serum from a healthy male volunteer was injected either: i) directly (after centrifugation to remove particulates) or: ii) following protein precipitation (2:1 (v/v) acetonitrile:serum) onto a TLX-2 TurboFlow system coupled to a TSQ Vantage™ mass spectrometer. Acetonitrile precipitation effectively displaces testosterone from its binding proteins, so gives a measure of total testosterone. The 'neat' sample reflects the concentration of the sample that is retained by the TurboFlow column. Recovery experiments were conducted to confirm that column capacity for testosterone was not exceeded (data not shown). 25% of the total testosterone is retained by the TurboFlow column and this is stable up to injection volumes of 70 µl of neat serum (*Figure 3b*). This will likely reflect the bio-available testosterone, as weakly bound testosterone will re-equilibrate within the column interstices during chromatography which the testosterone tightly bound to SHBG cannot.

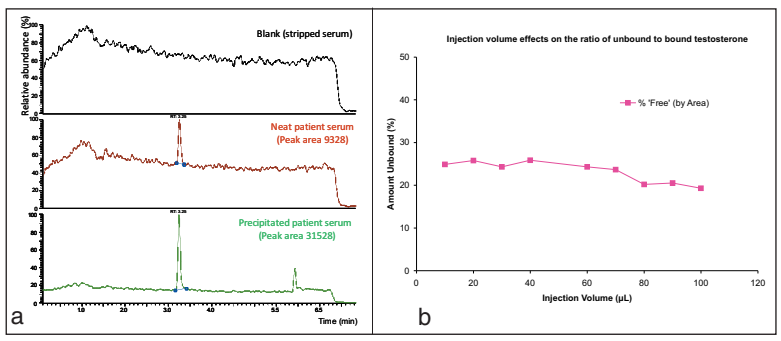

Figure 3. Measurement of non-bound and total testosterone in human serum. a) Testosterone SRM chromatograms of double charcoal-stripped serum, neat serum and serum precipitated with acetonitrile. This demonstrated the ability of the TurboFlow extraction column to exclude protein-bound testosterone. b) Ratio of non-bound:total testosterone at various injection volumes. The ratio of excluded testosterone is essentially independent of injection volume when below 70 µL.

Several theoretical predictions can be made regarding the nature of the retained fraction if this indeed reflects the bio-available species: firstly, if exogenous testosterone is added to the serum, the increase in the retained fraction should not reflect total testosterone as it will be complexed by the excess binding proteins found in serum and thus excluded from the column.

Secondly, addition of excess SHBG should increase the amount of excluded testosterone as it becomes sequestered by this high affinity testosterone binding globulin. Preliminary evidence suggests that this is indeed the case (*Figures 4 and 5*).

Given this initial data, we are engaged in a more detailed study to determine the nature of the excluded fraction and to examine the premise that the ratio of excluded to retained testosterone reflects the proportion of bio-available testosterone in vivo.

