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Clinical Applications

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1. Introduction

The first significant use of mass spectrometry in clinical diagnosis was the determination of inborn errors of metabolism using gas chromatography coupled to mass spectrometry or GC-MS. Biologically significant metabolites such as organic acids, amino acids and others were usually extracted from biological fluids such as urine, and then derivatized to make them volatile, volatility being a prerequisite to separation by gas chromatography. Such elaborate extraction and derivatization schemes have made the use of GC-MS difficult for biologically significant molecules because of their thermal instability. Separations of thermolabile biological molecules by HPLC is a far more straight forward process than by GC and the coupling of HPLC with mass spectrometry, or LC-MS, was a major breakthrough in the application of MS to the measurement of biologically significant molecules such as those measured for clinical diagnosis. Since the late 1970's there has been a great deal of research into coupling HPLC with mass spectrometry. Many different LC-to-MS interfaces had been developed over the last 3 decades including the Moving Belt FAB, Thermospray, Particle Beam, and Flow-FAB, all of which no longer exist today. All these once quite promising and even popular techniques, have been replaced by those involving the use of atmospheric pressure ionization (API) which was first introduced commercially in the late 1980's. The most prominent API LC-MS interfaces are Electrospray (ESI), Nebulizer Assisted Electrospray (a.k.a. IonSpray), Atmospheric Pressure Chemical Ionization (APCI) using a Heated Nebulizer, and Atmospheric Pressure Photo Ionization (APPI) also using a Heated Nebulizer. API facilitated the rapid adoption of LC-MS and a good review of API is available by Thomson ¹ and for clinical diagnostics using IonSpray by Henion ².

As the name implies, in API, ions are created at atmospheric pressure quite apart from the ultra-clean high vacuum analyzer. This means no analyzer contamination and no need to pump away potentially corrosive solvents and buffers using the MS system's expensive vacuum pumps. This alone has made LC-MS coupling exceptionally rugged as well as keeping the operational aspects simple allowing those less skilled in instrumentation but more focused on applications to be successful. This led to a flourishing of bioanalytical applications such as the analysis of a wide variety of biomolecules including biopolymers like polysaccharides, DNA/RNA, proteins/peptides and a plethora of heretofore intractable small molecule analytes such as sphingolipids, phospholipids, acylcarnitines, amino acids, biogenic amines, nucleotides, saccharides, polar and ionic pharmaceuticals, natural and exogenous metabolites, etc.

API techniques are defined as “soft” ionization methods producing primarily intact molecular or pseudo-molecular ions, i.e. ions are created without fragmentation. This presents a challenge in positively identifying compounds: one must subsequently employ tandem mass spectrometry (MS/MS) or high resolution/high mass accuracies or both to positively identify ionized species. Most clinical diagnostic applications today utilize MS/MS to identify and/or quantify trace analytes in complex biological matrices such as plasma and urine. Excellent review articles on this topic have recently been written by Dooley ³, Vogeser & Seger ⁴, and Shushan ⁵.

The most successful and widespread use of MS/MS in clinical diagnosis is in the area of newborn screening for congenital disorders such as amino acidopathies, fatty acid oxidation disorders and organic acidurias employing close to a thousand instruments worldwide ⁶⁻⁹. Other popular applications in the clinical diagnostic field are: the therapeutic drug monitoring (TDM) of cocktail therapies such as anti-viral treatments ¹⁰⁻¹¹ or immunosuppressants ¹²⁻¹⁴ or anti-cancer chemotherapies ¹⁵; the analysis of endogenous steroid hormone panels ¹⁶; the determination of peptide-based hormones especially where different isoforms are involved; and the screening and confirmation of drugs-of-abuse and toxicants ¹⁷⁻¹⁸. With respect to steroid analysis there has been an especially rapid adoption of MS/MS since there are now well documented cases of the superiority of LC-MS/MS assays versus immunoassays ¹⁹⁻²¹. The evidence of this superiority has led the American Endocrinology Society to issue a statement endorsing the use of LC-MS/MS for the measurement of low levels of endogenous steroids, such as testosterone in children and women, over traditional methods like immunoassays ²². There is a great deal of well founded interest in the application of MS/MS to endocrinology and the reader is also referred to a recent review article on this subject ²³.

In spite of the rapid advances made in the application of LC-MS/MS to clinical assays there are relatively few instruments employed in routine diagnostic labs compared to the traditional clinical analyzer systems which are based upon biochemical- and immunoassays. The advantages of LC-MS/MS are many including: no costly analyte specific reagents (ASRs); the ability to determine many analytes in a single run with the same low cost of analysis whether one or many analytes are determined; high specificity and sensitivity especially for small-molecule analytes in comparison to immunoassays; and relatively rapid assay development amenable to “homebrew”. There are however, significant disadvantages including: there are some classes of compounds, such as proteins, for which LC-MS/MS is not as sensitive as immunoassays; LC-MS/MS systems are complicated pieces of technology which require a great deal of training and skilled operators; the high capital cost of these instruments usually with no “reagent-rental” purchase options (no ASR’s); and finally, there is often a significant amount of pre-analytical sample treatment required frequently requiring external robotic liquid-handling systems. The above disadvantages are responsible for the relatively small uptake of LC-MS/MS into routine clinical diagnostic laboratories especially the latter where technicians are more used to simply loading instruments with samples without the requirement for sample pre-treatment.

2. Sample preparation

Perhaps the most important facet of using mass spectrometers for clinical applications is the sample preparation procedure. Dealing with biological matrices presents many unique

challenges to performing mass spectrometry and, in particular, using liquid chromatography in conjunction with MS. Consideration has to be made for matrix interferences that produce undesired signals in the channels being monitored as well as dealing with ion suppression effects. Matrix interferences can take the form of isobars that have the same molecular weight and similar (but not identical) structures (e.g. steroids with the same molecular weight), structural isomers of the same compound (i.e. 3-epi-25-hydroxy vitamin D and α -25-hydroxy vitamin D), fragmentation of metabolites back to the starting compound in the source (i.e. glucuronides fragmenting back to the hydroxyl precursor), source fragmentation of substrates to products (i.e. enzymatic profiling), or endogenous background materials that produce ions at the same masses as the compounds of interest. Ion suppression usually is from compounds that behave like a detergent or a surfactant such as endogenous fatty acids or formulation components like PEG or Tween. However; the suppression caused by the large amount of endogenous proteins or phospholipids found in the biological matrix are of primary concern. When analyzing small molecules (>1000 Da) by mass spectrometry the goal of the sample preparation is to remove as much of the proteins and lipids as possible. For large molecule analysis, such as proteins, isolating and purifying the peptide or protein usually must be done during sample preparation, and if not, lengthy chromatography must be used to separate the large number of compounds present in the biological matrix.

2.1 Off-line methods

Sample dilution to reduce the concentration of salts and endogenous materials is the simplest, but least efficient, method of sample clean-up. However, urine and saliva are relatively clean (compared to other biological matrices) and often the concentration of the analyte of interest is high enough ($\mu\text{g/mL}$) that by simply diluting the sample by a factor of 10 the matrix interferences are minimized enough that no further work-up is necessary.

Protein precipitation is also very straight forward and easy to perform. In this case, an organic solvent or pH change is used that causes the proteins to denature and become insolvent. The proteins precipitate out of solution and can either be filtered out of the “crashed” solutions or are centrifuged and the supernatant is removed for analysis. If an organic protein denaturant is chosen, it must be miscible in water. Protein precipitation is used on all biological matrices including whole blood, plasma and tissue homogenates. The key to whether a protein precipitation alone will work is the solubility of the compounds in the organic crashing solution at the pH chosen. Extremely hydrophilic compounds may precipitate due to a lack of solubility. The most common solvents are methanol or acetonitrile. Chilling the solvent will produce a more thorough clean-up. Typically at least a five to one ratio of organic solvent to matrix is required to “crash” the endogenous proteins, but 8 or 10 to 1 is preferred. Concentration of the sample can be done if the supernatant is dried and reconstituted in a smaller volume than the original sample. Another advantage of protein precipitation is that protein binding is destroyed when the proteins denature, so as long as the total fraction of the compound of interest is being measured, one does not need to worry about binding issues. If the free fraction is of interest, a different sample preparation method should be performed. The protein precipitation also destroys the enzymes present in the matrix, so for compounds that are susceptible to degradation due to enzymatic activity in the sample, protein precipitation will help with the overall stability of system.

Often the free or un-bound fraction of an analyte is desired. In this case one wants the compound bound to proteins to remain there (e.g. the analysis of free T4). Equilibrium dialysis or ultrafiltration is normally employed to measure the free fraction of an analyte. However, these methods can also be used to clean-up a sample since only molecules below a certain size can transport through the membranes. The majority of the biological matrix is usually large proteins that can not cross the membrane barriers and therefore the sample is cleaned for MS analysis.

Liquid-liquid extraction (LLE) is based on partitioning of an analyte between two liquids that are immiscible. Typically, an organic solvent that is not miscible in water is chosen where the compound of interest has a high solubility. In this way, the compounds of interest are extracted from the aqueous matrix because they prefer to be in the organic layer of the mixture. The organic layer is then separated from the aqueous layer and can either be directly injected or dried leaving the compound of interest behind. Samples that are dried can then be derivatized or reconstituted as needed for analysis. The samples can also be concentrated if the final reconstituted volume is less than the starting sample volume. LLE is also very good at removing salts since they prefer to stay in the aqueous phase. The disadvantage of LLE is that it is labor intensive and has many steps that can introduce experimental error. The process has been made more palatable with the advent of automated liquid handling systems, but there is a lot of hazardous waste material generated and there is a significant cost associated with all the disposable materials used in the process.

Solid-phase extraction (SPE) works in a similar fashion to LLE but the partitioning is between a solid and a liquid phase. SPE applies the same basic principles used in chromatography. Analytes of interest are absorbed to the solid phase during the clean-up step, which is usually under aqueous conditions. Under the right conditions, most of the matrix components will not be absorbed during the cleaning step and the analyte of interest is thus removed from the matrix. However, compounds with similar chemical properties to the analyte of interest are retained as well. Samples are then released or eluted from the solid phase with an organic solvent. The combination of the right pH and organic content can make the eluted solvent clean of all but a few relatively similar components, of which the analyte of interest is one. The process is completely automatable and has significantly less waste materials and cost than LLE. Once again, the samples can be directly injected or dried. Samples that are dried can then be derivatized or reconstituted as needed for analysis. The samples can also be concentrated if the final reconstituted volume is less than the starting sample volume and the process removes the salts from the sample.

2.2 On-line methods

Solid-phase extraction methods have also been developed on-line. In the majority of cases these methods employ two columns run in tandem, though single column methods exist. For two column approaches, the first column is an SPE column and the second is a normal HPLC column. Matrices are injected onto the HPLC system and samples are cleaned-up on-line because the analyte is retained on the SPE column while the unretained material is washed to waste. Once the analyte of interest has been extracted from the sample matrix, the analyte is eluted to a second analytical column for analysis. The process is done with either disposal cartridges that are changed after each sample injection, or with columns that are cleaned between each injection by the mobile phases. In both cases the on-line methods have the advantage of direct injection and elution of the analyte, which removes the time consuming off-line steps of evaporation, reconstitution, and preparation. Therefore, on-line

methods are more efficient, fully automated and require far less consumables than their off-line counterparts. An example of a two column on-line SPE configuration is shown in Figure 1. Isocratic focusing is often used to gain better peak shape with dual column methods. In this case the percent organic used to elute sample from the clean-up column is teed into a aqueous LC flow from a second pump to reduce the amount of organic the analytical column sees during the transfer step. The samples are focused at the head of the analytical column by the isocratic aqueous make up flow from the second pump making hydrophilic compounds easier to capture. Figure 2 illustrates the configuration used for “isocratic focusing”.

