Mass Spectrometry in the Clinical Laboratory

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I. Introduction

The utilization of mass spectrometers in clinical laboratories is undergoing considerable expansion at the outset of the 21st century.[1,2] This expansion is largely due to extraordinary advances in mass spectrometry (MS) developed in the previous decade.[3] A clear vision of the importance of MS in medicine was provided at the 11th Sanibel Conference on Mass Spectrometry[4] entitled "Mass Spectrometry in the Clinical Diagnosis of Disease". The consensus of the conference was that MS is poised to take an increasingly important role in clinical chemistry. MS is no longer the complex and laborious tool used exclusively by experienced mass spectrometrists. It is an accessible, versatile, and powerful technology that is best suited to solve research and analytical problems in an extensive number of scientific disciplines.[1] The rapid pace of developments in liquid chromatography and mass spectrometry (LC-MS) has profoundly influenced the potential number of applications of MS in the clinical laboratory.[5] Historically, most mass spectrometric applications were restricted to the analysis of small volatile molecules that were analyzed, using gas chromatography and mass spectrometry (GC/MS). These methods required extensive sample preparation that included sample extraction and derivatization. The analysis of intact high molecular weight or extremely polar biomolecules was either difficult or not possible in most laboratories just 15 yr ago. Today, new LC-MS applications provide solutions to the analysis of these biomarkers.

Most compounds that characterize human biochemistry are extremely hydrophilic, ranging in size from small molecules with molecular masses generally less than 500 Da (e.g. amino acids, fatty acids, bile acids, and steroids) to higher molecular weight molecules such as peptides and large biomolecules (e.g. proteins, glycoproteins and oligonucleotides). The advent of new ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI) together with improvements in mass analyzers such as time-of-flight (ToF) and benchtop

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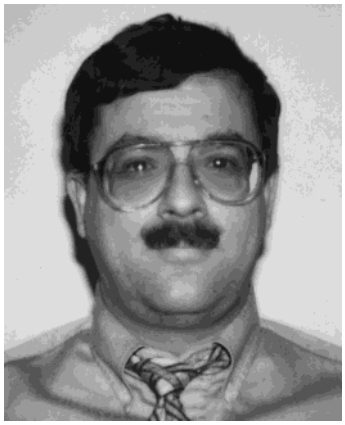

Dr. Donald Chace received his B.S. in Chemistry from Boston College in 1981. He earned two graduate degrees from The George Washington University. In 1984, Dr. Chace received a M.S. in Forensic Science specializing in forensic toxicology. In 1989, he received a Ph.D. in Pharmacology specializing in mass spectrometry and drug metabolism. A new technique for the selective detection of isotopically enriched drugs and their metabolites was developed using gas chromatography chemical reaction interface mass spectrometry (GC–CRIMS). In 1989, he began his postdoctoral research at the University of Maryland School of Pharmacy investigating the use of diamine reagents to improve the analysis of phosphate analgoues using thermospray MS. Dr. Chace joined the faculty at Duke University as a Medical Research Assistant Professor in the Department of Pediatrics in 1990. His research efforts included the application of tandem mass spectrometry for use in early identification of metabolic disease in newborns. In 1997, he joined Neo Gen Screening, Inc. to continue the development of mass spectrometry applications in newborn screening and clinical chemistry. He is currently the Director of the Division of Bio-Analytical Chemistry and Mass Spectrometry. This laboratory analyzes more than 250 000 blood samples obtained from newborns per year using tandem mass spectrometry for metabolic disease screening.

tandem quadrupole (MS/MS), quadrupole ion trap (QIT), and the newest hybrid mass spectrometers, e.g., quadrupole-time-of-flight (Q-ToF), have enabled clinical chemists to consider analyzing these very polar and very large compounds.[1,5−9] The number of potential clinical applications of MS is inestimable.

A. Clinical Chemistry and MS

Clinical[10] is defined as having to do with the direct treatment and observation of patients, as distinguished from experimental or laboratory study. The application of chemistry and clinical science therefore relates primarily to providing patient-derived chemically relevant data to the physician. The physician uses data obtained from the clinical chemistry laboratory and other physical assessments to make a hypothesis (diagnosis) regarding a disease state. The number of clinical results that will be provided to a physician that are based in MS will increase in this decade.

Bermes and Young[11] state that the objective of a clinical chemistry laboratory is "to perform qualitative and quantitative analyses on body fluids such as blood, urine, cerebrospinal fluid ..." and further state that "if the results are to be useful to the physician in the diagnosis and treatment of disease, the tests must be performed as accurately as possible. This [testing] requires the use of sound analytical methods and good instrumentation". Innovative sample preparation methods together with accurate, versatile, and robust analytical systems make mass spectrometric applications ideal for solving routine and complex clinical laboratory problems. However, as pointed out by L. Bowers,[4] the challenge for the clinical scientist is to present complex MS data in an easy to understand format. This format includes interpretation of complex mass spectra and the integration of these data with other laboratory results. The analytical chemist, who is not traditionally trained in medicine, is challenged to provide some form of interpretation of clinical data to a physician, certified geneticist, or other health professional. During this decade, chemists/mass spectrometrists trained in the clinical sciences or clinical specialists (physicians) trained in MS will provide expertise in a special area of MS defined as clinical mass spectrometry.

The most widely used application of MS in clinical chemistry is GC/MS.[12−15] Due to analyte volatility and size limitations, GC/MS analyses are restricted to derivatizable compounds such as fatty acids, organic acids, amino acids, monosaccharides, prostaglandins, bile acids, and steroids.[16] The most widely used GC/MS assay in clinical chemistry is the analysis of urine specimens from patients with suspected or known metabolic disturbances.[17−19] The power of GC/MS in analyzing complex urinary profiles and providing physicians with key evidence that assists in the diagnosis of a disease has led to its "acceptance" by clinical chemists, physicians, and other medical specialists.

Historically, the use of MS was limited to university and private specialty laboratories where the expertise was available to use this complex technology and clinically interpret these results. Why has the use and scope of MS recently increased in clinical laboratories as demonstrated by the recent surge in the use of tandem mass spectrometry (desginated here as MS/MS) throughout the world for newborn screening of metabolic disease? The answer[2] resides in the fact that MS has become simple enough to be operated by inexperience users in a variety of scientific disciplines. Although the machines have been made easier to use and operation simplified, mass spectrometers have retained, even dramatically improved, the power and flexibility demanded by experienced mass spectrometrist and analytical chemists. We may be experiencing a "renaissance" of mass spectrometric based analyses in the clinical laboratory. This reviewer would estimate[2] that more than 1 000 000 newborn blood samples will be analyzed using LC-MS/MS applications in 2000. LC-MS applications are replacing many of the older traditional specialty clinical laboratory methods[20,21] that used immunological, fluorometric, and biological techniques[18] as demonstrated by the newborn screening application of MS/MS for phenylketonuria.[22−24] Will MS completely replace these traditional clinical chemistry analyzers during this decade? It is unlikely. However, the number and scope of mass spectrometric applications will certainly increase, and MS will be an indispensable tool in the clinical laboratory.

The chemical diversity of compounds that are and will probably be routinely analyzed using MS is extensive. MS-based clinical assays that provide rapid, comprehensive, multicomponent analyses and maintain or improve sensitivity, selectivity, and accuracy[25] will likely lead the growth of MS in clinical laboratories. The use of stable isotopes in mass spectrometric quantitative analyses will confer a technical advantage over other methodologies used in the clinical laboratory because of its inherent accuracy and precision. Isotope dilution methods not only improve precision and accuracy in many clinical analyses but also potentially provide solutions for quality assurance, standardization, and interlaboratory comparisons.[26] LC-MS, MALDI-MS, and other MS-based systems will be important for the measurement of informative biomarkers such as protein and gene fragments[27] as GC/MS has proven to be a powerful clinical tool for qualitative and quantitative analysis of important small molecules.

B. Laboratory Testing and Diagnosis of Disease

A physician presumes that the laboratory data provided to him/her are accurate and precise, validated, and subjected to rigorous quality assurance. This presumption demands that the clinical chemist uses the most accurate methodologies available and that a protocol is followed exactly. Each MS analysis must be error-free because the information provided affects an individual's health and life. Obviously, the demands on the clinical mass spectrometrist are extraordinarily high. Every effort must be made to prevent analytical inconsistencies, and methods require extensive validation.[28] Furthermore, clinical assays must pass quality control and assurance assessments before data can be used in clinical practice.[29]

Raw MS results have no clinical significance to most physicians. Therefore, the clinical laboratory must develop extensive interpretation schemes that clearly communicate pertinent laboratory testing information to a physician before this information can be useful to diagnose a disease in a patient.

