Free Drug & Hormone Separations with Vivafree™ Filters

Background

Measurement of hormone and drug concentrations in clinical samples is critical to properly diagnose diseases and to determine correct medical dosages for therapy. However, such analyses can be affected by interactions with proteins in plasma and other bodily fluids. For example, hormones can bind to albumin and different globulins to varying degrees. Many drugs also bind to albumin as well as to a-1 acid glycoprotein (AGP) and other proteins *(1)*. While these molecules are bound to proteins, they are unable to pass through biological membranes to interact with receptors and produce the desired biochemical response. Only the unbound or free hormones and drugs are available for proper metabolism and pharmacological reactions. As a result, it is important to also measure free concentrations for accurate diagnosis and treatment of medical conditions.

Several procedures have been employed for analyzing free hormone and drug levels in serum samples. Some of these methods utilize radio-labeled analogues to bind and displace the hormone of interest *(2, 3)*. These tests are widely used because they are simple to automate but have been shown to be imprecise and to not correlate well with other methods *(4, 5, 6)*. Other procedures use algorithms to calculate free hormone concentrations based on measurement of the binding proteins *(7, 8, 9)*. However, results from these methods have also been shown to have issues *(10)*. Similarly, total drug levels have been used to estimate free concentrations but these assessments are not accurate if the inter-patient variability is significant *(11, 12)*.

Generally, the most accurate test methods separate the free from the bound analyte of interest before measurement of the unbound concentration. Use of ultrafiltration (UF) or equilibrium dialysis (ED) to isolate the free hormone or drug has been recognized as the "gold standard" method for many clinical tests *(2, 5, 10)*. Both processes use semi-permeable membranes to separate proteins with bound ligands from smaller free molecules. During ED, the free and sample solutions are both in contact with the membrane and the free analyte is allowed to diffuse through and re-equilibrate. With UF, the free drug or hormone filters through the membrane into a sample collection reservoir. UF devices use centrifugal force to process samples as quickly as 20 minutes compared to several hours or longer for ED *(1, 2, 13)*. It has been demonstrated that this separation does not impact the equilibrium during UF based on the binding kinetics of the free ligand and the binding protein. If K_D is the dissociation constant at equilibrium, the free ligand concentration is defined by:

$$[\text{Free Ligand}] = K_D \times [\text{Ligand-Protein Complex}] / [\text{Unbound Protein}]$$

Since the complexed and unbound proteins are concentrated at the same rate during UF, that ratio stays constant and the free ligand concentration would essentially be unchanged by volume *(14, 15)*. However, test results can be affected by temperature during sample preparation *(1, 16)*. Binding to the membrane can also be a concern as well as design of the UF device *(1, 13)*. Best results have been observed with cellulosic membranes placed horizontally instead of vertically *(17)*. Following separation of the free analyte, it may be measured by immunoassay or mass spectrometry *(2, 13, 15, 16, 17)*.

Vivafree™ Filters

Ultrafiltration (UF) membranes can retain proteins on the basis of their rated molecular weight cutoff (MWCO). While proteins with molecular weights larger than the MWCO are removed by the membrane, small molecules can pass through into the filtrate. Using centrifugal force, free drugs and hormones can be rapidly separated from those that are bound to plasma proteins (see Figure 1).

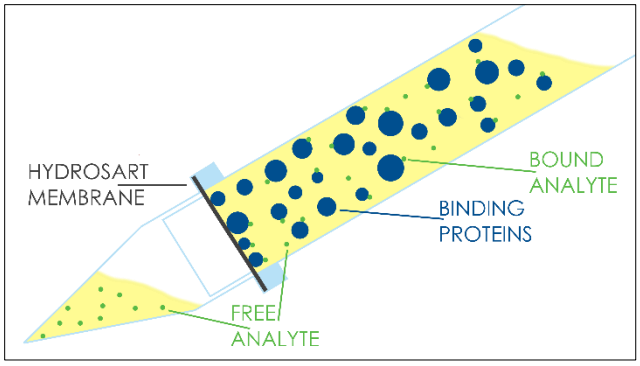

Figure 1

Diagram showing separation of free (unbound) molecules from those bound to serum proteins. Protein bound analytes are retained by the Hydrosart® UF membrane while those that are free pass through.