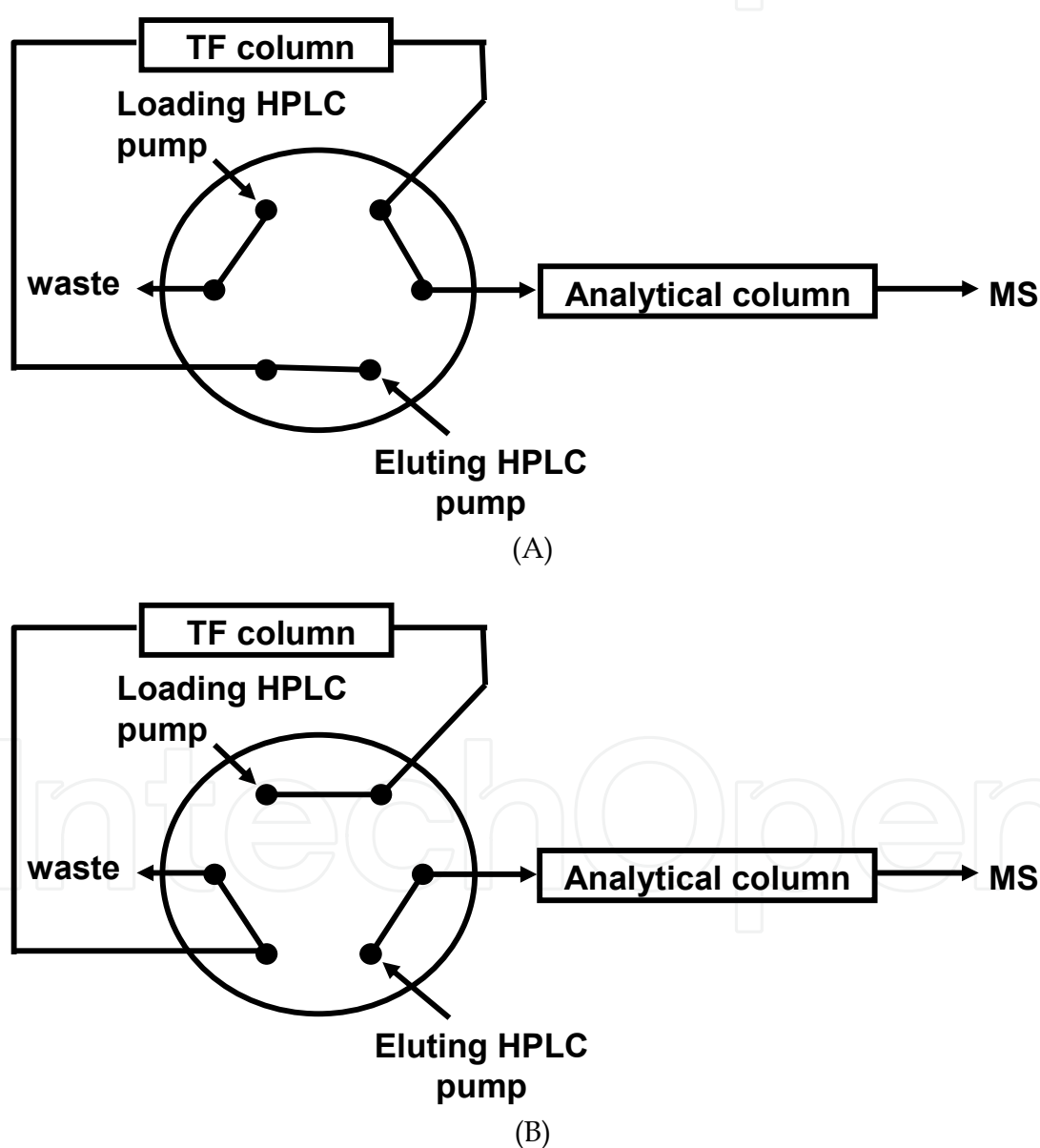


Fig. 1. Dual Column Method Configuration (A) Sample load and Clean-up (B) Sample Elute ²⁵.

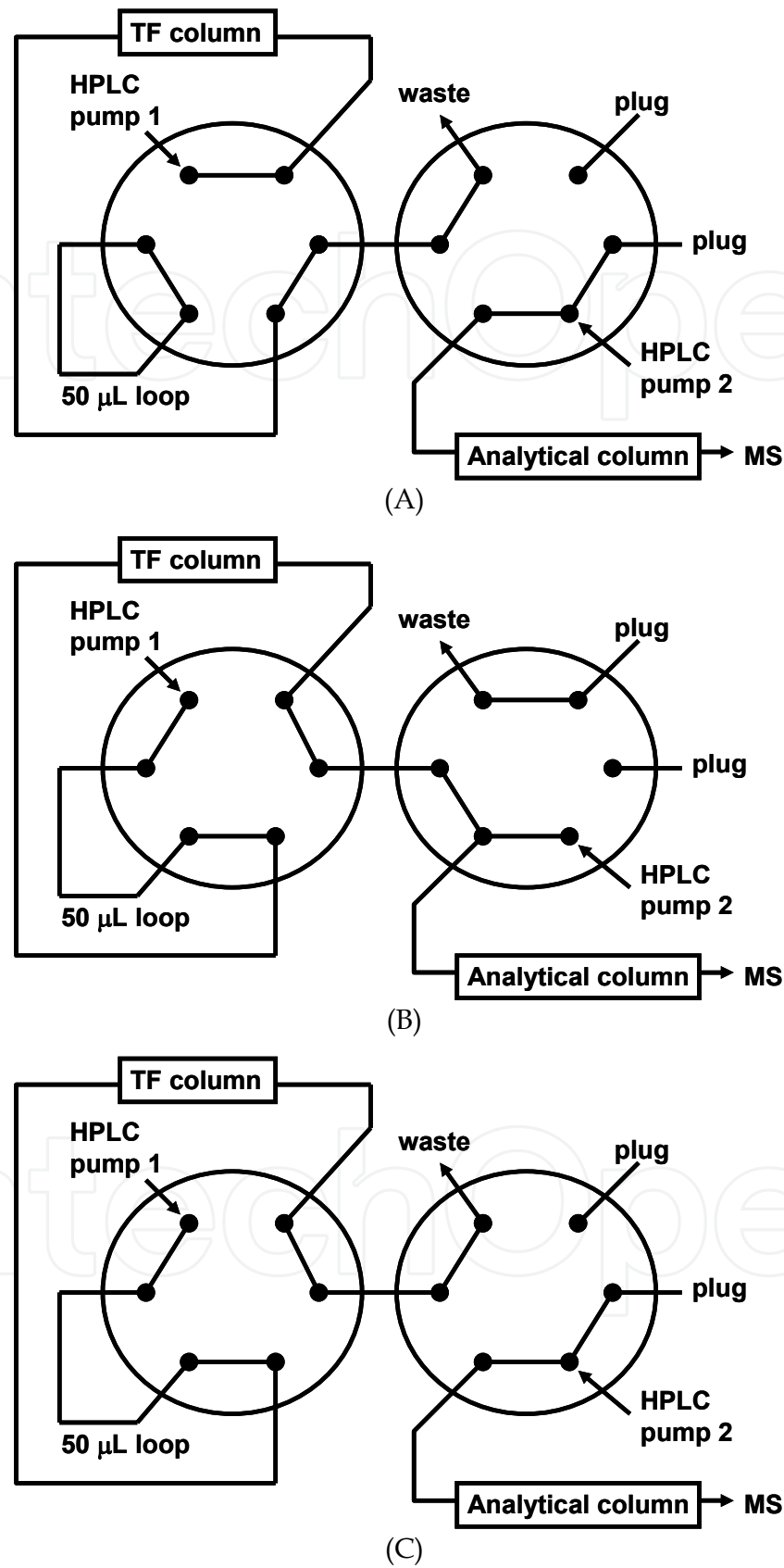


Fig. 2. Isocratic Focusing Method Configuration (A) Sample Clean-up (B) Sample Transfer (C) Sample Elute ²⁵.

Turbulent flow chromatography (TFC) is a second on-line methodology that is performed in a similar manner to SPE. The difference is in how the samples are separated from the matrix. Unlike traditional laminar flow HPLC systems, where the interaction between the stationary phase and the analytes is diffusion controlled, mass transfer is the primary mechanism of separation for turbulent flow. Since the diffusivity of a molecule is inversely proportional to its molecular weight, small molecules have high diffusivity and ready transport into the pores of a packed column under turbulent flow conditions. Large molecules, such as proteins, have low diffusivity and do not have time to enter the pores under turbulent flow conditions. The result is that large molecules are swept away by the turbulent flow while small molecules bind to the stationary phase inside the pores of the packed columns. Traditionally it was necessary to use high flow rates to achieve turbulent flow (4-5 mL/min), but 0.5 mm ID TurboFlow® columns are available that reach turbulent velocities at 1-1.5 mL/min. The molecular weight of the molecules excluded from the stationary phase can be adjusted by flow velocity. Figure 3 illustrates the effect of flow rate on the molecular weight exclusion using TFC²⁴. Since the primary matrix interferences are due to matrix proteins, separation of proteins in the biological matrix from the analyte of interest is the primary goal of online sample clean-up methods. In fact, when reading the literature on SPE it is often found that much better results are obtained at high flow rates (2-4 mL/min). This author believes that the higher flow rates achieve turbulent flow in the SPE column, even though they were not designed to do so, resulting in a far better mechanism for clean-up than the SPE partitioning alone. A comparison of the effects of ion suppression from protein precipitation, SPE and TFC is shown in Figure 4²⁵.

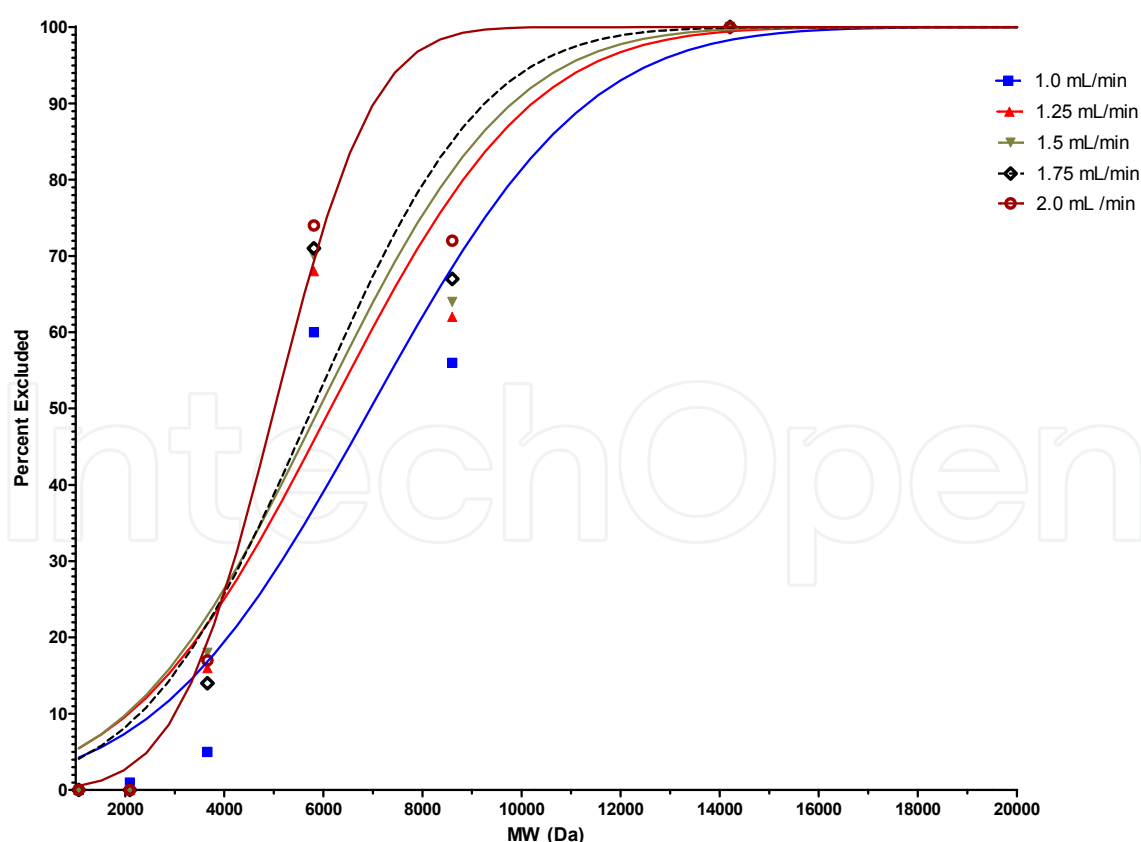


Fig. 3. Comparison of Molecular Weight Exclusion of Proteins by Turbulent Flow Chromatography as a Function of HPLC Flow Rate²⁴.

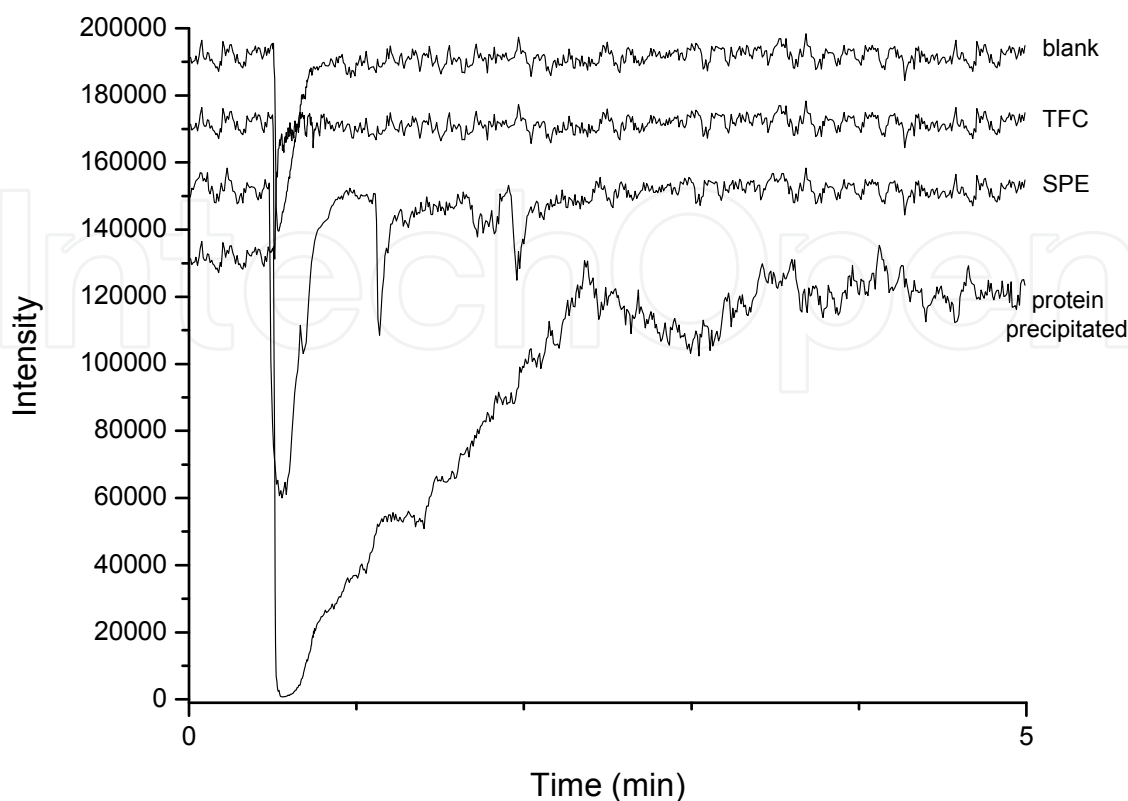


Fig. 4. Ion Suppression Effects from, Blank Injection (50/50 methanol:water), Injection of Protein Precipitated Rat Plasma (4:1 methanol: plasma, centrifuged), Injection of Extracted Rat Plasma in 50/50 methanol water, and TFC of Neat Rat Plasma ²⁵.