Expertise in the interpretation of mass spectrometric results does not reside in most routine clinical laboratories. The effort required to implement an interpretation and follow-up system for mass spectrometers is substantial. This is one reason that MS has primarily resided in small reference laboratories rather than large commercial clinical laboratories. In addition, information concerning false-positive and false-negative rates provide reliability indicators to the physician and should be provided with test results. Methods with low false-positive rates enable the clinician to respond more rapidly. Conversely, methods with high false-positive rates require time-consuming repeat analysis to confirm a result. Laboratory errors may result in a disease going undetected, delay a timely diagnosis, or raise health care costs by requiring further tests or patient referrals. Clearly, the power of MS-based analysis is its inherent accuracy. Precision will be the driving force for its use in the clinical laboratory. An Editorial by H. Levy that describes the use of MS/MS and newborn screening best espouses this concept.[23] When MS methods are used correctly, with proper safeguards and with communication of easily understood results to medical professionals, physicians will diagnose disease more accurately and rapidly with a lower cost and higher benefit, thus improving the state-of-the-art of health care delivery. These facts do not necessarily translate to increased use of MS in the clinical laboratory. Some potential barriers to implementation include reimbursement by health care payers, the availability of significantly less expensive and easy-to-use albeit less accurate techniques such as immunoassays, lack of physician acceptance or education, poor assay availability, high costs at low volumes, and other negative influences that are either economic or political.

C. Scope of Review

The number and type of clinically significant biomolecules and their analysis using MS are considerable. These MS applications range from elements such as iron and selenium to metabolites (such as phenylalanine and glucose) peptides, and proteins (such as insulin and hemoglobin) to large oligonucleotides (such as DNA and RNA fragments). Important biomarkers may be compounds of endogenous origin produced by intermediary metabolism or xenobiotics produced by exogenous metabolism following drug administration or environmental exposure. Clinical applications have also included biomedical research, clinical toxicology,[30,31] and other chemical disciplines such as immunology, virology, bacteriology, and oncology. It is impractical to cover every application of MS in clinical chemistry. Therefore, those applications that are currently practiced in clinical laboratories that will likely impact clinical analysis soon or that best illustrate the potential of mass spectrometric analysis will be emphasized. Referenced publications will primarily span 15 yr. Some older, historically important or frequently cited papers[32] will also be included, especially for applications using GC/MS. The clinical applications of MS will be arranged in three basic groups: small metabolites such as organic acids, amino acids, fatty acids, steroids and their conjugates;[33] peptides, proteins, and glycoproteins;[34,35] and oligonucleotides derived from biopolymers (DNA, RNA). A brief discussion of the quantitative aspects of MS, quality assurance, and integration of MS in the clinical laboratory will complete the review.

Of important note is the use of abbreviations and terminology. I have attempted to follow guidelines written by O. David Sparkman in his text "Mass Spec Desk Reference".[7] The use of standardized terminology is important for communication of meaning as seen in one recent example: MS/MS is now being used by several newborn screening laboratories, and there has been a recent trend among these users, many of whom are not classical chemists and mass spectrometrists, to abbreviate tandem mass spectrometry as TMS. TMS is the abbreviation for trimethylsilyl, a type of chemical derivative used frequently in the GC/MS analysis of organic acid metabolites found in urine. Therefore, the currently

accepted abbreviations developed by groups such as the American Society of Mass Spectrometry (ASMS)[36] (MS/MS for tandem mass spectrometry), the American Chemical Society, and IUPAC should be used to clearly communicate meaning. Users in other fields should apply these standard terms rather than create new ones.

II. Diagnostic Metabolites

A. Metabolome Analyses

Metabolome is a term used to characterize endogenous metabolites, i.e., those classes of compounds that are products or substrates of endogenous metabolism. In the clinical setting, the term metabolomics complements the terms proteomics and genomics. Metabolomics is more descriptive than the most common method for describing the laboratory investigation of low molecular weight substances as "small molecule analyses". The metabolome is to small organic molecules in cells and tissues as proteins and oligonucleotides are to the proteome and genome, respectively. Most mass spectrometric applications that are in clinical use today are characterized by the analysis of these small organic molecules, which includes the rapid analyses of multicomponent mixtures.

Nearly 200 genetic disorders of intermediary metabolism are characterized by a defect in a single enzyme.[37] Many of these inherited genetic diseases produce an abnormal protein with reduced or absent catalytic activity. If the defective enzyme plays a major role in the primary catabolic pathways of a substrate, then a significant reduction in that substrate's metabolic rate will occur. Results of decreased enzymatic activity may produce substrate A accumulation and product B deficiency when compared to normal. To maintain homeostasis, the excess substrate A will be metabolized by an alternative metabolic pathway. The product C of the alternative pathway, at high concentration, may produce toxicity, which includes cellular damage, impaired cellular function or inhibition of other metabolic pathways. Because the alternative metabolic pathway is generally less efficient than the primary pathway, the substrate A may also accumulate in a metabolic disorder. Substrate A, at very high concentrations, can also be toxic. With regard to product B of an abnormal enzyme, a deficiency can also have adverse metabolic consequence, especially if it participates in other anabolic or catabolic pathways.

The clinical effects of abnormal metabolism include organ malfunction or tissue damage, impaired development, delayed growth, mental retardation, physical disabilities, neurological disorders, cardiovascular disease, and premature death.[37] Identification and quantitation of these abnormal metabolites are critically important to the recognition and confirmation of a disorder of endogenous metabolism. Early detection through newborn screening prior to symptoms or clinical diagnostic testing after the onset of disease symptoms permits pharmacological and/or dietary intervention. Although the cures for inherited metabolic diseases are limited at this time, dietary alterations or vitamin supplements can be used to substantially reduce or prevent the most serious consequences in very many cases.

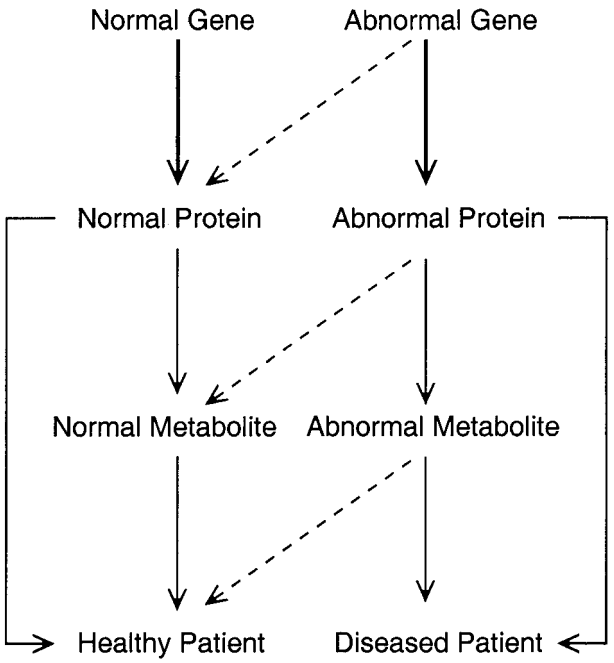

Figure 1. Simplified concept of the relationship between phenotypes and genotypes in genetic disease. In a "normal" state, genes (genotype) code for proteins, which produce metabolites and no disease expression. Abnormal genes (genotype) produce an abnormal phenotype if the gene is expressed as an abnormal protein and the abnormal protein alters metabolism. Alternatively, if the expressed protein is a functional protein, such as hemoglobin, then the disease is characterized by an abnormal protein rather than a metabolite. The relationships of gene to protein to metabolite to normal disease-free state are indicated by solid arrows. In some cases, an abnormal gene may not be expressed, an abnormal protein may not alter metabolism, or an abnormal metabolite not produce toxicity. For these examples, although an abnormal gene, protein, or metabolite concentration is present, no disease state is observed. This process is represented as a dashed arrow to the column representing a normal, disease-free condition.

The analysis of small substrates and products of enzymes systems (lower than 1000 Da molecular mass) can provide good evidence for disease detection because they often closely correlate with the disease (i.e., they are substrates or products of the abnormal enzyme produced by a defective gene). The analysis of larger proteins serving a functional role, i.e., hemoglobin and oxygen delivery to tissues, are biomarkers that also directly relate to disease detection, e.g., hemoglobin S and sickle cell anemia. The analysis of functional proteins, like hemoglobin, is used in a manner similar to measurement of metabolites in the diagnosis of metabolic disease. As observed in Figure 1, an abnormal gene product does not necessarily produce an abnormal protein (represented as a dashed line from the abnormal column to the column representing a normal, disease-free, expressed clinical condition). Likewise, an abnormal protein does not necessarily adversely affect metabolism. The accuracy of disease detection is directly related to the correlation between the causative agent and the presence of metabolite or protein. In addition,

it is unlikely that a single genetic marker will characterize all of the mutations that produce a particular disease. Furthermore, randomly occurring mutations may not be detected. The false-negative rate of current molecular methods that analyze genetic mutations can be high. Many mutations or known alterations in a gene fragment may have no clinical consequence if not expressed (benign polymorphisms).[37] This fact suggests a high false-positive rate. Nonetheless, some traditional biochemical tests may not be available or adequate for the analysis and detection of some inherited disorders. Detection of these genetic diseases will most likely be performed using primary DNA analysis.

The interplay among metabolome, proteome, and genome analyses will be the challenge of this and future decades. Most likely, clinical chemists will embrace all three forms of analysis. In some situations, the clinical chemist may actually use all these analyses sequentially to first identify the metabolome associated with the disorder, then to confirm this identification using protein and/or DNA analysis. With other clinical information, laboratory data will be used to characterize disease severity and treatment strategies. Currently, MS plays an important role in disorders of metabolism, where diseases are diagnosed by abnormal concentrations of biomarkers. These methods are described below.