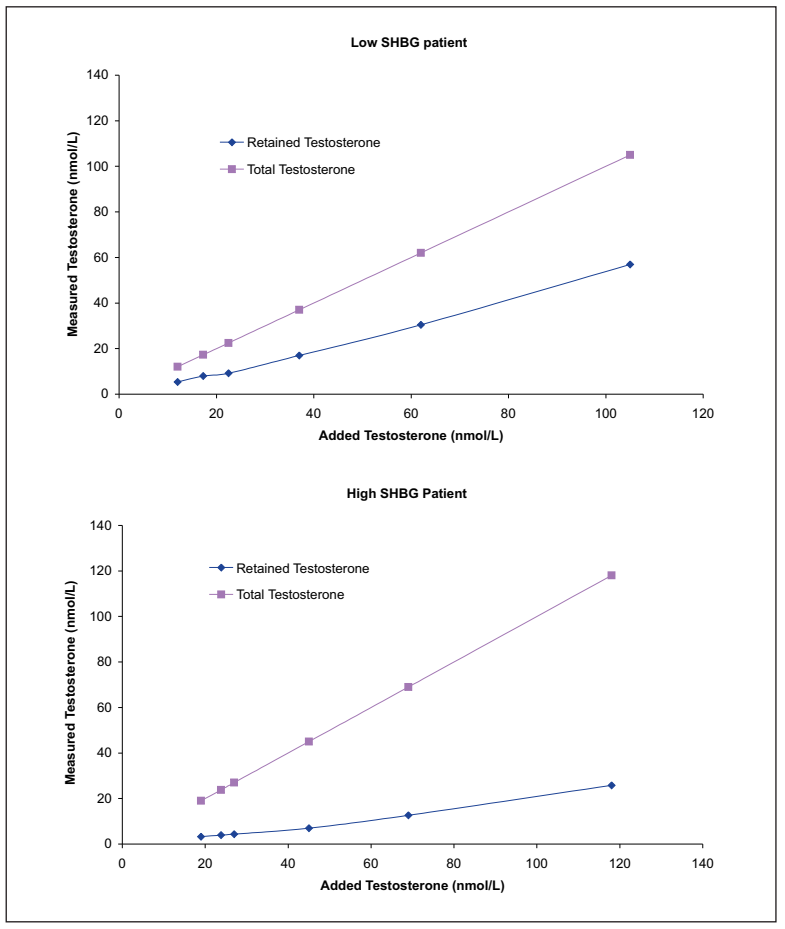

Figure 4. Demonstration of SHBG buffering effect on retention of exogenous testosterone by TurboFlow. Increasing concentrations of exogenous testosterone were added to two patient samples with identical serum albumin concentrations (44 g/L) but different serum SHBG concentrations (low 25.4 nmol/L; high 51.6 nmol/L). Whilst total testosterone increased linearly as expected, the fraction retained on the TurboFlow column from the neat injection did not increase stoichiometrically, as the exogenous testosterone was buffered by excess binding protein. Note that this effect was more dramatic in the patient with higher serum SHBG concentration.

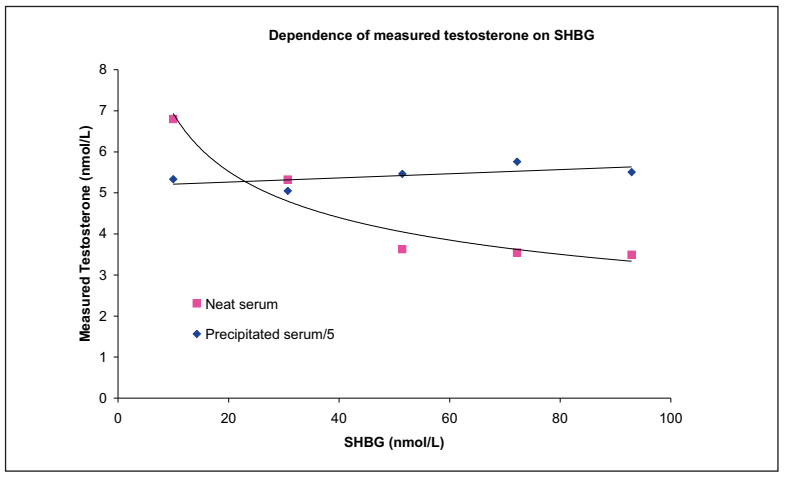

Figure 5. Dependence of measured testosterone on serum SHBG concentration. Patient pools were prepared with equivalent total testosterone and albumin concentrations but variable SHBG concentrations. Total testosterone concentration is independent of SHBG concentration, but the fraction retained by the TurboFlow column reduces as SHBG concentration increases due to SHBG sequestering of testosterone.

Conclusion

In conclusion, we have good preliminary data to show the potential of the TurboFlow sample extraction system coupled with MS/MS quantitative detection to determine the biologically available steroid hormone concentration in human serum. If further studies are encouraging, this methodology could be widely applicable for the study of protein: small molecule interactions in serum.

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