Another advantage of the two column methods is that they allow the use of multiplexing, which takes advantage of the time spent cleaning one sample to elute another. Two or more injections are staggered in time and can either have a selection valve that can divert multiple HPLC systems to a single MS, or has two clean-up columns where one is eluting to waste (clean-up) while the other is eluting either directly to the MS or to the MS through an analytical column (analysis). Figure 5 illustrates how multiple injections can be staggered in time to increase sample throughput. Samples are eluted to the mass spectrometer only during the part of the run when the analyte of interest is being eluted. During the HPLC method, when the samples are being cleaned, columns are being washed, or the systems are being equilibrated, flows from other streams are directed to the MS taking advantage of the time needed to perform these functions while not wasting any time on the mass spectrometer acquiring data when there is no analyte of interest eluting.

Restricted access media (RAM) is a third on-line column approach to sample clean-up that can handle neat biological matrices. The particles packed into a RAM column are designed to restrict the access of large macromolecules to the adsorption sites of the stationary phase by coating the normal HPLC packing materials with a second bonded phase that allows small molecules through but repels or excludes large molecules. The cleaning of the sample from the matrix is similar to the mechanism of TFC but accomplished in a different way under laminar flow velocities. However, since the barrier is physical rather than kinetic, RAM columns tend not to last as long as other on-line columns because the restricting layer

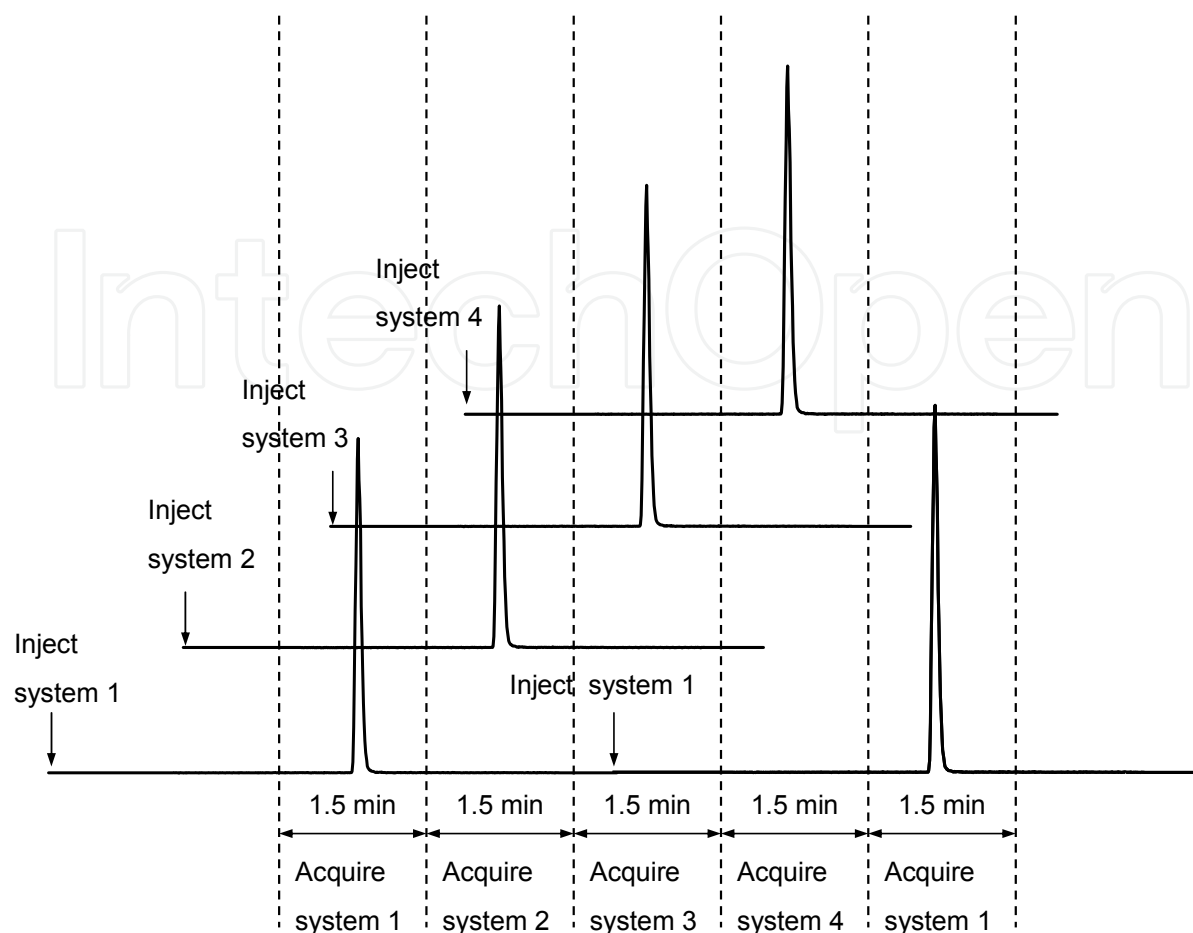


Fig. 5. Staggered multiple inlet methodology (Multiplexing) of several HPLC systems to a single mass spectrometer.

gets clogged over time. A comparison of TFC, SPE and RAM as a function of molecular weight exclusion of proteins is shown in Figure 6²⁴.

Immunoaffinity extraction (IAE) uses antibody-antigen interactions to capture analytes with very high selectivity. Antibodies are immobilized onto the stationary phase of an LC column to effect the removal of the sample matrix while leaving the antigen behind. The antigen is then released from the column for analysis. Often these antibodies will interact with a class of compounds rather than only a specific analyte. However, the cross reactivity of similar compounds are much easier to analyze than the original matrix. One of the primary drawbacks to clinical analyzers that do not employ LC/MS/MS is that the detectors can not separate the interferences from the cross-reactivity. The added dimension of separating analytes by their molecular weight allows MS to distinguish compounds within the same class from one another greatly improving the accuracy of the measurement. IAE can also be performed off-line similar to the way SPE is done both off-line and on-line. A problem with IAE columns is that they tend not to have long life times and are quite expensive.

2.3 Derivatization

Often it is desirable to derivatize an analyte in order to improve the sensitivity or assay performance. In GC/MS or LC/MS, derivatization is used primarily to improve the

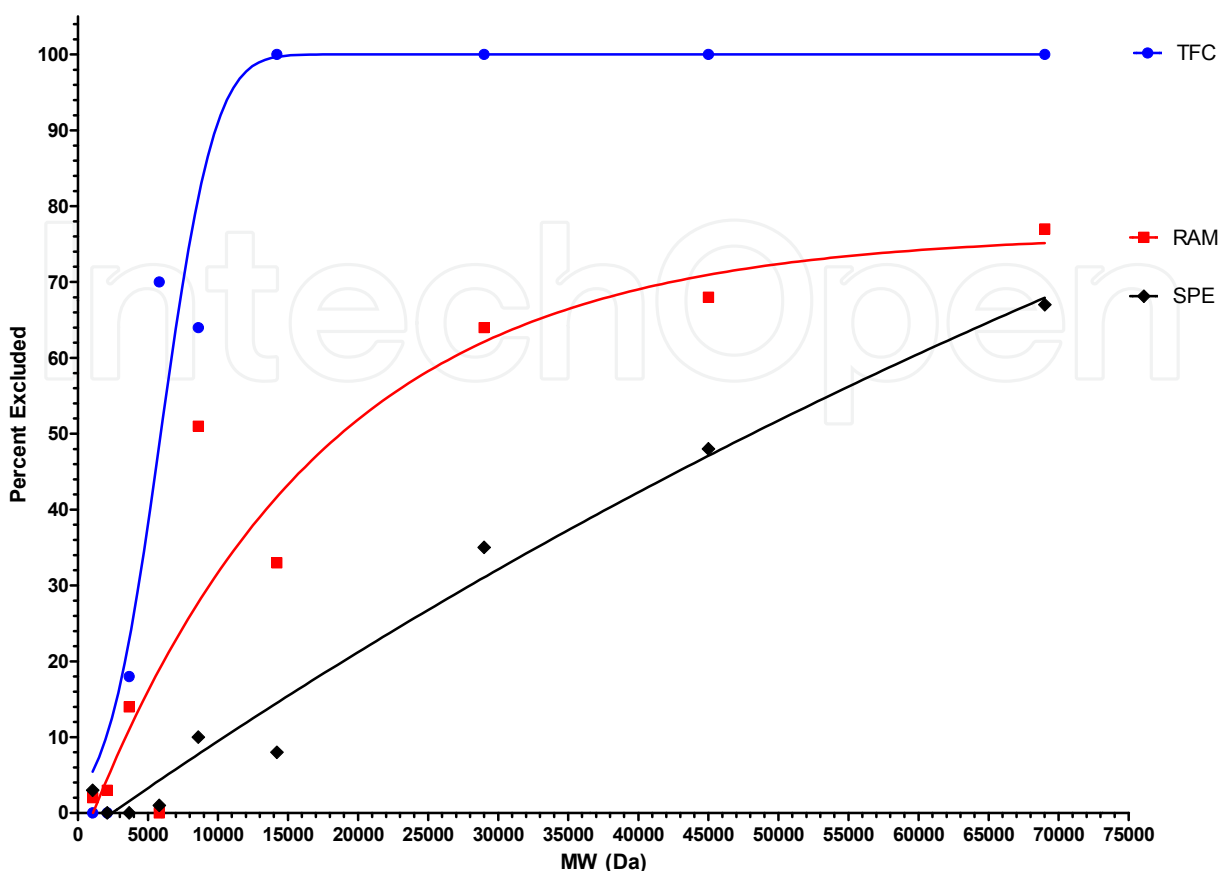


Fig. 6. Comparison of the Molecular Weight Exclusion of Proteins at 1.5 mL/min Flow Rates for Turbulent Flow Chromatography (TFC), Restricted Access Media (RAM), and Solid Phase Extraction (SPE) Clean-up Columns.

chromatographic separation or increase the ionizability of an analyte. Organic acids tend to give broad diffuse peaks by HPLC due to ionic interactions with the stationary phase. Low molecular weight compounds that are very hydrophilic, like amino acids, tend not to be retained on most reverse phased HPLC systems. Converting the acids to an ester not only removes the ionic interactions of the acids but also makes the compounds more hydrophobic resulting in stronger retention. The most common derivatization for both organic acids and amino acids is to form the butyl-ester by reaction with acidic butanol²⁶⁻²⁸. The enhanced chromatography results in better peak shape and better sensitivity. The sensitivity increases because the peaks are narrower, and the analytes elute with more organic in the mobile phase, which promotes desolvation in the source. In other cases, the chromatography is good but the compound just doesn't ionize well. Adding a functional group that promotes ionization can dramatically increase sensitivity. Vitamin D is often derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to increase sensitivity²⁹⁻³⁰. Drugs of abuse screening is usually preformed by hydrolyzing the samples. The hydrolysis converts all phase two metabolites back to their precursors increasing the likelihood of detecting small concentrations by summing the signals from several sources of the drug into one signal.

The majority of the derivatization preformed in a clinical laboratory is done off-line, is labor intensive, and is time consuming. However, automated liquid handling system can be used

to automate the derivatization. If fast kinetics exists, the derivatization can be performed on-line. One issue with derivatization is determining how complete the reaction is and what affect that has on what is being measured.

3. Clinical diagnostics

3.1 Vitamin D

Vitamin D deficiency has been linked to several skeletal disease conditions³¹⁻³² prompting an increase in laboratory testing of serum vitamin D levels over the last several years. The Mayo Clinic has reported increases in vitamin D testing of over 80% per year³³. In fact, Vitamin D analysis is easily the most frequently used LC/MS/MS assay in the clinical laboratory today.

In order to understand vitamin D analysis, it is important to distinguish the various analogs of vitamin D that are analyzed and their relationship and functions, since different laboratories perform the analysis by different methodologies. Figure 7 illustrates the nomenclature for the physiologically relevant vitamin D analogs. Both vitamin D₃ and vitamin D₂ are active, and while both can be absorbed from diet, only vitamin D₃ can be made in vitro from exposure to UV light. The active form of vitamin D is the 1,25-hydroxyvitamin D metabolite, but historically it has been difficult to measure. Therefore, the primary measurement in the clinically laboratory has been the 25-hydroxyvitamin D metabolites because they have higher circulating serum levels and much longer half lives.