B. Organic Acids

Chalmers[19] defines organic acids as carboxylic acids that may contain hydroxyl, oxo, and non-amino functional groups. This definition includes short and medium-chain fatty acids and excludes α-amino acids. Unconjugated organic acids are characterized by their acidity, high water solubility, and relatively low molecular mass (<250 Da). Nearly 250 organic acids have been identified in urine.[33] Some important organic acids frequently analyzed in clinical laboratories are shown in Figure 2.

Metabolic disorders that produce high concentrations of organic acids in blood and urine are described as organic acidurias.[5,19,33] Organic acids are involved in nearly all pathways of intermediary metabolism. Their analysis provides important diagnostic information for many inherited diseases.[38] GC/MS has served a vital role in the diagnosis and recognition of many metabolic disorders[39] and remains today a cornerstone of routine clinical laboratory analyses.

1. GC/MS Applications

Applications of GC/MS to the analysis of urinary organic acids are numerous. Several excellent books, chapters, and reviews have been written.[16,18,19,21,33,39−42] Many of these reviews include clinical correlations to disease diagnosis.[18,38] A notable fact in the review of organic acid assays using GC/MS is the substantial number of publications that differ slightly in the method of analysis (i.e., sample preparation, chromatographic separation) or pertain to a highly specific analysis for an individual disorder. This fact is important if one considers that most clinical analyses rely upon standardization or harmonization of methodology. Harmonization of methods provides the basis

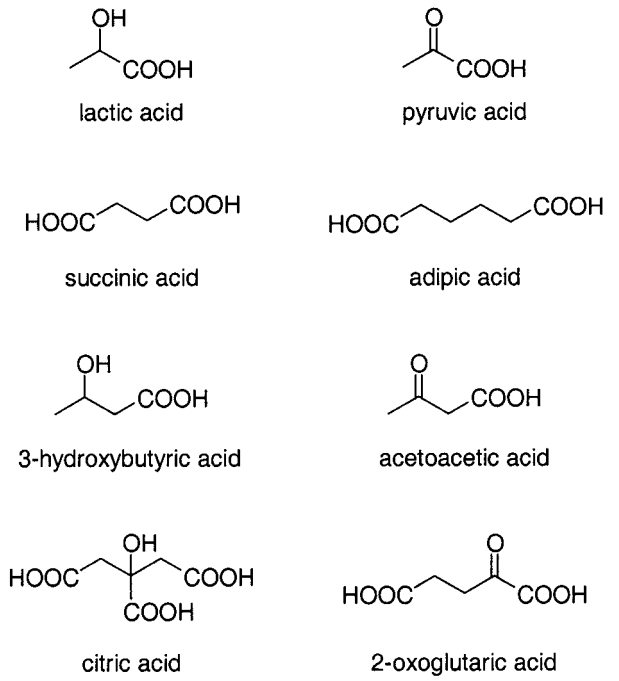

Figure 2. Organic acids commonly measured in urine and plasma by GC/MS for the diagnosis of inherited disorders of organic acid metabolism.

for interpretation of results provided by different laboratories. A key metabolite measured in laboratory A might not be measured in laboratory B. If this key metabolite is diagnostic for a particular disease and a physician orders the analysis from laboratory B, then a strong likelihood exists for a false diagnosis. This lack of standardization may certainly increase with the rapid expansion of MS into clinical laboratories. Further discussion of issues in the standardization of MS methods is provided in Section V. Fortunately, for organic acid analysis, there already is a core set of diagnostic metabolites that is measured in most GC/MS assays.

a. Metabolic Profiling. Historically, GC/MS has proven to be the most valuable clinical chemistry tool to diagnose disorders of organic acid metabolism.[18,43] GC/MS provides two means for assisting in the identification of key metabolites in derivatized urine extracts: high-resolution capillary gas chromatography and MS. The combination of chromatography and mass spectrometric detection is a major reason for reduced numbers of false-positives and for the high accuracy for these assays. Another advantage of combined GC/MS techniques is a diagnostic approach known as metabolic profiling.[16] Metabolic profiling is a method of diagnosing a disease by identifying and quantifying important disease markers or metabolites in a single analysis. In this manner, metabolite relationships, i.e., concentration of metabolite A relative to metabolites B and C, indicate a specific disorder. In addition, metabolic profiling provides a visual pattern of metabolite relationships, and this pattern has not been fully integrated in computer-assisted data interpretation. For some metabolic disorders, interpretation is very simple because a key metabolite is present in urine at an extremely high concentration. Conversely, other disorders are characterized by subtle or mild increases of several

different metabolites. Some physicians and clinical laboratory experts that are experienced in complex pattern recognition may observe subtle changes in metabolite concentrations;[44] however, many other clinical chemists will not detect these differences. The need for computer-assisted interpretation will be important in detecting subtle metabolic patterns in several ways. These may include metabolite ratios and integration of patient physical data such as age, clinical data such as liver disease, cardiomyopathy, or other laboratory tests that detect vitamin deficiencies, hypoglycemia, or hyperammonemia. Some computer-assisted interpretation systems have been developed.[45−47] Recently, neural networks have been used to assist in identification of new patterns of metabolic profiles that indicate disease.[48]

b. Urine Organic Acids. Organic acids are isolated from urine by solvent extraction or anion-exchange chromatography.[42,49−51] In addition, organic acids have been directly extracted from urine that has been applied to filter paper.[52] The most common methods for extraction of organic acids use ethyl acetate or diethyl ether and salt-saturated, acidified urine specimens. Recently, ion-exchange chromatography[51] has been used to extract urine organic acids. This technique is based on the retention of organic acids, while neutral and basic compounds are washed with aqueous buffers or distilled water from the column. Organic acids are eluted from the ion-exchange resins with organic solvent. Some researchers have used robotic workstations for sample preparation but have had limited success.[53] Due to increased volatility following many derivatization techniques, short-chain and medium-chain free fatty acids (acetic through decanoic) and other very volatile organic compounds are not detected because they coelute with solvent peaks or are lost during sample preparation.

Quantitation of urinary organic acids is nearly always expressed relative to the concentration of creatinine. This method of quantifying creatinine is important because the concentration of organic acids will vary substantially, depending upon the volume of urine produced in a defined time period (typically 24 h). Creatinine is an excellent reference because it is produced in plasma at a constant rate and within a narrow concentration range. Creatinine has been used to estimate the glomerular filtration rate and, as such, is an excellent reference standard for urine output. Creatinine is most often measured by non-MS-based assays,[54] although one procedure used GC/MS.[55]

Following extraction, organic acids are derivatized most commonly by trimethylsilylation. TMS derivatives are characterized by their ease of preparation, good chromatographic separation, and characteristic M − 15 fragment ion (loss of CH_3) as shown in Figure 3. The most frequent alternative to TMS derivatization is *tert*-butyldimethylsilylation. This derivative offers improvements in hydrolytic stability and better GC separation. This method of derivatization is helpful in the analysis of more volatile organic acids and short-chain fatty acids by increasing their retention time and making them slightly less volatile. An alternative to forming silyl derivatives is methylation, using diazomethane and other reagents.[56] These derivatives are very stable and produce informative mass spectra. Other derivatization schemes have been used frequently.[41,57,58] Certain classes of organic acids are difficult to derivatize with standard techniques. For example, ketoacids are unstable and require methods that convert these compounds to methoxime and ethoxime TMS derivatives.[59] These derivatives provide characteristic fragment ions, M − 15, M − 31, or M − 45 that help to identify the molecular ions and keto groups.

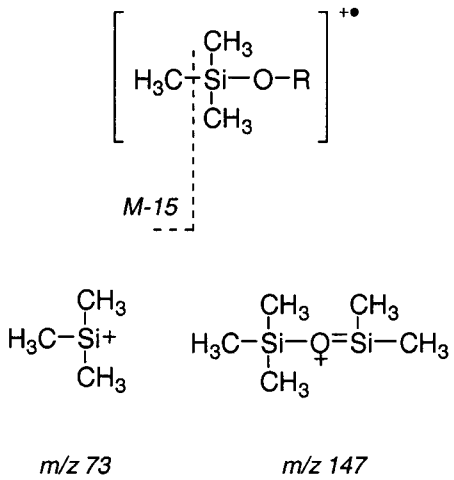

Figure 3. Illustration of common fragments produced by electron ionization of TMS derivatives of organic acids.