Vivafree™ centrifugal filters should be used with fixed angle rotors as filtration times with swing-out rotors may be 2-3 times longer. They are available in two sizes for use with sample volumes up to 0.5 ml (Vivafree™ 500) or up to 2 ml (Vivafree™ 2). For most separations, the 30,000 MWCO is recommended. However, for some larger hormones, such as insulin, the 125,000 MWCO provides better separation. Samples may be filtered as quickly as 20 minutes depending on the volume and centrifuge speeds. All Vivafree™ filters are intended for *in vitro* diagnostic (IVD) use and are supplied non-sterile for single use only.

Device Size	MWCO	Test Sample (all from serum)	Initial Volume	Filtrate Volume	Time	Temp.	Centrifugal Force
Vivafree™ 500	125,000	Free Insulin	500 µl	150 µl	30 min.	37° C	2,000 x g
Vivafree™ 500	30,000	Free Piperacillin	500 µl	100 µl	30 min.	25° C	2,500 x g
Vivafree™ 500	30,000	Free Fluconazole	500 µl	90 µl	20 min.	37° C	1,000 x g
Vivafree™ 2	30,000	Free Cortisol	500 µl	100 µl	20 min.	37° C	2,500 x g
Vivafree™ 2	30,000	Free Phenytoin	1000 µl	200 µl	20 min.	25° C	1,500 x g
Vivafree™ 2	30,000	Free Testosterone	300 µl	100 µl	20 min.	37° C	4,500 x g
Vivafree™ 2	30,000	Free Testosterone *	1000 µl *	500 µl	60 min.	25° C	4,500 x g

Table 1. Vivafree™ Filter Performance Characteristics (All data from fixed angle rotors)

**** This sample starts with 250 µl of serum diluted to 1000 µl. Free Testosterone analyzed by mass spectrometry.***

Note: Filtration times may vary according to sample volume and quality. Lipemic samples may require longer spin times.

Figure 2
Vivafree™ 500 Filters

Figure 3
Vivafree™ 2 Filters

Membranes for Vivafree™ filters are formulated from cellulose polymers to minimize non-specific absorption of drugs and hormones. Such interactions can reduce the recovery of the free analyte of interest and can affect clinical results. The 30,000 MWCO membrane uses the proprietary Hydrosart® regenerated cellulose polymer while the 125,000 MWCO has a cellulose triacetate membrane.

Testosterone

Testosterone is generally present in the blood tightly bound to Sex-Hormone Binding Globulin (SHBG) or loosely bound to albumin. Only about 1-3% is found unbound or free in serum and this concentration has been determined to be most important for diagnosing the androgen status of a patient *(5, 17)*. This is useful in testing for Hypogonadism in males as well as Hirsutism and Polycystic Ovary Syndrome in females *(18)*. In some cases where albumin levels fluctuate, such as in older men, bioavailable testosterone (BT) is measured. The BT level includes the free hormone as well as that bound to albumin from which it can easily dissociate. The BT test is usually performed by protein precipitation using ammonium sulfate *(3, 7)*. Concentrations of serum free testosterone (FT) and BT have been calculated by algorithms based on measured levels of total testosterone, albumin and SHBG and their binding characteristics *(7, 8)*. A study of 5 such algorithms by de Ronde et al *(10)* found that results of these calculations can have issues.

Serum levels of FT have been measured clinically by several methods. Direct analogue radioimmunoassay (RIA) tests are widely used by clinical labs because they are simple to automate. However, these kits do not physically separate free from bound testosterone. Instead, they rely on a radio-labeled analogue to compete with free serum testosterone for binding to specific antibody sites *(3)*. The amount of radioactivity on the binding sites is correlated to the FT level using a calibration curve. However, these analogue assays have repeatedly been shown to be inaccurate and to not correlate well to calculated FT values and other methods *(4, 5)*. Fritz et al *(20)* showed that FT results from these assays were a function of total testosterone while Vermeulen reported they were related to SHBG concentrations *(7)*.

Other tests involve separation of FT from serum by ultrafiltration (UF) or by equilibrium dialysis (ED). Following isolation of FT, analysis has been done by immunoassay *(18, 19, 20)*, gas chromatography-mass spectrometry *(17)* or liquid chromatography-mass spectrometry *(13)*. Both separation methods correlate well to calculated FT values *(13, 19)* but UF has a large advantage in speed. Samples can be processed by centrifugal UF in an hour or less while ED can take as long as 16 hours. Some reports indicate that FT recovery can be reduced due to absorption to the UF membrane, but it has been shown that this is not a factor if the proper membrane is used *(13, 17)*.