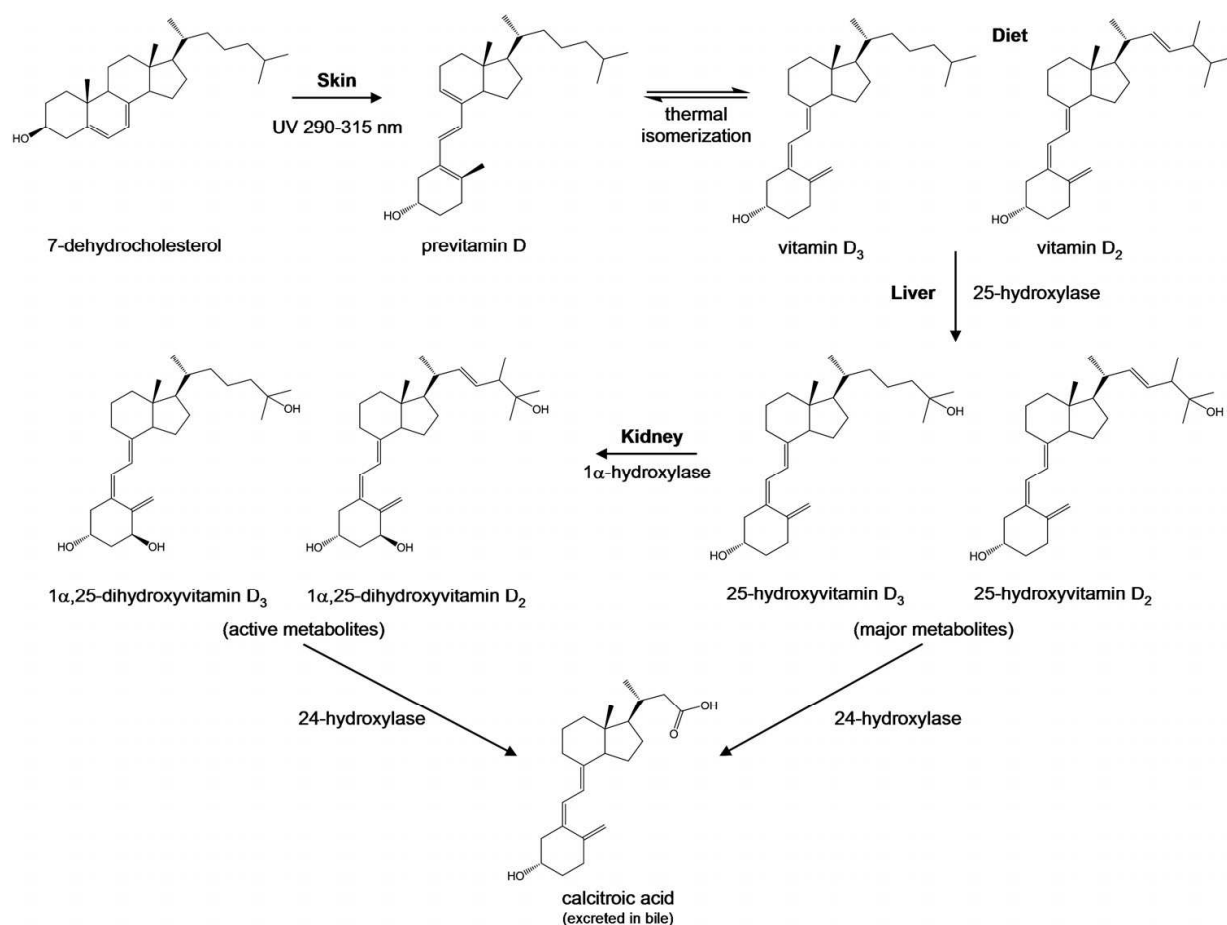


Fig. 7. Vitamin D nomenclature.

Vitamin D₃ (cholecalciferol) is endogenously produced from 7-dehydrocholesterol in the skin from exposure to UV radiation at 290-315 nm. A pre-vitamin D precursor is formed that rapidly isomerizes to Vitamin D₃. Significant variability in the amount of vitamin D₃ produced in this manner is observed because of differences in sun exposure due to climate and/or social behavior³⁴⁻³⁵. Both vitamin D₂ (ergocalciferol) and vitamin D₃ are present in food such that diet becomes a significant factor in disease on-set and prevention. Dietary supplements for both vitamin D₂ and D₃ are available as well.

To become physiologically active, both vitamin D₂ and D₃ must be metabolized. While there are several inactive metabolites, it is the 1 α ,25-dihydroxyvitamin D that is active with the D₃ analog having about twice the activity of the D₂ analog. To form 1 α ,25-dihydroxyvitamin D, vitamin D is first metabolized to 25-hydroxyvitamin D by the liver. The kidney then metabolizes the 25-hydroxyvitamin D to 1 α ,25-dihydroxyvitamin D. Regardless of which form of Vitamin D is present, most of the circulating Vitamin D is bound to vitamin D binding protein (DBP).

Clinically, the total amount of 25-hydroxyvitamin D₂ and D₃ is usually reported. There are several reasons both analytically and physiologically for evaluating the 25-hydroxy metabolites. First is that both vitamin D₂ and D₃ have short half-life times ($t_{1/2}$) in circulating blood of 24 hours so their concentrations are greatly influenced by recent sun exposure and diet. Therefore, someone who is vitamin D deficient that just happens to go out in the sun for few hours right before their test would appear to be okay. The 25-hydroxyvitamin Ds have $t_{1/2}$ of 3 weeks and; therefore, are much more representative of the individuals overall vitamin D levels. Several authors have made the case that only the 1,25-hydroxyvitamin D levels are important since that is the active moiety. There is laboratory testing available for 1,25-hydroxyvitamin D but the analysis is more difficult, requires larger sample volumes, the $t_{1/2}$ is only a few hours (though exposure to sun light and diet are no longer regulating factors), and the complexity of the sample preparation takes more time and resources making the assay less cost effective. However, for certain indications the analysis 1,25-hydroxyvitamin D is clearly more useful, such as patients with chronic renal failure, vitamin D dependent rickets, 1,25-hydroxyvitamin D intoxication, lymphoma, and hyper or hypo parathyroidism³³.

Historically 25-hydroxy vitamin D levels were measured using immunoassays. Immunoassays are unable to distinguish between 25-hydroxy vitamin D₂ and D₃ such that only the total 25-hydroxy vitamin level is reported. The LC/MS methodologies are able to measure the levels of 25-hydroxy vitamin D₂ and D₃ separately so the contribution of each to the total can be determined, which is useful in diagnosing the effectiveness of supplemental treatment of vitamin D₂ to patients that are vitamin D deficient. In addition, there are problems with protein binding in the immunoassays that produce results that are lower than the corresponding LC/MS assays³⁶⁻³⁷. There is also some controversy as to whether immunoassays give accurate results due to nonspecific binding of other vitamin D metabolites and or matrix effects³⁸⁻⁴⁰. Many experts consider LC/MS to be the most accurate method for vitamin D analysis^{39, 41} but there is a lot of resistance to changing reference levels that were established by the previous techniques.

Early attempts to run 25(OH) vitamin D by mass spectrometry was challenging due to the fact that it is lipophilic, which makes it's difficult to ionize by API methodologies. Furthermore, fragmentation is also difficult with the loss of water being the only ion formed easily. Other fragment ions are formed at high collision energies; however, under these conditions, multiple fragment ions are formed such that no one particular ion is formed

with any reasonable abundance. Early attempts to overcome the low ionizability of 25(OH) vitamin Ds involved various types of derivatization. Vreeken et al ⁴² and others ⁴³⁻⁴⁵ used a Diels Alder reaction to enhance sensitivity in API of 25(OH) vitamin D. The method also increased the mass of the 25(OH) vitamin Ds resulting in lower background interferences. Hiagashi et al ⁴³⁻⁴⁴ used a Cookson-type reagent to produce a 15 fold increase in the sensitivity over the native form for measuring vitamin D in plasma. However, these derivatization methods are time consuming and cumbersome. Improvements in the sensitivity of mass spectrometers in recent years have made it unnecessary to derivatize vitamin D in order to achieve the desired sensitivity.

Once the mass spectrometers became sensitive enough to measure vitamin D at the concentration required for clinical analysis the main obstacle to LC/MS/MS becoming a routine method for clinic diagnostics was making it high throughput. Vogeser et al ⁴⁶ reported a rapid LC/MS/MS method in serum employing on-line solid phase extraction (SPE) with a run time of 9 minutes. Chen et al ⁴⁷ improved upon this method and reduced the total run time to 7 minutes. Knox et al ⁴⁸ used protein precipitation followed by off-line SPE to run 160 samples per day. The off-line methods produce much faster LC/MS/MS run times but require more preparation in advance. However, these methods can be automated making them an attractive alternate. Hojskov et al ⁴⁹ used protein precipitation followed by liquid/liquid extraction to get the total run times down to 4 minutes. However, the biggest improvement in sample throughput was realized by combining on-line sample clean-up with multiplexing capabilities. Taylor et al ⁵⁰ demonstrated the use of on-line clean-up using turbulent flow chromatography with a Cyclone column and a Cohesive Technologies (now part of ThermoFisher Scientific) TX4 multiplexing system to get a sample throughput of 40 samples per hour. Turbulent Flow allows fast and efficient on-line clean-up of biological sample while multiplexing allows coupling of multiple LC systems to a single mass spectrometer, greatly reducing time between injections. Several other authors have reported increased sensitivity in addition to increased sample throughput using TX2 or TX4 on-line multiplexing systems ⁵¹. Vitamin D analyses on these systems are routinely running injection to injection times of one minute (60 samples per hour). Table 1 summarizes the current LC/MS/MS vitamin D assays found in the literature.

3.2 Immunosuppressant drug monitoring

Therapeutic drug monitoring of immunosuppressant drugs (ISDs) is well established ⁵⁶. The importance of monitoring these drugs is due to their narrow therapeutic window. Elevated dosing of the immunosuppressant drugs can cause significant toxicity while under dosing can result in transplant rejection. Because of this narrow therapeutic window, the immunosuppressant drugs are considered critical dose drugs, requiring individualized drug therapy by measuring the actual drug concentrations in each patient to maximize the therapeutic response and minimize adverse side effects.

Currently there are two main choices in the clinical laboratory for monitoring immunosuppressant drugs; immunoassay or chromatography. However, issues with non-specific binding of the antibody resulting in over estimation with immunoassays ⁵⁷⁻⁶⁰ as well as the long sample run times, complicated sample preparation procedures and lack of sufficient chromophores with HPLC-UV detection ⁶¹⁻⁶³, have made LC/MS/MS methodologies the assay of choice when available. Most of the large-scale Contract Research Organizations (CROs), which operate as central laboratories for clinical diagnostics, analyze immunosuppressants by LC/MS/MS ^{57, 64}.

Reference	Analyte	Matrix	PPT	Sample Prep		Run time	date
				Extraction	Derivatization		
Higashi (43)	25(OH)D2 and 25(OH)D3	human plasma	ACN	LLE (AcOEt)	DMEQTAD	7 min	2001
Higashi (45)	25(OH)D2 and 25(OH)D3	human plasma	ACN	LLE (AcOEt)	NPTAD	7 min	2003
Vogeser (46)	25(OH)D3	human serum	ACN	on-line SPE		9 min	2004
Singh (52)	25(OH)D2 and 25(OH)D3	human serum	ACN	TFC		7 min	2006
Chen (47)	25(OH)D2 and 25(OH)D3	human serum	ACN	on-line SPE	PTAD	7 min	2008
Knox (48)	25(OH)D2 and 25(OH)D3	human serum/plasma	MeOH	off-line SPE		5 min	2009
Bunch (51)	25(OH)D2 and 25(OH)D3	human serum	ACN	TFC		3 min	2009
Newman (53)	25(OH)D2 and 25(OH)D3	dried blood spots	MeOH	LLE (hexane)		10 min	2009
Eyles (54)	25(OH)D2 and 25(OH)D3	dried blood spots	ACN			3 min	2009
Hojskov (49)	25(OH)D2 and 25(OH)D3	human serum	ACN	LLE (heptane)		4 min	2010
Casetta (55)	1,25(OH) ₂ D3	human serum	ACN	on-line SPE		18 min	2010

Table 1. Selected Summary of LC/MS/MS Vitamin D Methods.

The four primary immunosuppressant drugs analyzed today are Sirolimus, Tacrolimus, Everolimus and Cyclosporin A. Clinical Assay kits are available from several vendors for the analysis of these immunosuppressants. These Kits include all the controls, calibrators, sample preparation reagents, internal standards, columns and all the necessary instructions to perform the analysis. Table 2 summarizes the performance of the currently available assay kits for LC/MS/MS analysis of ISD’s.

Drug	Linear Range
Cyclosporin A	10-2000
Tacrolimus	1-50
Everolimus	1-50
Sirolimus	1-50

Table 2. A Summary of the Current Analytical Ranges for Immunosuppressant Drugs CV/IVD Kits.

Both electrospray ionization (ESI) and atmospheric chemical ionization (ApCI) are used to analyze the immunosuppressants. Usually the precursor ion is not the protonated molecular ion (M+H)⁺ but is formed from the ammonium adduct (M+NH₄)⁺ produced by having ammonium formate or ammonium acetate in the HPLC mobile phases ⁶⁵. The use of negative ions has also been reported for cyclosporine A and tacrolimus ⁶⁶. ApCI has the advantage of producing fewer matrix effects but sensitivity is reduced due to more fragmentation in the source. However, most mass spectrometers available today have more than enough sensitivity to measure the ISD’s using either ionization method. The chromatography of cyclosporine A normally requires elevated temperatures be used on the columns to produce good peak shape. Column temperatures are normally between 60-75°C.