Chromatography[40] of organic acids is performed using a variety of capillary columns and stationary phases, including DB-1, SE-30, OV-1, and OV-17. Mass spectrometric analyses are most commonly performed on quadrupole MS instruments with electron ionization. This use of quadrupole MS analysis is significant because most searchable MS reference libraries are composed of EI spectra generated using quadrupole mass spectrometers. Practitioners of GC/MS routinely use these libraries as a reference in the identification of organic acids. GC quadrupole ion trap MS (GC-QIT) and GC/MS/MS methods are both used in several laboratories.[60] Other techniques rely on different methods of ionization such as chemical ionization (CI),[57,61,62] which offers the advantage of producing observable molecular ions. Selected ion monitoring (SIM)[63] is the standard method for compound specific analysis with improved sensitivity. Isotope dilution mass spectrometry (IDMS) techniques provide more accurate quantitative results for many analytes.[64] Other improvements in the quantitation of urine organic acids have also been described.[65]

c. Plasma Organic Acids and Free Fatty Acids by GC/MS. Organic acids are isolated from biological fluids which contain protein, e.g., plasma. These sample preparation methods require deproteinization either by solvent precipitation, membrane ultra filtration,[66] or other techniques.[67] The analysis of short-chain and very long-chain fatty acids in plasma, using GC/MS, has been described,[68−70] with recent improvement in plasma free fatty acid analysis described by Jones et al.[71]

d. Clinical Applications. Ozand and Gascon define an organic acidemia as the accumulation of organic acids in cells or body fluids and is indicative of a disorder of intermediary metabolism.[72,73] More than 50 phenotypically different organic acidemias[37] (organic acidurias) are known primarily because of metabolic profiling with GC/MS. Organic acidemias arise from (i) defects in branched-chain amino acid metabolism, e.g., isovaleric acidemia, propionic acidemia, β-ketothiolase deficiency, and 3-methylcrotonyl-CoA carboxylase deficiency; (ii) vitamin metabolism, e.g., methylmalonic acidemia, vitamin B12 deficiency, and other cobalamin defects; (iii) flavoprotein metabolism disorders, e.g., short-, medium-, and very long-chain acyl-CoA dehydrogenase deficiencies, glutaric acidurias type I and II; (iv) lipid metabolism disorders; (v) disorders in glycolysis, e.g., pyruvate dehydrogenase deficiency; (vi) gluconeogenesis; (vii) citric acid cycle disorders, e.g., fumaric aciduria, 2-ketoglutaric aciduria; and (viii) disorders of glutathione metabolism.

Propionic acidemia is an inherited metabolic disorder of propionyl CoA metabolism caused by a deficiency of propionyl CoA carboxylase.[37] Symptoms of the acute disorder that presents early in infants are lethargy, vomiting, acidosis, hypogylcemia, hyperammonemia and possibly death.[37] Patients with chronic propionic acidemia present symptoms of hyperglycinemia, ketoacidosis, and vomiting after protein ingestion, developmental delay, and seizures. Treatment involves protein restriction and administration of biotin and L-carnitine. Propionyl-CoA is an intermediate in the catabolic pathway of leucine, isoleucine, valine, methionine, and threonine. Propionyl-CoA is converted to methylmalonyl-CoA by propionyl-CoA carboxylase (an enzyme that requires biotin as a coenzyme). Deficiency of this enzyme results in a marked accumulation of propionyl-CoA and propionic acid. The accumulation of propionic acid causes acidosis in blood. Propionic acid is removed by metabolism through other pathways. The disorder is diagnosed by using an organic acid profile obtained by GC/MS, as shown in Figure 4, when compared to control urine specimens. The most diagnostic compounds for this disease are propionic acid, methylcitric acid, and 3-hydroxypropionic acid derivatized as their TMS derivatives (Figure 5); their respective mass spectra are provided in Figure 6. The molecular ions of these compounds are generally small or absent, with (M − 15) ions produced by the loss of $CH_3^{\bullet}$ indicating the molecular weight (Figure 3). In addition, timethylsilyl derivatives are characterized by an intense ion (Figure 3) at *m*/*z* 73, $(CH_3)_3Si^+$. Compounds with two or more silyl groups produce an ion at *m*/*z* 147 that is formed by rearrangement (Figure 3)[19] to give $TMS{-}O^+{=}Si(CH_3)_2$. Propionic acid is not normally detected as a TMS derivative because of its high volatility. However, propionic acid can be indirectly detected as a glycine conjugate using GC/MS.

Early detection of organic acidemias can prevent severe complications. Some investigators have used GC/MS in newborn screening by developing a rapid simplified GC/MS analysis of urine samples.[74] In addition to identifying organic acidemias, GC/MS analysis of organic acids has been helpful in the diagnosis of vitamin B12 deficiency[75] and in the management of diabetes.[76] The analysis of fatty acids, using GC/MS, has been helpful in prostate cancer diagnosis.[77]

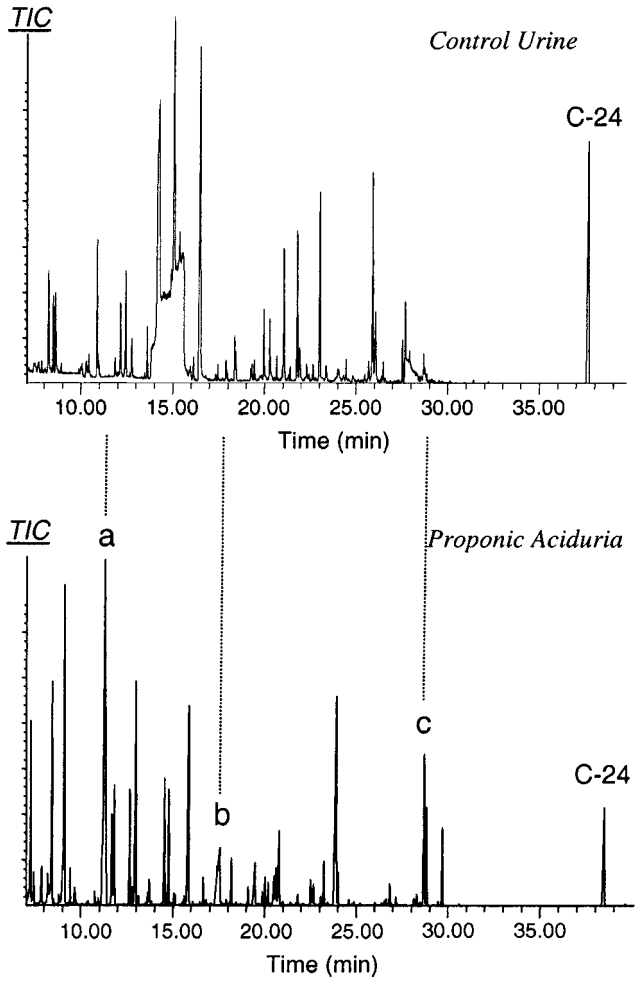

Figure 4. Total ion chromatograms (TIC) from derivatized urine extracts of a control patient and a patient with propionic acidemia. TMS derivatives of 3-hydroxypropionic acid (a), propionylglycine (b), and methylcitric acid (c) are diagnostic metabolites identified by retention time and mass spectra. C-24 (*n*-tetracosane) is the reference standard. Laboratory data from E. Prence, E. Naylor, and S. Singleton.

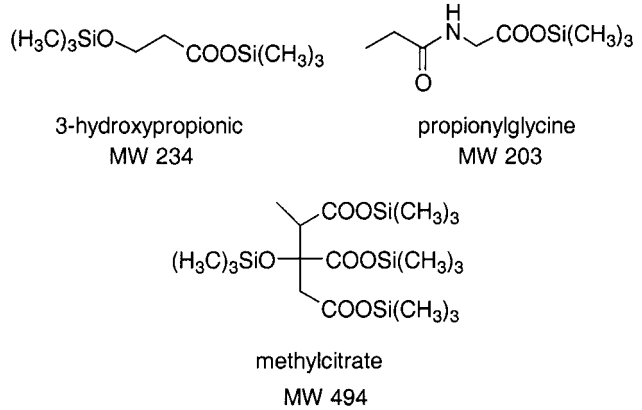

Figure 5. Structures of TMS derivatives of 3-hydroxypropionic acid, propionylglycine, and methylcitric acid.

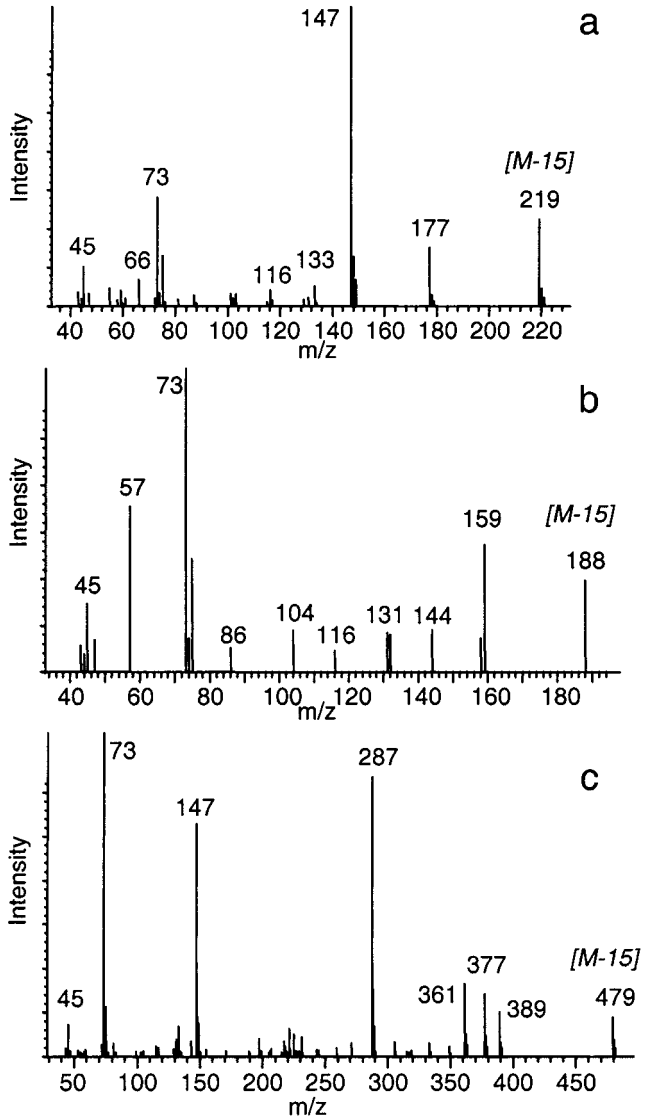

Figure 6. Mass spectra of the TMS derivatives of 3-hydroxypropionic acid (a), propionylglycine (b), and methylcitric acid (c). Molecular ions are characterized by loss of methyl groups (CH_3). Laboratory data from E. Prence, E. Naylor, and S. Singleton.