Olson et al of the Cleveland Clinic *(18)* reported that Vivafree™ UF filters were able to process their 300 µl samples for FT. Results were measured by RIA and compared to another centrifugal UF device. Initial tests were performed with a centrifugation time of 15 minutes but showed an average bias of -9.71. After increasing the time to 20 minutes, an average bias of 5.78 was calculated. Comparison by scatter plot (see Figure 4) showed a slope of 1.047 and correlation coefficient of 0.9748. Cleveland Clinic reduced costs 35% by changing to the Vivafree™ filters.

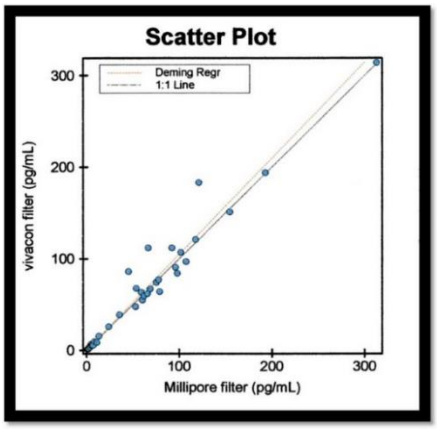

Figure 4 Vivafree™ 2 compared to another UF filter for FT *(18)*

Insulin

Insulin is produced by the pancreas and regulates glucose uptake and utilization. Antibodies can develop after insulin treatment and can directly bind to the insulin to make it unavailable for metabolic activity. Free (bioactive) insulin can be measured by immunoassay after removal of insulin antibodies and bound insulin *(21)*. This can be done by precipitation with polyethylene glycol (PEG) *(22)* or by ultrafiltration. ARUP Laboratories utilizes quantitative ultrafiltration / quantitative chemiluminescent immunoassay to perform testing for free insulin in serum *(23)*.

Cortisol

About 70% of the cortisol in plasma is tightly bound to corticosteroid binding globulin (CBG) and about 20% to albumin from which it can dissociate easily *(24)*. Only the free cortisol (FC) is physiologically active and should be measured in the diagnosis of hypercortisolism (Cushing's Disease) and hypocortisolism (Addison's Disease) *(25)*. FC levels in serum have been estimated based on total cortisol along with CBG and albumin concentrations *(26)*. Direct measurements separate the FC from the bound fraction using methods such as ultrafiltration (UF) or equilibrium dialysis (ED). Subsequent measurement of the FC concentration can be performed by immunoassay or mass spectrometry *(24, 27)*.

Therapeutic Drug Monitoring (TDM)

The measurement of free drug concentrations has been useful in cases where the drug has a narrow therapeutic range. While insufficient levels may not provide the proper benefit to the patient, excess drug amounts can be toxic *(15, 28)*. Since many drugs will bind to and interact with plasma proteins, monitoring of the unbound concentration is important to provide the proper pharmacologic effect *(1, 28)*.

Phenytoin and other anti-epileptic drugs can bind highly (~90%) in the bloodstream and free levels can vary widely (7-35%) due to clinical conditions *(12)*. Ultrafiltration (UF) has been widely used by reference labs such as Quest Diagnostics to separate these free drugs for testing by immunoassay *(15, 29)*. UF has also been reported to be the easiest method to perform this separation *(28)*. It has been shown temperature during UF is important *(1, 36)* and that UF should be performed prior to freezing samples for storage *(28)*.

TDM is also required for many types of antibiotic and antifungal drugs *(30)*. UF has been used extensively to separate free fractions of these medications but results can vary according to operating conditions. Recovery of free drug can decrease significantly at high centrifugal force (10,000 x g) and if temperature and pH are not maintained at physiological conditions (37°C and pH 7.4)*(31, 36)*. Membrane type is also critical in maximizing recovery. Cellulose based membranes have been shown to provide superior recovery when compared to polysulfone filters *(32)*. Vivafree™ filters have been reported to work well for these tests *(35)* showing low non-specific binding when compared to other centrifugal filters *(36)*.

The cardiac drug Digoxin has also been shown to have a specific therapeutic range where toxicity due to overdose can occur. This toxicity can be reversed by treatment with anti-digoxin immune fragments such as DIGIBIND®. Monitoring free Digoxin levels after such therapy is critical for proper dosing. UF devices have been reported to be the best strategy for separating free digoxin *(33)* and 30-kD centrifugal UF filters are used by Mayo Medical Laboratories in their test methodology *(34)*.

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