Initial LC/MS/MS methods for the ISDs used off-line liquid-liquid extraction for clean-up or solid phase extraction (SPE) to isolate the drugs from the plasma ⁶⁷⁻⁶⁹. Off-line methods are rather tedious, involve many manual steps that each can be a source of error, and take time. Automated liquid handling systems are available to reduce the tedium and operator errors but most laboratories have switched to on-line sample preparation processes. The on-line methods usually follow protein precipitation and are of two general types: two-dimensional chromatography using turbulent flow or on-line SPE columns for matrix removal followed by a reverse phase HPLC analytical column to get good chromatographic peak shape ⁶⁹; or direct injections that rely on the analytical column to effect suitable chromatographic separation ⁷⁰⁻⁷¹. Sample run times using two-dimensional chromatography with as little as 1 minute per sample run times are achievable when combined with multiplexing systems ⁶⁹. Whole blood analysis is the most common method used due to the high protein binding of the ISDs. Cell lyses and denaturing of the proteins to release the bound drug is accomplished by using an organic such as methanol or acetonitrile in combination with zinc sulfate ⁶⁷⁻⁷².

The importance of choosing the right internal standards has been demonstrated in the literature ⁷³. The availability of stable labeled isotope internal standards is critical because the ISDs are known to be more susceptible to matrix effects ⁷³⁻⁷⁵ and co-elute with many of the commonly found phospholipids. A study on cyclosporine by Taylor et al ⁷⁴ clearly demonstrated that the isotope labeled cyclosporine had better analytical performance than any other of the analogs of cyclosporine that were tested.

3.3 Steroids

Steroid analysis is used for diagnosing several endocrine disorders. Traditionally the measurement of steroids was accomplished with immunoassays and radioimmunoassay. However, as stated previously, recent studies have shown the use of immunoassays is problematic ⁷⁶⁻⁷⁹ suffering from a lack of specificity, limited dynamic range and matrix effects. In fact the use of immunoassays has resulted in poor clinical correlation of the test results and substantial disagreement between different manufacturers of the assay kits ^{22, 76-80}.

The first successful use of mass spectrometry to steroid analysis in the clinical laboratory was achieved with GC/MS ⁸⁰⁻⁸². Significant improvement in steroid measurement was made because of the high resolution separation capabilities of capillary gas chromatography coupled to the high specificity of mass spectrometer. However, in order to get the required sensitivity for steroid analysis, low throughput, labor intensive derivatizations, and other sample preparation requirements resulted in GC/MS methodologies not becoming widely used as a routine technique for steroid measurement in the clinical laboratory.

The development of LC/MS/MS over the last 15 years has made it the technique of choice for analyzing steroids because of the high specificity of the mass spectrometer, the separation capabilities of the liquid chromatography, its wide dynamic range, and the availability of simple sample automated preparation procedures. Steroid analysis is commonly performed from serum, plasma, urine and saliva. The choice of sample preparation procedure is somewhat dependent on whether increasing the concentration of the steroid is necessary for detection. If that is required, off-line LLE or SPE is desirable so that the reconstitution volumes are lower than the amount of starting matrix used; alternatively derivatization can be employed to enhance detection. Both these methods

require labor intensive methodologies and significant cost per sample that are not desirable in the clinical setting. All of methods outlined previously are used today including extraction from dried blood spots. Table 3 summarizes the current steroid assays found in the literature.

Aldosterone	113, 81, 119, 120
Androstenedione	81, 83, 96, 88, 117
Androsterone-sulfate	90
Corticosterone	114, 81, 83
Cortisol	108, 112, 114, 81, 116, 83, 88
Cortisone	108, 114, 116, 122
11-deoxycorticosterone	114, 116
11-deoxycortisol	110, 114, 81, 83
21-deoxycortisol	114, 83
DHEA	81, 96, 118
DHEA-sulfate	81, 90
Dihydrotestosterone	83
Epiandrosterone sulfate	90
Estradiol	101, 81, 82, 102, 121
Estriol	82
Estrone	101, 82, 102, 121
17-hydroxypregnenolone	110, 115
17-hydroxyprogesterone	110, 114, 81, 83, 88, 117
Pregnenolone	110
Progesterone	81, 83
Testosterone	109, 111, 81, 82, 83, 96, 117, 118

Table 3. Steroid assay references.

The biggest problem with analyzing steroids by mass spectrometry is that there are isobaric interferences and considerable cross talk due to the similar fragmentation patterns of other endogenous steroids. Figure 8 illustrates the complexity of the various steroid pathways. By looking at the molecular weights, the loss of water (which easily occurs in the source), and the isotopic distributions, it becomes obvious that there is the potential for a large amount of cross talk between the steroids in the mass spectrometer. Table 4 lists the possible cross talk channels between the steroids. Therefore, the use of chromatography becomes critical to the unambiguous measurement of the various steroids.

Another issue with steroid analysis is protein binding. When the total amount of steroid present is desired simple techniques like protein precipitation easily release the bound steroid. However, if the free fraction measurement is needed, dialysis or ultra-filtration methods are usually used. It is possible to measure the free and total separately if the sensitivity of the method is sufficient to measure the lower levels of the free fraction. Great care must be taken not to release any of the bound protein if the unbound steroid is measured.

Often it is more desirable to measure steroid panels rather than individual steroids. The advantage of steroid panel profiling is not just to simplify the analytical methodology, but monitoring multiple steroid pathways often has clinical relevance ⁸³⁻⁸⁷. Diagnostics for CAH

Steroid	Nominal Mass (Da)	isobars	loss of water interference	loss of two waters interference	loss of acetyl group interference	isotope interference	loss of water isotope interference	loss of two waters isotope interference
cholesterol	386							
cortisol	362					cortisone, aldosterone		
cortisone	360	aldosterone						
aldosterone	360	cortisone						
corticosterone	346	11-deoxycortisol						
11-deoxycortisol	346	corticosterone						
17-OH-pregnenolone	332					17-OH-progesterone, 11-deoxycorticosterone		
11-deoxycorticosterone	330	17-OH-progesterone		cortisol			corticosterone	cortisone, aldosterone
17-OH-progesterone	330	11-deoxycorticosterone		cortisol			corticosterone	cortisone, aldosterone
pregnenolone	316					progesterone	17-OH-pregnenolone	11-deoxycortisol, corticosterone
progesterone	314		17-OH-pregnenolone	11-deoxycortisol, corticosterone			17-OH-progesterone, 11-deoxycorticosterone	
dihydrotestosterone	290				17-OH-pregnenolone	testosterone, estriol, dehydroepiandrosterone		
testosterone	288	estriol, dehydroepiandrosterone			17-OH-progesterone	androstenedione		
estriol	288	testosterone, dehydroepiandrosterone			17-OH-progesterone	androstenedione		
dehydroepiandrosterone	288	testosterone, estriol			17-OH-progesterone	androstenedione		
androstenedione	286					androstanedione		pregnenolone
androstanedione	284			pregnenolone				
estradiol	272				progesterone	estrone	testosterone, estriol, dehydroepiandrosterone	
estrone	270		testosterone, estriol, dehydroepiandrosterone					

Table 4. Mass interferences between the steroids.

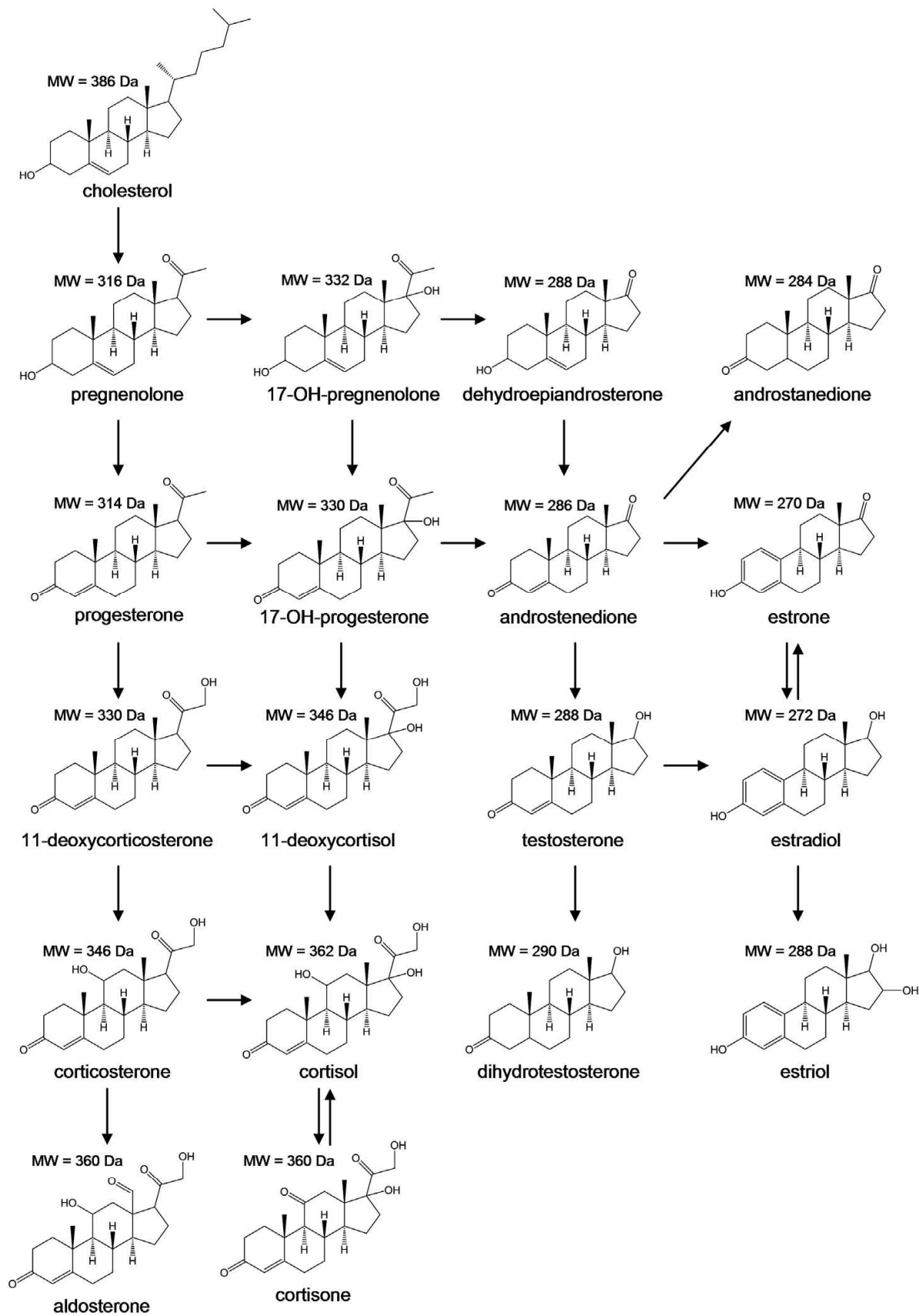


Fig. 8. Complexity of the steroid pathways.

and PCOS, fertility treatment, tumor location, and patient monitoring after gonadal or adrenal surgery are routinely performed by LC/MS/MS⁸⁸⁻⁹⁰.

Measurement of adrenal steroids is important for the differential diagnosis of CAH and evaluation of adrenal function. The CAH panel includes the following steroids; 17OH-progesterone, androstenedione, cortisol, adrenocorticotrophic hormone (ACTH) and pregnenolone. Congenital adrenal hyperplasia is characterized by elevated levels of ACTH, which is the most common disorder associated with the assay. Cross reactivity and poor clinical correlations are observed with immunoassays making agreement with LC/MS/MS methods difficult. Method specificity is also critical to the population being tested and LC/MS/MS has been shown to be much more reliable than other methods especially for newborns, infants and the elderly^{83, 91-92}. LC/MS/MS methods for CAH have much lower false positive rates^{83, 91-92} than the immunoassays and have been adapted to saliva⁹³⁻⁹⁶ and dried blood spots^{91, 97}, which are far less invasive sampling techniques than drawing blood. Measurement of testosterone in men is used to diagnose hypogonadism and to monitor its treatment as well as to monitor androgen suppression therapy during prostate cancer treatment. In women, testosterone levels are markers of alopecia, acne, hirsutism, osteoporosis, tumor screening, late on-set CAH, PCOS, and other endocrine and reproductive diseases⁹⁸. In children, testosterone is analyzed for gender assignment in infants with ambiguous genitalia, delayed onset puberty, and CAH⁸⁸⁻⁹⁰. The analysis of testosterone is also complicated by the need to measure both the free and total testosterone levels.

Immunoassays for testosterone work well for healthy normal men but their lack of specificity makes them unreliable for the low concentrations found in women and children⁷⁶⁻⁷⁸. Methods for testosterone that utilize LC/MS/MS have demonstrated accurate measurement down to 10 pg/mL^{84, 86, 89-100}. Diagnostic methods for other androgens by LC/MS/MS have been reported with similar results including dihydrotestosterone (DHT), androstenedione, and DHEA^{84, 86, 89, 100-102}. Methods for the androgens often require derivatization or large sample volumes. However, recent improvements in mass spectrometer sensitivity have allowed analysis without these requirements.

Low concentration of estradiol in females is associated with disturbed puberty, oligoamenorrhea and menopause^{88, 98}. Estradiol suffers from the same issues discussed previously for steroids in that there are several endogenous and matrix-related interferences. Problems with cross reactivity with immunoassays is a major problem once again, making LC/MS/MS the method of choice analytically. Like testosterone, estradiol does not ionize well and often derivatization is performed with dansyl chloride or an amine containing sulfonyl halide¹⁰³⁻¹⁰⁶. Two dimensional chromatography methods have been able to measure estradiol without the need for derivatization^{85, 105}.