2. LC-MS Applications

The term and abbreviation, LC-MS, is used to broadly categorize methods that use a mass spectrometer and a liquid interface or delivery system. Liquid chromatography is clearly implied in the abbreviation LC and creates confusion because many "LC-MS" methods use direct flow injection techniques without chromatography. In addition, liquid mobile phases may be delivered via a syringe pump rather than a high-pressure liquid chromatographic "pump". Flow injection techniques use a high-pressure, pulseless delivery of mobile phases composed of aqueous and organic solvents to the mass spectrometer through an uncoated, deactivated, fused-silica capillary, PEEK, or other small inner diameter tubing. No chromatography is intended in these analyses. Furthermore, older ionization techniques such as fast atom or ion bombardment (FAB or FIB), also known as liquid secondary ionization (LSI), and matrix-assisted laser desorption (MALDI) are often characterized as LC-MS methods even though their analysis is often performed on a stationary viscous liquid (LSI) or crystalline matrix (MALDI) and placed in the end of a stainless steel probe or target and manually inserted into a mass spectrometer. To reduce some confusion, where possible, HPLC will be used to broadly categorize those methods in which chromatography is performed. Methods that use flow injection analysis or liquid secondary ionization will be part of the general categories of either LC-MS or MS/MS. Where possible, the ionization mode will be included, e.g., electrospray (ESI), ionspray, atmospheric pressure chemical ionization (APCI), and liquid secondary ionization methods (FAB or FIB). One final note concerning LC-MS methods. The commonality between LSI, FAB, FIB, ESI, MALDI, etc. is that they are soft ionization techniques rather than out-and-out LC-MS methods. These techniques gave mass spectrometrists the first possibility to analyze biological molecules directly without derivatization to aid volatility.

a. Organic Acids. Methods that use HPLC-MS techniques to analyze organic acids are few because HPLC offers little improvement over clinical analysis of organic acids by GC/MS. Liquid chromatographic resolution is relatively poor as compared to capillary GC. Mass spectra formed by LC-MS analysis are primarily protonated molecules and do not contain any important fragment ion information. The use of LC-MS/MS to generate these fragment ions is limited because of the unavailability of product ion libraries of organic acids. Because organic acids generate a negative charge at neutral pH, thermospray or electrospray ionization produces negative ions that are not detected with the same degree of sensitivity as positive ions. To overcome the limitations of negative ions, very acidic or ammonium acetate-based mobile phases[78] have been used to assist in protonation. Negative ionization methods that produce intense $(M - H)^-$ molecular ions are not sensitive and yield little fragmentation data.[79] Recently, Johnson[80] developed a new derivatization scheme for use in positive ion electrospray MS. This scheme produced dimethylaminoethyl esters of carboxylic acids. This application primarily focused on fatty acids. However, this technique may potentially lead to new methods developed with LC-MS in the analysis of organic acids. Recently, Johnson described a method to elucidate peroxisomal disorders,[81] using dimethylaminoethyl esters of long-chain fatty acids. Organic acid disorders have also been detected with LC-MS techniques in other approaches that measured glycine conjugates in urine or acylcarnitines in blood. These methods are described in Section II.D.

C. Amino Acids

Amino acids are compounds characterized by amino and carboxylic acid functional groups.[82] Physiologically important amino acids are known as α-amino acids. Structurally, α-amino acids are composed of an amine, a carboxylic acid, and a side chain denoted as the "R" group, which is attached to a common "central" carbon atom in the α-position relative to the carboxylic acid. These α-amino acids are essential

components of all peptides and proteins. In solution at physiological pH, amino acids are dipolar (zwitter) ions. Amino acids are categorized as neutral, basic, or acidic by either their pK_a or pK_b values, which directly correlate with the composition of the R group. Amino acids are the essential components of proteins. Twenty-two different amino acids have been found in varying amounts in human proteins. These and other important amino acids participate in intermediary metabolism and are precursors to active biomolecules such as vitamins, nucleic acids, and neurotransmitters.

The primary source of amino acids for protein synthesis is dietary protein. Proteins are enzymatically converted to amino acids in the gastrointestinal tract (proteolysis), and are absorbed in blood, where they are available to participate in catabolic and anabolic pathways. Approximately 10 of the 20 most common amino acids are essential amino acids and must be obtained in the diet. Many amino acids are readily interconverted to other amino acids by transamination. Metabolism includes conversion of amino acids to ammonia and organic acids by deamination. Many inherited disorders of amino acid metabolism are characterized by a significant elevation in the concentration of certain amino acids in blood (amino acidemias) and urine (amino acidurias), ammonia, and organic acids (organic acidemias).[12,19,20] Abnormal concentrations of amino acids may also be found in blood or urine as a result of organ failure and impaired function from disease or immature development.[21,82]

HPLC and ion-exchange chromatography are the predominant methods for quantitative amino acid analysis in clinical laboratories.[11,21,38] Improved specificity can be achieved by combining chromatographic and mass analysis with GC/MS. This approach is similar to that for organic acid analysis and requires extensive sample preparation and derivatization. More recently, MS/MS has been applied to the very rapid screening for amino acids.[54] This method of analysis requires no chromatography and is accurate, sensitive, and selective. IDMS techniques are used in quantitation. The method is limited to screening or analyte-specific quantitation because it does not detect all important amino acids. Special sample preparation techniques are required prior to the analysis of disulfide-forming amino acids, e.g., homocysteine and cysteine. Chromatography is necessary to separately measure isomeric amino acids, e.g., leucine, isoleucine, alloisoleucine, and isobaric amino acids (e.g., hydroxyproline). Recently, some development toward rapid capillary HPLC methods with MS/MS detection has occurred as reported at the 4th International Society of Neonatal Screening Conference.[83]

1. GC/MS Applications

Amino acids are often analyzed by GC/MS by using methods similar to that for organic acids[47,74] Most amino, carboxylic acid, and hydroxyl functional groups of amino acids easily form TMS derivatives.[47] Analysis of amino acids in urine has been problematic because of the high concentration of urea. Large amounts of urea adversely affect chromatographic and mass spectrometric analysis of several amino acids. A solution to this problem has been the pretreatment of urine with urease, which reduces the concentration of urea in a specimen.[47,74] Due to their similarity and common derivatization schemes, organic acid and amino acid analysis have been combined in a single analytic run.[84]

In addition to forming TMS derivatives, amino acids can be converted to their *tert*-butyldimethylsilyl (TBDMS) derivatives.[85] Also, a single-step esterification procedure, using ethyl chloroformate, has been developed.[86] Many GC/MS methods employ isotope dilution techniques for quantitative amino acid analyses[52,87−90] with excellent reproducibility and precision. Ionization methods include electron impact (EI) and chemical ionization (CI) for positive and negative ions.[91] Modified amino acids have also been found in disease states, and their measurement was obtained with GC/MS; for example, identification of *N*-acetyl amino acids in urine.[92]

2. LC-MS Applications

a. MS/MS Applications. MS/MS was first used in the clinical analysis of amino acids in plasma and blood, using liquid secondary ionization (fast ion bombardment, FIB).[24,93−95] The early studies required the use of manual sample introduction techniques. Semi-automated flow injection,[96] using FIB ionization, was developed to facilitate automated sample introduction and higher throughput. However, this method was somewhat tedious because of problems regarding sample retention on the probe tip and frequent blockage in the capillary at the probe surface. With the introduction of electrospray ionization, significant improvement in sample throughput and automated sample analysis was realized.[97−99] ESI-MS/MS has been subsequently shown to be a robust, rapid, and accurate method for rapid throughput, high sample volume, and amino acid analyses as demonstrated by validated methods used successfully in newborn screening or clinical amino acid analyses.[2,98−105]

Historically, GC/MS has played the primary role in clinical chemistry for the diagnosis of inherited metabolic disorders. However, applications of MS/MS in the analysis of dried filter paper blood samples for purposes of screening inborn errors of metabolism is now sharing in this primary role. Clearly, MS/MS applications are making an extraordinary impact in increasing the number of metabolic disorders screened in newborns.[2,102,103] The MS/MS analysis of amino acids requires no chromatographic separation and is extremely rapid (~2 min per sample). It is highly accurate, selective, and precise. For example, the MS/MS analyses of newborn blood samples for PKU has been shown to significantly lower the false-positive rate by at least 10-fold as compared to other techniques that use HPLC, fluorometry, and bacterial inhibition.[83,106] MS/MS also demonstrated the ability to accurately detect PKU in samples collected within the first 24 h. Blood specimens collected from infants less than 24 h after birth increase the risk of a false-negative result because the diagnostic metabolite,

phenylalanine, may not have reach sufficient concentration to be diagnostic for PKU. Time of collection is also important for other disorders characterized by the accumulation of toxic metabolites, e.g., MSUD, homocystinuria, etc. For MS/MS techniques, the threshold concentration, i.e., the concentration that is used to decide whether a sample result is presumptive for a disease, can be altered in such a manner to reduce false negatives without raising the false-positive rates substantially.