Another important steroid that is analyzed by tandem mass spectroscopy is cortisol. Cortisol measurement is used to diagnose adrenal hyperfunction (Cushing's syndrome) and adrenal insufficiency. Cushing's syndrome results from over expression of cortisol by the adrenal gland and can be caused by pituitary hyperplasia, cancer of the adrenal or pituitary gland, or production of ACTH outside the pituitary gland. Cortisol measurements are done in plasma, serum, saliva and urine depending on the diagnoses.

3.4 Thyroid hormones

Thyroxine (3,3',5,5'-tetraiodo-L-thyronine or T4) and triiodothyroxine (3,3',5-triiodo-L-thyronine or T3) are tyrosine-based hormones produced by the thyroid gland that are essential

for regulation of cell metabolism. Both excess and deficiency of thyroxine can cause clinical disorders. Hyperthyroidism is caused by the over production of T3, T4 or both. The most common example is Graves' disease in which both the T3 and T4 levels are elevated. Hypothyroidism is a result of T3 and T4 deficiency and is related to Hashimoto's disease. Both hyper and hypothyroidism can be caused by diet from either not enough or too much intake of iodine. Many thyroid cancers result in the over or under production of T3 and T4 as well.

T4 is produced in the thyroid gland and is the primary circulating form of thyroid hormone. The T4 is converted to T3 by tissue deiodinases that remove iodine from the 5' position of T4. T3 is the active thyroid hormone. Both T3 and T4 are extremely protein bound (>99%), which makes the measurement of the free amounts difficult. The free hormone is considered the clinically relevant concentration because it measures the amount available to the cells.

Free thyroid hormone measurements require separation of the free hormone from the protein bound hormone. It is critical that the separation does not disturb the endogenous equilibrium^{107, 123}. Equilibrium dialysis (ED) is the preferred method¹²⁴⁻¹²⁶ but ultrafiltration (UF) is also used¹²⁷⁻¹²⁸. ED is labor intensive, time consuming (overnight, 17-24 hrs), technically demanding, and expensive, which make it unattractive to all but the large, well equipped and well staffed clinical laboratories. UF is much less time consuming (30-40 min), easier to use and has better reproducibility, but is also prone to leakage through the UF-membrane¹²⁴.

The use of tandem mass spectrometry following ED or UF has overcome the issues associated with measuring thyroid hormones with immunoassays¹²⁹⁻¹³⁹. The first T3 and T4 assays to use tandem mass spectrometry were developed for GC/MS and isotope dilution^{129-131, 133, 139-142}. These methods required extensive sample clean-up and derivatization. Development of LC/MS methods eliminated the need for derivatization and allowed the introduction of on-line sample clean-up, greatly increasing the ease of use and removing many sources of experimental error¹²⁵.

4. Toxicology

Analytical toxicology is the detection, identification and measurement of drugs, or other foreign compounds (xenobiotics), and their metabolites in biological specimens. Toxicological measurements in the clinical laboratory can be both quantitative and qualitative depending on what question needs to be answered. Therefore, unlike the previous discussions where only quantitative measurements were needed and triple quadrupole MS/MS is the preferred methodology, toxicology screening uses many more types of mass spectrometers on a routine basis. There are several areas of interest for toxicological screening. First is screening of a biological specimen to detect and identify compounds in patients admitted to the hospital with acute intoxication of unknown origin. Intoxication can result from using drugs of abuse, both known and unknown, from accidental exposure to hazardous chemicals in the environment, from bacterial or viral infections, or from disease states that produce toxic compounds in vivo. Second is the screening for illegal drug use of known origins where intoxication has not occurred but the substances are regulated, like steroid use in athletes. In the US, there are also programs to monitor pain management for compliance of prescribed drug use. The latter is mostly a legal issue to identify patients that take advantage of the medical system and to identify doctors that abuse their privileges and prescribe drugs in manners not consistent with medical doctrine. Third is therapeutic drug monitoring (TDM) and occupational/environmental toxicology. There is considerable overlap between all of the toxicological assays.

One issue with the use of SRM transitions is the presence of isobaric interferences. When only one SRM transition is used there may not be enough specificity in the MS to distinguish between isobars. Chromatographic separation of the isobars is often the easiest approach to resolving isobars. However sometimes it is not possible to separate isobars chromatographically or the time taken to do so is not optimal for laboratory throughput needs or the time to result required is too long for an emergency case. Interference during the analysis of tramadol by LC-MS/MS arising from ingestion of the antidepressant venlafaxine is an example of this problem ¹⁴³. In order to overcome isobaric interferences, the use of multiple transitions for a single compound and the ratio between the signal intensities for each transition is used ¹⁴⁴⁻¹⁴⁶. The application of product ion ratios is obviously limited for compounds which do not fragment reproducibly. Interference may also arise from metabolites, or other compounds, which fragment or thermally degrade in-source back to the parent compound. In these cases, chromatographic separation is the only option, but since the metabolites tend to be more hydrophobic than the parent, it is not difficult to achieve. Therefore, to minimize the risk of interference, multiple transitions that avoid non specific transitions such as water loss or low molecular weight fragments should be used whenever possible. For compounds which do not fragment well, or have only one major fragment, interferences must be separated by the chromatography.

In forensic and post-mortem toxicology, systematic toxicological analysis (STA) and general unknown screening (GUS) is the starting point from which further, targeted quantitative analyses follows. Good sensitivity and reliability on as wide a range of compounds as possible is required. For many years GC/MS, despite the problems associated with larger, non-volatile and thermally labile compounds, was considered the best strategy for these analyses. The reproducibility of GC/MS ionization/fragmentation allowed for the development of comprehensive mass spectra libraries for reliable structural identification. There are no equivalent LC/MS spectral libraries due to poor instrument to instrument reproducibility of LC-MS fragmentation. Therefore, in house libraries are needed for LC/MS application of SRM methods ^{17, 147-157}. SRM methods also suffer from the fact that only known compounds can be searched and that a limited number of transitions can be monitored at one time. For these reasons, a move toward full scanning instruments for screening for both known and unknown compounds is more practical ¹⁵⁸⁻¹⁶⁴.

Information or data dependent acquisitions are used to find compounds above a background threshold when acquiring full scan data. Identification is accomplished by triggering MS/MS acquisitions on the precursor ions found above the threshold ¹¹⁶⁵⁻¹⁶⁷. Linear ion traps, orbital traps, Qtraps and QTOF are better suited to full scan acquisitions because they acquire full scan data much faster and with no loss of sensitivity compared to triple quadrupoles. However, once the identity of a compound is known, the triple quad is still the best quantitative instrument such that screening is often done by ion traps but conformation and quantification is usually done by triple quads.

An emerging approach to STA analysis is the use of accurate mass (exact mass, or high-resolution) MS. Full-scan MS experiments with 0.1 mDa accuracy are possible that filter the full-scan data and extract analyte chromatograms with very low background noise. Compounds which have the same nominal mass, but different exact masses, can be resolved by the mass spectrometer ¹⁶⁸⁻¹⁶⁹. TOF-HRMS is of interest in the application of empirical formula-based data libraries, with isotope pattern-matching software, and the potential to screen for unknown compounds (and identify their metabolites) by knowledge of elemental composition alone, without the absolute need for reference material ¹⁷⁰. Exact mass

identification of specific metabolites and systematic fragmentation approaches have shown that even structural isomers can be distinguished using accurate mass ¹⁶⁹. Further, retrospective interrogation of full-scan data can be useful to investigate the presence of new compounds/metabolites (such as new 'designer drugs') without re-analysis. Newer Orbitrap®/Exactive™ technology (ThermoFisher Scientific), also capable of HRMS, is finding toxicologically relevant applications ¹⁷¹⁻¹⁷³. "In house" library matching should be carried out for unequivocal compound identification because mass spectral libraries and compound databases may not be completely transferable between instruments. Furthermore, library data matching does not give any information about chromatographic retention time, which is just as important as the mass spectral information ^{156, 174-177}. Examples of current assays preformed using tandem mass spectrometry for toxicology are listed in Table 5.

4.1 New born screening

Newborn screening programs are designed to identify disease states in infants due to inborn errors in metabolism or genetic defects before they become symptomatic. One problem as the ability to detect more and more disorders in newborns increases is that often there is no treatment for the disease. Researchers need to be cognizant of the implications of knowing a patient has a disease and not be able provide treatment.

Newborn Screening (NBS) for metabolic disorders started in the 1960's with a test for phenylketonuria using bacterial inhibition from dried blood spots ²⁶. This method was used exclusively for phenylketonuria (PKU) for several decades without many additional aminoacidopathies being added. The beginning of the use of electrospray tandem mass spectrometry changed all that, heralding an explosion of new tests beginning in the mid 1990's where the development of high throughput screening from dried spots using ESI-MS/MS was demonstrated for a large number of inherited metabolic disorders by several authors ^{26-27, 178-182}. Using ESI-MS/MS, PKU was determined from newborn dried bloodspots (DBS), taken between 24 and 72 hours after birth, by first extracting the amino acids then derivatizing phenylalanine (Phe) and tyrosine (Tyr) to form butylated esters. The butylation reaction was simple and quick making it easily adaptable to high throughput screening. The MS/MS analysis of butylated amino acids was very simple and specific and the use of isotopically labelled internal standards allowed for absolute quantification of the analytes in the DBS sample. Diagnosis of PKU was further improved by taking the ratio of concentrations of Phe to Tyr which is a more sensitive measure of the activity of the enzyme phenylalanine dehydrogenase. The same extraction/derivatization and ESI-MS/MS methodology was then extended to diagnose other aminoacidopathies such as maple syrup urine disease, by forming and detecting the butylated esters of leucine, isoleucine, alloisoleucine and valine¹⁷⁸. Once again, absolute quantification was made by the use of isotopically labelled internal standards for each amino acid tested. The same technology was then extended to methionine for diagnoses homocystinuria and hypermethiononemia¹⁷⁹.

In addition to amino acid screens, it was discovered that the butylation reaction was compatible with the simultaneous determination of acylcarnitines from the same DBS sample. The test for medium chain length acylcarnitines was thus developed and used to diagnose medium-chain acyl-CoA dehydrogenase deficiency (MCAD) ^{27, 180}. The added advantage that the tests for the amino acids and the acylcarnitines could be performed in the same 2 minute assay on the same DBS sample, meant that more than 30 different diseases

could be routinely screened by ESI-MS/MS including aminoacidopathies, organic acidurias and fatty acid oxidation disorders.

One problem with performing amino acid analysis by MS is the inability to distinguish isobars such as Leu and Ile. Iosbars must be separated by the chromatography but that often can extend the run times making the method unsuitable for high throughput screening. Another issue with the use of butyl esterification derivatives is that it destroys glutamine which is the best marker for ornithine transcarbamylase deficiency (OTCD). Glutamine is mostly converted to the glutamic acid butyl ester during the derivatization and the remaining glutamine butyl ester formed is deaminated in the electrospray source ¹⁸³.

An alternative derivatization method that has been shown to produce more stable ions than the corresponding butyl esters is the formation of formamidene butyl esters of amino acids ^{28, 184}. The method was further optimized to include glutamine and achieved an increase in sensitivity of 50% by forming the isobutyl esters instead of the n-butyl esters. Unfortunately this method can not be used as a substitute in newborn screening assays because the acylcarinitines are not fully derivatized at room temperature with this reagent.

In addition to amino acid and acylcaritine analysis, newborn screening is also performed on free methylmalonic acid and 3-OH propionic acid using the butylated ester derivatization ¹⁸⁵. There are some authors attempting to remove the derivatization step from the procedure but no routine screening is being done without it.

Hypothyroidism, which was described previously, is also measured in routine newborn screening; however, the assay is usually performed using dried blood spots and an immunoassay to test for the thyroid stimulating hormone (TSH) ¹⁸⁶.

Lysosomal storage disorders (LSD) are a group of rare inherited metabolic diseases that result from deficiency or absence of specific enzymes that breakdown unwanted substances in cells. The resulting build up of undesirable materials leads to disability or death. Recently, treatment for some of the more abundant LSDs has made the need for screening in newborns necessary and is now required in several states and in many European countries. Even though the individual disorders have low incidence their combined abundance is 1 in 3,000 ¹⁸⁷⁻¹⁹¹ warranting their screening in newborns.

Screening for enzymatic deficiencies is done by incubation of samples, in this case dried blood spots, with an added substrate that is known to be converted to a specific product by the enzyme of interest. Normal patient samples will form the product because the enzyme is present. Patients deficient in the enzyme will produce much less or no product from the incubation. Measurement of the product formation over time is then used to diagnose those with enzyme deficiencies.

The initial method for LSDs was designed to analyze Fabry, Gaucher, Krabbe, Neimann-Pick and Pompe disease simultaneously and comprised a 24 hr incubation step prior to mass spectral analysis ¹⁸⁷⁻¹⁹¹. The method was designed for quick LC/MS/MS analysis to facilitate high throughput and had a sample runtime (injection to injection) of 2 minutes. The drawback of the method was that in order to have fast analysis time on the mass spectrometer no real chromatographic separation was used. The result is that there were interferences from the substrates on the product ion spectra. To reduce the cross talk from the substrate, extensive off-line extractions are conducted to separate the substrates from the products. These extractions used a lot of materials increasing the cost, all the extra manipulation increases the odds of experimental error, and the process took an entire day to perform. More recently, a method that employs turbulent flow chromatography for on-line clean-up to remove matrix effects and UHPLC to separate all the substrates from all the products has

been reported by Kasper et al.¹⁹⁰ This method is 4 minutes injection to injection but is multiplexed to reduce the system run time to 2 minutes per sample. Another advantage to the improved method is that the initial method needed to de-tune the ion source conditions to reduce fragmentation in the source to guarantee no signal from the substrates in the product ion channels. In doing this the authors also reduced the signal strength. The UHPLC step employed in the new method separates the substrates and products chromatographically such that no detuning is necessary. The result is that less product formation is needed and reduced incubation times are possible as well. Recently a sixth LSD was added to the LSD panel to screen for MPS 1 disease¹⁹¹. Additionally, cost analysis comparing on-line TFC clean-up to off-line extraction methods calculated the total cost per sample for LSD screening to be 0.29 Euro using the on-line method and 0.96 Euro using off-line extraction.

Most of the disorders in the expanded or supplemental newborn screening list used today that are performed by mass spectrometry are shown in Table 10. This expanded screening is not yet universally mandated.

4.2 Proteomics

Barr et.al.¹⁹² were the first to demonstrate the use of proteotypic peptides, usually tryptic peptides of proteins targeted for quantification, as quantifiable surrogate markers for the intact protein. Gerber et.al.¹⁹³ employed isotopically labelled proteotypic peptides and LC-SRM. They called these peptides “AQUA” short for Absolute QUAntitation when employing these isotopically labeled peptides as internal standards (ISs) for quantifying the surrogate proteotypic peptides. The main advantage of this method of quantification is that the AQUA peptides are chemically identical to the proteotypic peptides making them ideal ISs since they coelute with the analyte of interest. This is important since both IS and analyte peptide are ionized and detected under identical conditions eliminating issues like ion-suppression brought about by co-eluting matrix. The method of detection is SRM or selected reaction monitoring where the precursor molecular ion is transmitted by the first analyzer (“Q1” usually a quadrupole mass filter in a triple quad instrument), fragmented in the quadrupole collision cell (2nd quadrupole) and the appropriate product ion(s) are then monitored by the final quadrupole mass filter (“Q3”). The fragment ions formed are dictated by the proteotypic peptide’s amino acid sequence making the SRM process very specific for the analyte and corresponding IS. If more specificity is required, additional fragment ions can be monitored per proteotypic peptide.

The group at the Plasma Proteome Institute improved this process adding an immunoaffinity step called SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies), to concentrate the proteotypic and AQUA peptides enhancing their detection by LC-SRM¹⁹⁴. These SISCAPA techniques have evolved to where they now employ integrated systems incorporating magnetic beads coated with immunoaffinity agents implemented in low-volume trap-wash-elute apparatus suitable for the efficient transfer of highly concentrated analytes to low-flow LC-SRM systems. It is estimated that such systems are capable of enrichments of analytes up to 20,000 times providing detection limits of proteotypic peptides and the proteins they represent down to low ng/mL levels rivaling those of some ELISA assays¹⁹⁵.

One significant advantage of this approach is that the cost of analysis of many analytes by LC-SRM is the same as that of a single analyte which could be especially important when multiple biomarker assays are employed. Anderson and Hunter¹⁹⁶ demonstrated the

principle where over 100 SRM's were monitored in a single LC-SRM experiment representing the quantitation of over 50 proteins in a single assay.

Certain immunoassays have poor performance due to a variety of factors including potential interference from endogenous immunoglobulins and imperfect concordance across platforms due to antigen microheterogeneity across patient populations¹⁹⁷. In such cases the performance of LC-SRM may have significantly better performance as described in the example using SISCAPA to quantify thyroglobulin, a well characterized tumour marker for which interference from endogenous immunoglobulins affects results for 10% to 25% of all patients. Thyroglobulin was successfully quantified down to picomolar levels (3 ng/mL) in non-depleted plasma by SISCAPA LC-SRM. One advantage of the assay is that the trypsinization step digests endogenous immunoglobulins that could potentially interfere with immunoaffinity. LC-SRM overcomes these serious problems exhibited by commercially available immunoassays for thyroglobulin.

Carr¹⁹⁸ recently demonstrated that SISCAPA LC-SRM can be used to quantify a "panel" of protein biomarkers in plasma. In the present example plasma samples were taken at timed intervals during a planned myocardial infarction (PMI) used to treat hypertrophic obstructive cardiomyopathy. It was thought that these samples would be representative of myocardial infarction (MI) and that monitoring well recognized biomarkers of cardiovascular disease such as interleukin-33 (IL-33), for which there is no well-validated immunoassay, and cardiac troponin I (cTnI) would provide insight into the pathophysiology of MI. The authors suggested that SISCAPA LC-SRM would be better suited to multiple-biomarker assays since there is less likelihood of non-specific binding between the capture and detection antibodies. With SISCAPA each analyte (proteotypic peptide) requires only a capture antibody since the detection is done by LC-SRM. The results of the study indicated a poor correlation between the immunoassay for cTnI and SISCAPA LC-SRM. They speculated that non-specific interactions with endogenous antigens in the immunoassay could account for the poor correlations where LC-SRM would not be as susceptible to these interferences. Even though the SISCAPA LC-SRM exhibited impressive detection limits for IL-33 in spiked serum (low ng/mL), signals for endogenous levels of this analyte in plasma samples were below detection limits. Sensitivity is a common problem for the SISCAPA LC-SRM approach where protein analytes are required to have plasma concentrations above the low ng/mL where many important protein analytes are present at pg/mL levels.

There are some recent examples of the use of LC-SRM to determine endogenous protein biomarkers at higher concentrations. A group at Mayo used LC-SRM of proteotypic peptides to quantify serum Zn- α 2 glycoprotein (ZAG), a putative biomarker for prostate cancer¹⁹⁹. Serum samples were trypsinized without the need for the depletion of abundant proteins and relatively high flow-rate LC-SRM was used instead of nano-flow HPLC. The use of 2 mm ID HPLC columns and higher flow rates greatly enhances the ease-of-use of this method as well as ruggedness and reliability of the assay. Prostate cancer patients showed an average ZAG concentration of 7.6 mg/mL where control subjects had an average ZAG concentration of 3.7 mg/mL demonstrating a clear differentiation. The method itself was validated between 0.32 and 10.2 mg/mL.

Another interesting example involves the determination of human serum albumin (HSA) in urine as a biomarker for renal failure. Renal disease is steadily rising as a complication of type-2 diabetes which is reaching epidemic proportions in North America. Urinary Albumin is a sensitive prognostic and diagnostic biomarker for renal disease. Early diagnosis of the onset of renal disease can prevent much more expensive interventions by giving patients a

chance to change diet or life-styles to avoid more serious manifestations of the disease. Accurate and precise methods of rapidly screening for renal disease will become important in population screening to avoid false positives and false negatives; such methods must also be inexpensive to permit their widespread use as disease-screening modalities. A group at the Mayo clinic ²⁰⁰ recently published a method for HSA in serum using LC-SRM and the detection of 2 proteotypic peptides representative of HSA and employing isotopically labeled AQUA peptides as ISs generated in a novel fashion. ISs were introduced into the serum sample as a precisely known amount of intact recombinant ¹⁵N-labelled HSA prior to tryptic digestion. During trypsinization both endogenous and labeled HSA forms are degraded into tryptic peptides where the ¹⁵N labeled peptides act as ISs for the endogenous HSA. The high flow rate LC-SRM method was validated over the linear range of 3 to 200 mg/mL spanning the clinical requirements. HSA at these levels requires no preconcentration or nano-scale chromatography to be detectable. The run time, however, was 30 minutes making it too long for a population-screening modality. This long analysis time could be significantly shortened using multiplexed sample introduction as described above. The LC-SRM method compared well with immunoturbidity but without the need for sample dilution to cover the full analytical range like immunoturbidity requires.

There is significant interest in the use of SISCAPA LC-SRM for the detection of proteins in biological fluids as evidenced by the following references by Ackerman and others ²⁰¹⁻²⁰⁴. There have been multi-site trials to evaluate the reliability of these types of assays in various laboratories across different LC-SRM platforms ²⁰⁵. There is controversy as to whether such tests will be transferable to the clinical diagnostic laboratory given the complexity of these tests. This controversy was recently discussed in an article by Diamandis ²⁰⁶ based upon interviews of a panel of experts in the field. The consensus opinion was that MS will best be used in situations where good immunoassays do not exist and that the greatest application for LC-SRM will be in the validation of putative protein biomarkers; while this is not clinical diagnosis, this application cannot be undervalued. Over the past 15 years of intense proteomic research thousands of putative protein biomarkers have been identified in human plasma or serum, yet no significant increase in FDA-approved protein diagnostics has followed ²⁰⁷. Articles have been written decrying this terrible record and the blame lies with a lack of rigorous validation of putative biomarkers involving clinical studies of sufficiently large cohorts of patient samples. Such studies would require the high throughput quantitative analysis of trace protein biomarkers and there is much hope that MS-based quantitative techniques such as SISCAPA LC-SRM will help in this validation process ^{184, 208}. One area where MS may also be very useful is for the determination of heterogeneous proteins whose isoforms respond similarly to the immunoassay but whose diagnostic values differ greatly. The sources of protein biomarker microheterogeneity could be genetic, environmental, or a mixture of both. This kind of variability in populations is precisely why large-scale multi-center clinical trials are performed on new diagnostic tests to ensure that this natural protein variability does not confound the interpretation of results. However, what is being ignored here is that the variability itself is likely a source of information about disease and possibly diagnostic of it.

Nelson ²⁰⁹ and Niederkofler ²¹⁰ pioneered the application of Mass Spectrometry Immuno Assays (MSIA) combining immunoaffinity of intact protein targets with a direct mass spectrometry readout usually using MALDI-TOF to determine the various protein isoforms captured by their proprietary immunoaffinity pipettor tips. This has led to some very informative and thought provoking observations of significant correlations of

protein variants and isoforms with disease states. Lopez and Nelson ²¹¹ combined MSIA with LC-SRM in the determination of PTH isoforms and their potential correlation with renal disease. The full length form of PTH secreted from the thyroid gland comprises 84 amino acids referred to as PTH(1-84). The measurement of PTH(1-84) and its N-terminal truncated form PTH(7-84) are currently assayed to assist in the diagnosis of hypo/hyperparathyroidism and hypercalcemia and in monitoring for renal osteodystrophy in patients with end-stage renal failure. The PTH(1-84)-to-PTH(7-84) paradigm is a recent example of the biological and clinical utility of microheterogeneity within the PTH protein. These authors began their investigation with an MSIA MALDI-TOF experiment that identified a significant number of PTH variants including the previously unidentified PTH variants PTH(28-84), PTH(34-84), PTH(37-84), PTH(38-84), PTH(48-84), PTH(34-77), PTH(37-77), and PTH(38-77). Quantitative MSIA LC-SRM assays were developed for PTH(1-84), PTH(7-84) and the newly identified variant PTH(34-84), where immunoaffinity captured PTH isoforms were tryptically digested postcapture prior to analysis by LC-SRM using a variety of proteotypic peptides and their corresponding isotopically labeled analogues as internal standards. In all, 32 SRM transitions were simultaneously monitored throughout the analytical run. The peptides monitored by LC-SRM exhibited linear responses (R^2 0.90–0.99) relative to recombinant human PTH concentration, limits of detection for intact PTH of 8 ng/L and limits of quantification of 16–31 ng/L depending on the peptide. Many of these peptides showed good correlations with renal disease and could be used as a basis for differentiating patients with renal disease from healthy controls.

The determination of LSDs, as discussed above in the section on Newborn Screening, is basically the indirect determination of proteins (lysosomal enzymes) by monitoring the conversion of small molecule substrates into products. Many other newborn screening (NBS) methods are designed to assay proteins, usually from dried blood spots (DBS). The most well-known of these is the measurement of Thyroid Stimulating Hormone (TSH) by immunoassay. TSH concentrations from newborn blood spots are significantly below the detection limits of LC-SRM methods; however, there are some recent examples of how mass spectrometry can be employed to screen diseases in newborns employing the measurement of protein biomarkers.

Wilson's Disease (WD) is characterized by reduced ceruloplasmin (CP) levels in blood leading to abnormal transport of copper resulting in dangerous elevations of copper in the body's tissues. The excess copper damages the liver and nervous system and WD affects ca. 1 in 30,000 newborns. Wilson's disease has an autosomal recessive pattern of inheritance characterized by progressive deterioration of the liver and the brain. Consequently liver disease and neuropsychiatric symptoms are the main features that lead to diagnosis. A significant percentage of those diagnosed with WD already require a liver transplant but relatively inexpensive effective treatments are available if diagnosed early ²¹²⁻²¹³. The measurement of intact proteins directly is often impractical by LC-MS because of their low concentrations such as TSH. As discussed previously, proteotypic tryptic peptides, which are surrogates for the intact protein, are much easier to detect and quantify by LC-MS/MS. This methodology was employed to determine CP extracted from DBS. The results of the study demonstrated clear differentiation between affected newborns, heterozygous carriers of WD and normal newborns. The levels of CP for the unaffected carrier newborns was ca. 250 mg/L and in a cohort of affected newborns ranged from 10 to 20 mg/L. The LC/MS/MS method was rather lengthy for a newborn screening modality (ca. 7 min);

however, this could be made faster using a multiplexed multi-column system, as suggested by the authors.