The sample preparation required for the MS/MS analysis of amino acids in blood specimens is relatively simple. The use of methanol to extract amino acids and other organic compounds from the dried blood imbedded in a filter paper matrix results in high extraction efficiencies. This extract is also free of proteins and high salt concentrations that could otherwise form adducts with important analytes, thereby reducing sensitivity.[24] Furthermore, stable isotope-labeled amino acid standards are added to the methanol extract and are subsequently used to quantify amino acids.[24,83] Note, however, that methods that use extraction solvent that contains internal standards will not account for the losses of analyte during the extraction process. Therefore, this method of quantitation of amino acids is not entirely the same as traditional IDMS methods, in which the internal standard is mixed with liquid samples. The use of the term, pseudo-isotope dilution mass spectrometry (pseudo-IDMS) should be considered as a way to indicate that the manner in which stable isotopes are used in filter paper blood specimen analyses is a variation of traditional IDMS methods.

b. Filter Paper Blood Specimens. Although the analysis of dried filter paper blood samples with pseudo-IDMS is less accurate than liquid specimens with IDMS, there are some advantages in their use. These advantages include ease of collection, reduced biohazards for some bacteria and viruses that are killed on exposure to air, and improved storage and pre-sample cleanup for methods that use organic solvent extraction. Nevertheless, there are some limitations that require mention. The volume of dried blood samples on filter paper is imprecise.[107,108] Therefore, much of the high accuracy of MS methods that use isotope dilution techniques, i.e., addition of known concentrations of internal standard to known volumes of liquid, is lost. The estimated volume of blood obtained from a dried filter blood sample can be calculated from the area of the excised sample and the mean blood volume per square inch or square centimeter. This estimated volume is based on a 50% hematocrit and a quantity of liquid blood applied to the paper.[109] Blood spot diameters and blood hematocrit that are different from the NCCLS reference standard[109] will produce errors in the calculated blood volume of the excised filter paper dot.[108] Even with these limitations, the use of filter paper blood specimens is increasing, especially in the age of the genome, where DNA can be extracted and analyzed.

Following amino acid extraction, analytes are derivatized to butyl esters with acidified butanol (3 N HCl in butanol or butanol with acetyl chloride). Butylation of the amino acids that contain mono- and

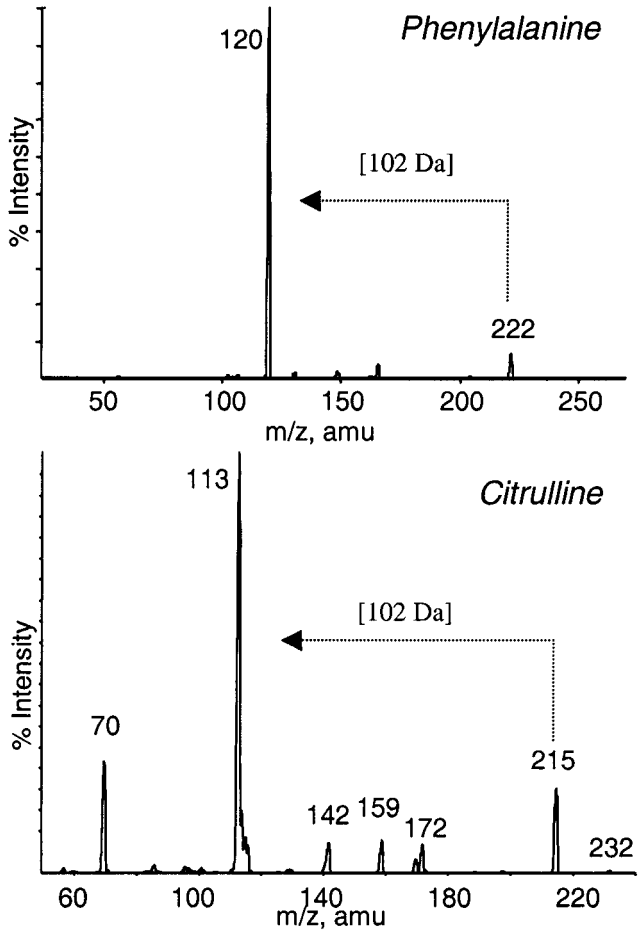

Figure 7. Product ion ESI-MS/MS of butyl esters of phenylalanine $(M + H)^+ = 222$ Da and citrulline $(M + H^+) = 232$ Da. Laboratory data from D. Chace and E. Naylor.

dicarboxylic acid groups greatly improves ionization efficiencies and hence analytical sensitivity, especially for LSI-MS methods. Furthermore, esterification improves the mass differentiation of dicarboxylic acids such as glutamate and aspartate from other amino acids. Reconstitution of analytes in acetonitrile:water (50:50) with 0−1% formic acid has been used as the mobile phase in most procedures.[83]

The selective MS/MS analysis of several diagnostically important amino acids is obtained via a neutral loss of 102 Da (NL 102) scan function. Butyl formate (102 Da) and a product ion that is 102 Da less than its precursor ion are produced by collision-induced dissociation (CID) of protonated butylated α-amino acids. An example of the product ion spectra of the butyl esters of amino acids, e.g., phenylalanine, is shown in Figure 7. A schematic of the CID for phenylalanine is shown Figure 8. The neutral loss of 102 Da MS/MS profile of a normal newborn and a newborn with the inherited metabolic disorder PKU (phenylketonuria) is presented in Figure 9. PKU is an inherited disorder produced by the absence of phenylalanine hyrdoxylase, resulting in a reduction in phenylalanine (Phe) metabolism and a subsequent hyperphenylalaninemia. Tyrosine (Tyr) is the end product of phenylalanine hydroxylase, and the tyrosine concentration in blood is potentially reduced in conditions where phenylalanine hydroxylase is deficient. However, this expected tyrosine deficiency

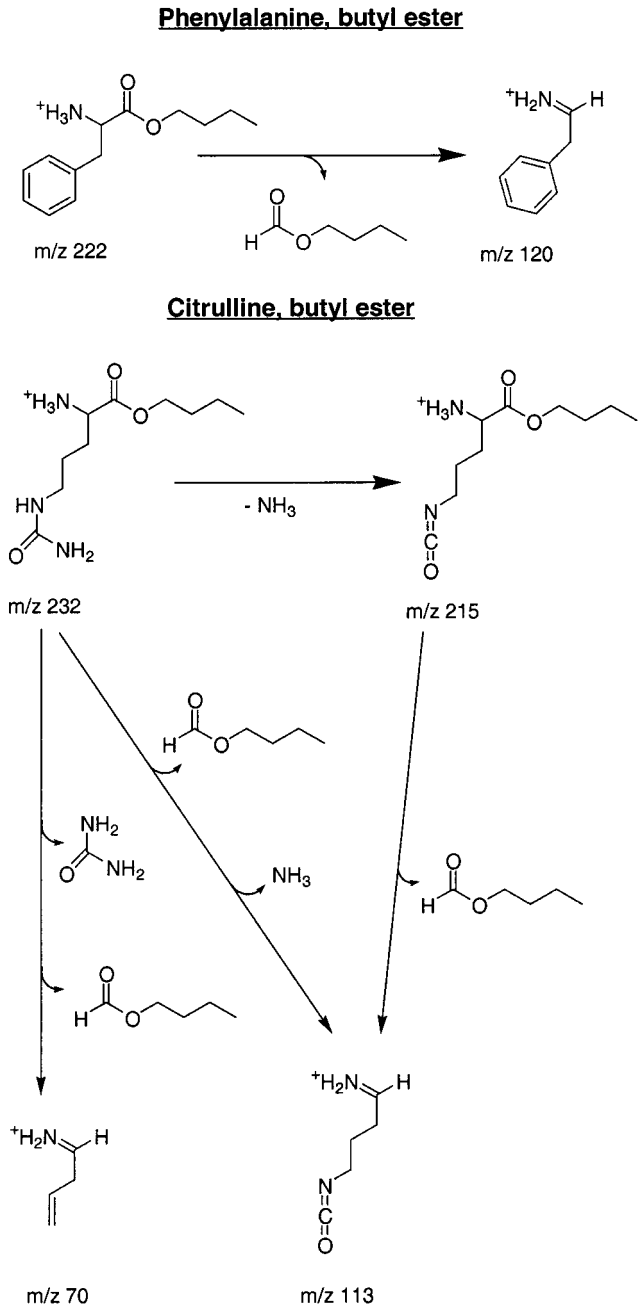

Figure 8. Schematic of collision-induced dissociation (CID) of the protonated butyl esters of phenylalanine and citrulline.

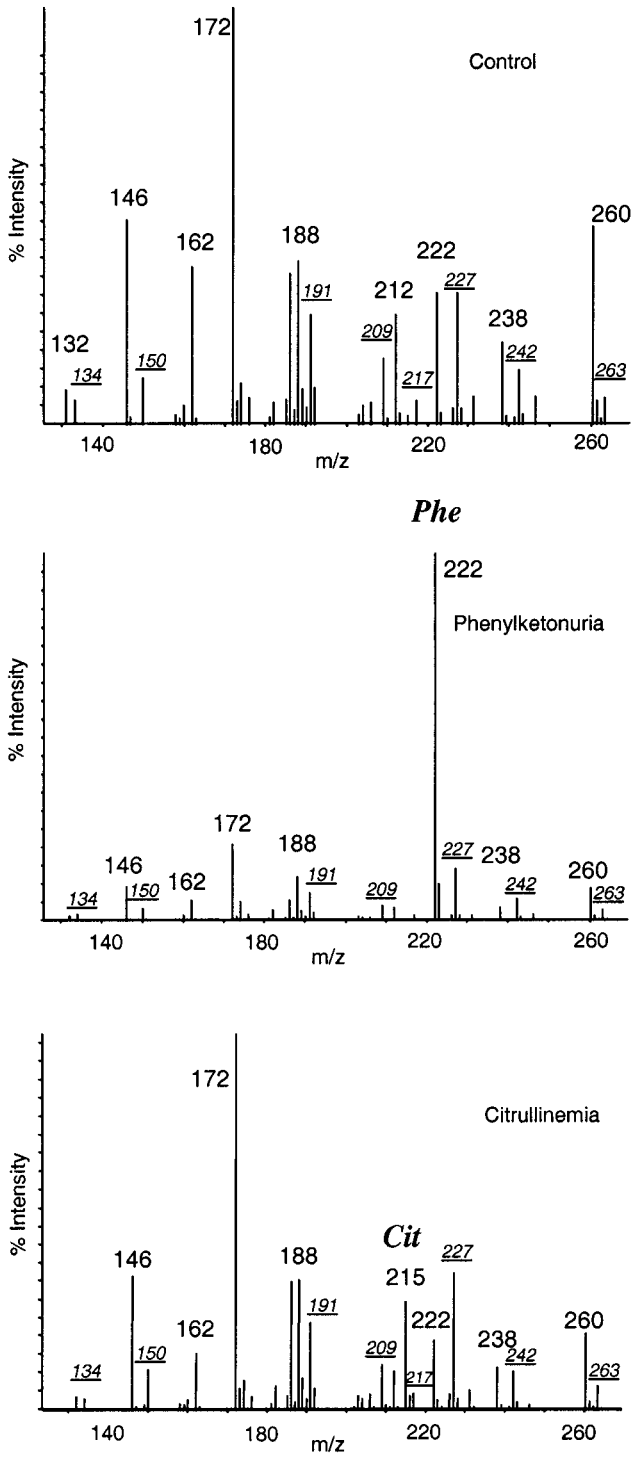

Figure 9. Neutral loss of 102 ESI-MS/MS of butylated, dried-blood spot extracted amino acids from a normal newborn (top) and newborns diagnosed with phenylketonuria (middle) and acute neonatal citrullinemia (bottom). Stable isotope internal standards are underlined italic. Laboratory data from D. Chace, E. Naylor, and J. DiPerna.

may not be substantial if significant amounts of tyrosine are derived from dietary sources. Because these two amino acids are the substrate and product of phenylalanine hydroxylase, their simultaneous measurement and concentration ratio (Phe/Tyr) is a very sensitive indicator of PKU.[106] In the analysis of a sample obtained from a patient with PKU, a significant elevation in phenylalanine (*m/z* 222) relative to its internal standard, phenylalanine-d_5 (*m/z* 227) is observed in Figure 9. Other amino acids are also measured, including tyrosine (*m/z* 238), alanine (*m/z* 146), leucine + isoleucine (*m/z* 188), glutamic acid (*m/z* 260), and their respective internal standards.

Some basic amino acids are analyzed with alternative scan functions. These scans account for the loss of ammonia or other basic side chains in addition to butyl formate. A NL 119 Da scan function is used to detect ornithine, citrulline, and homocitrulline. Citrulline can also be detected in a NL 102 scan from the $(M + H - 17)^+$ produced by nozzle-skimmer or orifice-induced dissociation in the electrospray source. The product ion scan for citrulline butyl ester (*m/z* 232) is presented in Figure 7. A representation of CID of citrulline is shown in Figure 8. An ion at *m/z* 113

(Figure 7) represents the loss of 119 Da from the precursor ion of citrulline at *m/z* 232, and its CID is represented in Figure 8. Other important fragments of citrulline include *m/z* 215, representing the neutral loss of ammonia (M − 17), and *m/z* 70, representing the neutral loss of 162 Da produced by losses of butyl formate[104] and $H_2N-CO-NH_2$. Interestingly, citrulline is also detected in a NL 102 scan analyses at *m/z* 215. Citrulline loses ammonia in the electrospray source prior to analysis in the first quadrupole. Therefore, the source-induced fragment ion with a mass/charge ratio of 215 is detected in MS-1. This ion is subject to a neutral loss of 102 Da in the collision cell producing a product ion at *m/z* 113.

Acute neonatal citrullinemia is characterized by a substantial increase in the concentration of citrulline in blood and plasma. A MS/MS of a NL 102 profile of citrulline, using the electrospray source-induced fragmentation pathway described above, is shown in Figure 9. A significant elevation of citrulline butyl ester (M − 17, *m/z* 215) is shown relative to its internal standard, d_2-citrulline butyl ester (M − 17, *m/z* 217). In addition to a NL of 102 profile, a NL 119 profile or MRM analysis of the ions 232/113 (precursor/product or Pre/Pro) can be used to measure citrulline. Other amino acids, including ornithine and arginine, are detected via similar approaches, e.g., ornithine using NL 119 and arginine using NL 161 scan functions.[2,102]

c. HPLC-MS and HPLC-MS/MS. In addition to MS/MS approaches that use flow injection analysis, other methods use chromatographic separation prior to MS or MS/MS analyses. Tuchmann[110] described a pseudo-IDMS method for the analysis of phenylalanine and tyrosine in filter paper blood samples by selected ion monitoring. A benzoylation derivatization procedure has been used prior to HPLC-MS analysis[111,112] to simplify the analysis of liquid specimens. This procedure avoids many tedious steps such as protein precipitation, drying, and the use of pyridine. The combination of MS/MS and HPLC is under development in several laboratories and may be an even more powerful technique for the quantitative clinical analysis of amino acids.

3. Homocysteine

Homocysteine is a branch-point metabolic intermediate in a pathway that produces cysteine from methionine or reconversion back to methionine. Homocysteine is unstable in solution. In excess, homocysteine undergoes conversion to its disulfide, homocystine. Structures of homocysteine and homocystine are presented in Figure 10. Homocysteine is the main biochemical marker for inborn errors of transulfuration. Homocystinurias are disorders characterized by increased concentrations of homocysteine in urine and plasma. The most common homocystinuria is produced by cysteine β-synthase deficiency. This enzyme deficiency produces a metabolic block of the conversion of homocysteine to cystathionine,[82] resulting in the accumulation of homocystine and methionine in plasma. Clinical symptoms that include ocular, skeletal, and vascular abnormalities are not detectable in the newborn

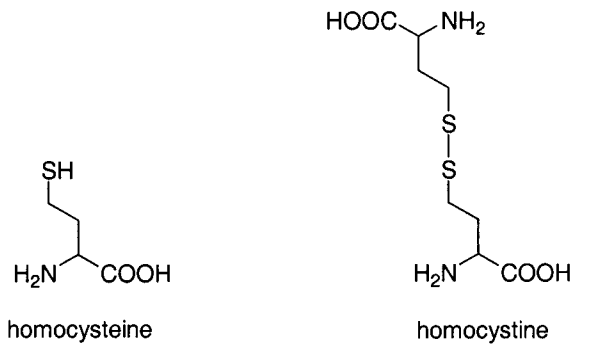

Figure 10. Chemical structures of homocysteine and homocystine.

period but do become more apparent with age. Premature death can arise from venous and arterial thrombosis. Disorders of the folate-dependent methylation of homocysteine, part of the methionine recycling pathway, produce mental retardation. Neonatal screening is the only practical means to screen for these disorders early.