Hemoglobinopathies represent a tremendous opportunity for MS/MS. These are rare disorders but the combined incidence can be quite high and every jurisdiction in the USA and many others throughout the world test newborns for these disorders. Outcomes can be significantly improved if treatments are commenced early after birth. Currently used methods to detect hemoglobinopathies include ion exchange chromatography (IEX) and isoelectric focusing (IEF) both of which are time consuming and generally exhibit poor specificity when unusual post-translational modifications occur. Many articles have been published on the use of MS and MS/MS for the determination of hemoglobin (Hb) variants via molecular weight determinations on intact β -globin chains²¹⁴⁻²¹⁶. These techniques suffer from situations where the mutation involves a zero (nominal) mass change such as Lys \leftrightarrow Gln or Leu \leftrightarrow Ileu. Most labs use a combination of ESI-MS, IEX and IEF followed by DNA analysis to screen and confirm hemoglobinopathies. MS/MS analysis on its own using proteolytic (tryptic) peptides was suggested as a means of analyzing all mutations²¹⁴. Dooley was the first to suggest that all hemoglobinopathies could be screened and confirmed using only MS/MS of tryptic peptides derived from DBS extracts²¹⁷. Recent work by Dalton and Turner²¹⁸⁻²¹⁹ demonstrated that MS/MS could be used to screen all the clinically important hemoglobinopathies including HbS, HbC, HbE, HbD^{Punjab}, HbO^{Arab}, Hb^{Lepore}, and β -Thalassemia as well as other clinically important conditions such as delta β -Thalassemia, hereditary persistence of fetal hemoglobin trait (HPFH) and alpha zero thalassemia trait. The important finding of this work was that all these conditions could be screened in a 1 to 2 minute MS/MS experiment using SRM, monitoring 1 SRM per proteotypic peptide and employing proteotypic peptides of wild-type globins as endogenous internal standards. The method employed simple trypsinization of the DBS extract followed by a 1 to 2 minute flow injection analysis (FIA) MS/MS experiment ideal for high throughput screening. These authors demonstrated that by using SRM-triggered full scan MS/MS they were also able to confirm the amino acid sequence of the variant peptide without the need for costly genetic (i.e. DNA) analysis. It was demonstrated that confirmation could easily be built into the screening assay without paying a penalty in time or sensitivity. This work was validated by Boemer, et.al.²²⁰ employing 2 SRM channels per proteotypic peptide and a different brand of MS/MS instrument. Both assays demonstrated 100% sensitivity and specificity using this approach for a wide variety of hemoglobinopathies and even the ability to detect heterozygous genotypes. Dalton and Turner went on to perform a clinical trial²²¹ on a cohort of 40,000 newborns comparing MS/MS to the predicate IEF method. The results indicated complete concordance between the two methods detecting 199 HbS (including 8 HbS/HbF and 3 HbSC variants), 39 HbC, 52 HbD^{Punjab}, and 48 HbE.

4.3 Metabolomics

As discussed in the previous section, there has been almost 15 years of intensive proteomics research with relatively little to show for it in terms of novel protein diagnostics due mainly to a lack of biological validation of the putative diagnostic targets. Similarly, there is intensive research into metabolomics as a source of new disease biomarkers. Many of the putative metabolomic biomarkers are actually panels of endogenous metabolites for which mass spectrometry has been demonstrated to be very well suited as both a discovery and detection modality.

A recent review by Ellis ²²² concluded that of all the “omics” areas of research to discover novel biomarkers, metabolomics is probably the most achievable. There are probably on the order of <5,000 metabolites ²²³ circulating in blood, where there are about 30,000 expressed genes with hundreds of thousands of single nucleotide polymorphisms (SNPs) and probably well over 1,000,000 proteins considering post-translational modifications and splice variants. With only several thousand metabolites that need consideration, metabolomics is likely to be a more achievable source of new diagnostics than either genetics or proteomics posing much less of a challenge to identify, quantify and validate diagnostic potential. Apart from their potential diagnostic value, metabolite-based biomarkers could also be useful in patient stratification in clinical trials or in disease-treatment where it would be used to monitor whether patients are responding well or poorly to pharmaceuticals or even simple life-style changes. Just as in proteomics research, the majority of recent metabolomic work using mass spectrometry has concentrated on the discovery of novel disease-related biomarkers involving metabolome-wide studies (hundreds or even thousands of metabolites) on cohorts of only relatively few patients and controls. The result has been the creation of panels of many putative metabolomic disease biomarkers without the requisite clinical or biological validation involving broader population studies. To facilitate such studies one group in particular (an Innsbruck-based company called Biocrates, pronounced like “Socrates”) has developed CE-IVD approved kits that can be applied in anyone’s laboratory to economically perform these rigorously quantitative metabolomic validation studies across relatively large sample sets. Such studies are very important for the translation of metabolomic research into clinical practice.

An interesting example of this application is a collaborative study by systems biology researchers and Biocrates investigating a diabetes mouse model ²²⁴ employing a proprietary kit of reagents including all required reactants, standards and protocols to quantitatively determine over 800 endogenous metabolites by LC-MS/MS including: 18 amino acids; 50 reducing mono-, di-, and oligosaccharides, uronic acid, and N-acetylglucosamine; 16 acylcarnitines; 5 hydroxyacylcarnitines; 5 dicarboxylacylcarnitines; free carnitine; and 707 lipids. These lipids were subdivided into 82 different ceramides and glucosylceramides, 110 different sphingomyelins and sphingomyelin derivatives, 95 glycerophosphatidic acids, 85 glycerophosphatidylcholines, 103 glycerophosphatidylethanolamines, 11 glycerophosphatidylglycerols, 177 glycerophosphatidylinositols, glycerophosphatidylinositol-bisphosphates and -triphosphates, and 44 glycerophosphatidylserines. Glycerophospholipids were further differentiated with respect to the presence of ester and ether bonds in the glycerol moiety. Using these kits and flow injection analysis (FIA)-MS/MS (SRM) researchers correlated plasma levels of analytes with diabetes treatments; accurate concentrations were derived from the use of isotopically labeled internal standards (ISs) included in the kits. Metabolite concentrations and ratios of concentrations were derived and subjected to unsupervised statistical analysis revealing novel diabetic metabolic phenotypes and the impact medication has on them. Many metabolites were oppositely impacted by treatment which reduced noise in the data to reveal new potential biomarkers of diabetes. This study was subsequently extended to human subjects ²²⁵ comparing the Biocrates kit results to other approaches using a cohort of 100 subjects comprising 40 individuals with self-reported diabetes and 60 controls. The other LC-MS approach in this study was a proprietary workflow undertaken by Metabolon as well as an NMR study of the same samples done by Chenomx. In this pilot study a total of 482 metabolites were quantified. The results of this human trial were far less clear than those using the inbred murine subjects of the above earlier study for a variety of reasons including heterogeneity of

the human subjects with respect to genetic and environmental factors including varying degrees of glycemic control. The authors concluded that this multi-platform approach to the metabolome-wide analyses of diabetes in a general population was helpful in the identification of a series of known, and also some novel, deregulated metabolites that associate with diabetes under sub-clinical conditions in the general population. However, given the above limitations they observed that a much larger cohort of subjects would be necessary.

In related articles by the same authors ²²⁶⁻²²⁷ human genotypes were evaluated against metabolomic data creating what the authors deemed as the first genome-wide association (GWA) study using metabolic traits as phenotypic traits. In these studies 363 serum metabolites from 284 male subjects who were participants in the KORA study ²²⁸ were rigorously quantitated using the Biocrates kits and (FIA)-MS/MS (SRM). In parallel, single nucleotide polymorphisms (SNP's) were measured using gene-expression chips and correlated with the metabolomic data. Genetically determined variants in the metabolic phenotype (so called "metabotype") were observed and enhanced using ratios of metabolites with opposite correlations with genotypes. Using this approach, almost 30% of genetic variability correlated with the metabolic data with high statistical significance. The authors conclude that the investigation of the genetically determined metabotypes in their biochemical context may help to better understand the pathogenesis of common diseases and gene-environment interactions. These findings could result in a step toward personalized health care and early nutritional or other life-style interventions based on a combination of genotyping and metabolic characterization.

The above examples of metabolomic applications featured the use of mainly triple quadrupole MS/MS systems, the commonly accepted "workhorse" of quantitative bioanalysis. Over the last 20 years the performance of the triple quad platform has risen steadily due to the incorporation of new technologies and larger, more efficient pumping systems. In fact, like Moore's Law for computing, the performance of the triple quad platform increased by about an order of magnitude every 2 to 3 years more or less in a linear fashion until the last 5 years or so when the triple quad began to asymptotically reach its maximum performance with respect to signal-to-noise and limits of detection.

As the triple quad platform begins to mature technologically, other areas of mass spectrometer performance are beginning to be exploited in the market; specifically there has been a rapid evolution of performance in the area of mass resolving power and mass accuracies. This started with the development of the hybrid MS technologies based upon quadrupoles and time-of-flight systems, the so called Q-ToF instruments, as well as the introduction of linear ion-traps coupled to Fourier transform mass spectrometers (LTQ-FT-MS systems) and its successor the ion-trap coupled to an Orbitrap system (LTQ-Orbitrap systems). These MS/MS systems are capable of high mass accuracies (1-2 ppm) and high mass resolutions (20,000 to 300,000 $m/\Delta m$) which greatly enhances analytical specificity. Generally these hybrid systems are very large, expensive and quite difficult to operate making their incorporation into routine clinical diagnostics quite limited. However, recently lower cost benchtop versions of the Orbitrap instruments are beginning to appear ²²⁹⁻²³¹. The significance of this new type of high resolution/mass accuracy (HRMA) system is just beginning to be appreciated since the specificity of HRMA of a single MS system could rival that of lower resolution MS/MS systems like the triple quadrupole. The simplicity of a single mass analyzer versus MS/MS could provide the impetus to use benchtop HRMA systems for routine bioanalytical assays instead of MS/MS.

One recent study that exemplifies the use of this technology in metabolomics was recently published by Volmer, et.al.²³² where lipids extracted from plasma samples for a cohort of 10 healthy volunteers were analyzed by UHPLC-MS on a benchtop Orbitrap system. About 240 separate data analyses were undertaken to develop a statistically meaningful data set obtained using 50,000 resolution, and mass accuracies typically well below 2.5 ppm. Like previous lipidomic studies²³³ Volmer found that setting m/z ion-extraction windows to ≤ 2.5 ppm provided unambiguous lipid identification based upon elemental composition. Moreover, the re-constructed ion chromatograms were used for quantitation without significant isobaric interferences using the analyzer constraints of 50,000 resolution and ~ 1 –2 ppm mass accuracy. This large data set was subjected to unsupervised statistical analysis and demonstrated the ability to differentiate subjects' plasma samples and to also pick out those samples enriched with a particular endogenous or exogenous analyte. The study demonstrated that, with high resolving powers and mass accuracies, both targeted and untargeted data analysis can be accomplished on the same data set. Another recent example of the application of this technology was published by Rabinowitz, et.al.²³⁴ involving the UHPLC-MS analysis of anionic metabolites in yeast extract identifying 137 metabolites in a single experiment. This type of platform could be especially useful for metabolomic studies involving human subjects where high numbers of analytes must be rigorously quantitated in a single run but with sufficient flexibility to be able to provide information on unexpected analytes such as exogenous compounds and their metabolites that may be present due to environmental factors (e.g. disease treatment with pharmaceuticals). The translation of the Orbitrap from a research validation tool to a routine instrument for clinical diagnostic measurements is inevitable given the analytical power of this novel technology and the results that have been published to date.

5. Conclusion

The use of tandem mass spectrometry is well documented in the clinical laboratory. In particular, the development of atmospheric pressure ionization techniques to perform LC/MS has allowed the clinical chemist to address new areas of research and drastically improve the quality of clinical assays due to higher specificity and better sensitivity than other approaches. The main draw back to clinical applications of LC/MS/MS is the high initial cost and the need for dedicated highly trained personnel to run and maintain the instruments. The result is that centralized laboratories, either CROs or large medical facilities, where samples are sent for analysis are usually needed in order to take advantage of the emerging technologies. As our understanding of the use of LC/MS/MS and the impact of analyzing biological matrices improves, and mass spectrometers become more and more user friendly, the development of a fully automated clinical analyzer should be possible. However, until an LC/MS/MS clinical analyzer is commercially available, the use of LC/MS/MS in the clinic will remain a specialized tool available by out-sourcing or development core facilitates.

6. References

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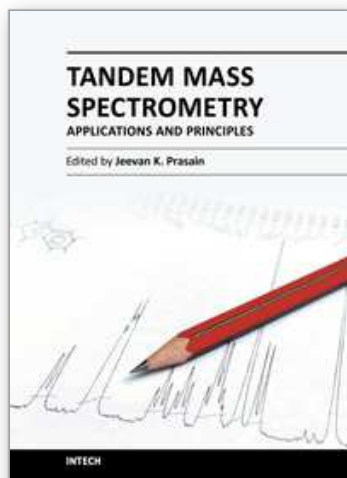
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Tandem Mass Spectrometry - Applications and Principles

Edited by Dr Jeevan Prasain

ISBN 978-953-51-0141-3

Hard cover, 794 pages

Publisher InTech

Published online 29, February, 2012

Published in print edition February, 2012

Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Joseph Herman and Bori Shushan (2012). Clinical Applications, Tandem Mass Spectrometry - Applications and Principles, Dr Jeevan Prasain (Ed.), ISBN: 978-953-51-0141-3, InTech, Available from:
<http://www.intechopen.com/books/tandem-mass-spectrometry-applications-and-principles/clinical-applications>

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