Recently, the importance of plasma levels of homocysteine has emerged as an indicator of risk for cardiovascular disease, although the relationship between elevated homocysteine and cardiovascular disease is not completely understood.[82] In addition, elevated plasma homocysteine may indicate folate and cobalamin cofactor deficiencies, which can contribute to neural tube defects. Increasing folic acid in the diet is believed to decrease levels of homocysteine in plasma and thereby reduce the risk for cardiovascular disease and neural tube defects. With either newborn screening for inherited metabolic defects or adult population screening for risk factors in cardiovascular disease, MS is emerging as an accurate and precise method for measuring homocysteine in plasma and (potentially) newborn blood samples.

a. GC/MS and LC-MS Applications. More than 70% of homocysteine is bound to plasma proteins via disulfide bonds with other thiol-containing amino acids. It is also present as free circulating disulfide (homocystine) and as a mixed disulfide with cysteine. Measurement of homocysteine requires reduction of the disulfide bonds that are formed by homocysteine and other thiols prior to analysis. Preventing reformation of the disulfide bond is necessary with GC/MS and LC-MS methods.[113]

Several GC/MS methods used to quantify homocysteine in plasma and urine have been published.[114−120] Many of these assays use β-mercaptoethanol[118,119] to reduce disulfides to the free thiol compounds. Sample purification includes the use of cation- or anion-exchange chromatography. Isotope dilution techniques and selected ion monitoring are used to provide accurate, precise, and sensitive quantitation. Ubbink[121] compared several homocysteine assays and found poor agreement of a comparison between GC/MS and other techniques such as HPLC, fluorescence, and enzyme immunoassays. These methods expressed different biases in homocysteine quantitation for fasted patients or those subjected to methionine loading. The study concluded that the results of each method could not be interchanged. The

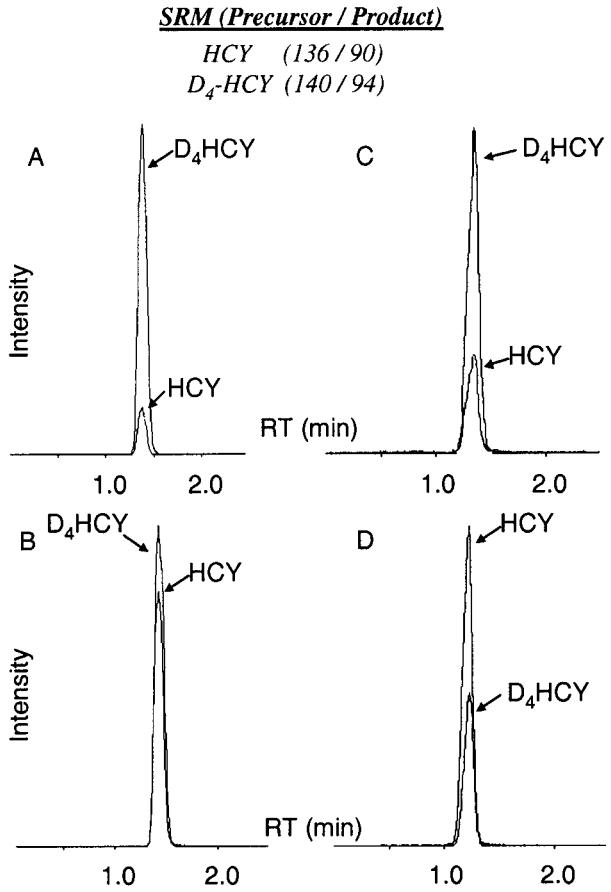

Figure 11. MRM flow injection profiles of homocysteine (HCY) and its internal standard (IS), D_4HCY, for normal plasma (a), abnormal plasma (b), normal urine (c), and (d) abnormal urine. Laboratory data from P. Rinaldo and M. Magera.

Centers for Disease Control (CDC)[122,123] and others[124] produced a similar report and conclusions.

Newer developments have been made to improve the analysis of homocysteine[125] and related metabolites.[126] Recent publications by Magera et al.[125] and Gempel[127] used continuous flow ESI-MS/MS to analyze total homocysteine from plasma and urine samples with isotope dilution techniques. Disulfides were reduced with dithiothreitol. The product ion tandem mass spectrum of underivatized homocysteine (*m*/*z* 136) is characterized by a prominent fragment ion at *m*/*z* 90 (neutral loss of formic acid). Other ions represent the additional loss of either ammonia or hydrogen sulfide and loss of formic acid, resulting in product ions at *m*/*z* 73 and 56, respectively. This CID is similar to the fragmentation of α-amino acids shown in Figures 7 and 8, with the exception that homocysteine is underivatized in this application.[125] Data were acquired in the SRM mode with the transitions (Pre/Pro) of 136/90 for homocysteine (HCY) and 140/94 for d_4-homocysteine (D_4HCY). Figure 11 shows an overlay of SRM-extracted ion chromatograms of HCY and the internal standard D_4-HCY for control (A) and abnormal (B) plasma samples and control (C) and abnormal urine samples (D).[125] This MS method[125] demonstrated good correlation with the other methods that are used routinely in clinical laboratories.

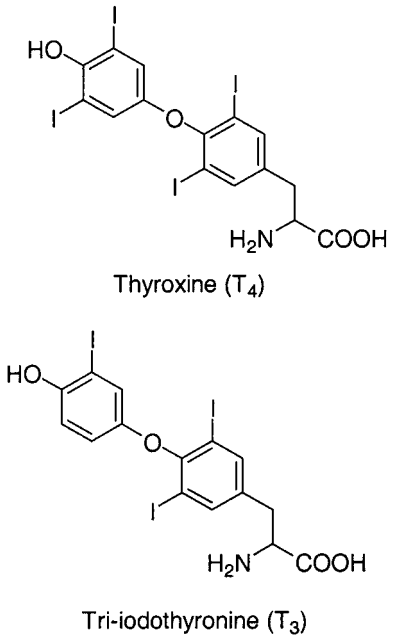

Figure 12. Chemical structures of thyroid hormones T_3 and T_4.

4. Thyroid Hormones

The thyroid gland produces two major hormones, thyroxine (T_4, tetraiodothyronine) and triiodothyronine (T_3). The chemical structures of these compounds are shown in Figure 12. T_3 is the most biologically active of these hormones and is 5-fold more potent than T_4. The thyroid gland primarily secretes T_4; peripheral deiodination of T_4 accounts for nearly 80% of T_3 production.[128] These thyroid hormones have many important actions, including regulation of metabolic rate, growth, maturation, and development.

Disorders of thyroid hormone metabolism include hyper- and hypothyroidism.[128] Each metabolic disorder is expressed by different clinical symptoms and produces characteristic physiological effects. Hypothyroidism is defined as a deficiency of thyroid activity. It is relatively common in mild or severe forms and occurs predominantly in women with advancing age. Primary hypothyroidism occurs as a result of decreased production in T_3 and T_4. Deficiencies of T_3 and T_4 cause hypersecretion of thyroid-stimulating hormone (TSH). Congenital hypothyroidism is produced from inherited defects in the synthesis of thyroid hormones or the absence of a thyroid gland.[37] Irreversible neurological damage will occur if this disorder is not detected in the newborn period. Detection of hypothyroidism relies upon measurement of high TSH levels or low thyroid hormone (T_3 and T_4) concentrations in blood. Hyperthyroidism is characterized by elevated levels of thyroid hormones together with suppression of TSH concentrations in blood. It is a relatively rare disorder with hyper "stress" symptoms such as weight loss, fatigue, nervousness, and restlessness.

The clinical analysis of thyroid hormones in plasma is used to diagnose thyroid diseases.[38] Total T_4 is the sum of free T_4 plus T_4 bound to plasma proteins. The determination of total T_4 reflects thyroid hormone

production.[128] However, measurement of free T_4 provides improved diagnostic information because it is unaffected by the elevated concentrations of plasma proteins that occur in various clinical states such as pregnancy. T_3 is a poor measurement of thyroid activity because its concentration in blood is highly variable; it fluctuates rapidly in situations of physiological stress or other diseases. TSH analysis may be a better indicator for detection of thyroid disorders because TSH reflects the integrative action of all thyroid hormones. Many laboratories measure plasma thyroid hormone and TSH concentrations to provide the most complete diagnostic information.

Mass spectrometric methods have been developed to analyze the thyroid hormones T_3 and T_4 primarily by GC/MS[129−139] and most recently by LC-MS.[131,140] The GC/MS methods used in T_3 and T_4 quantification are similar to procedures use to analyze amino acids. Derivatization methods include trimethylsilylation,[136] trifluoroacetylation,[134,135] methylation,[132,133] and the formation of *N*,*O*-diheptafluorobutyryl methyl esters.[129,130] Mass spectrometric analysis is primarily characterized by isotope dilution GC/MS with SIM of the thryoid hormones and their associated isotopically labeled internal standards. The results from these studies demonstrate excellent precision and compare well to other methods such as radioimmunossay.[133] MS has been suggested as a reference method for thyroid hormone analysis.[132]

Recently, the use of LC-MS/MS has been applied to the analyses of thyroid hormones. Thienpont[131,140] describes a flow injection ESI-MS/MS analysis following protein precipitation and HPLC column chromatography for sample purification. In both methods, an SRM analysis is used to quantify T_3 by obtaining mass spectra of T_3 (*m*/*z* 652/661) and its internal standards $^{13}C_9$-T_3 (*m*/*z* 606/614). T_4 quantification is obtained similarly by measuring T_4 (*m*/*z* 777/731) and its internal standard, $^{13}C_9$-T_4, (*m*/*z* 783/737). A comparison of GC/MS and LC-MS/MS demonstrated limits of detection of 100 and 18 pg, respectively, with excellent recoveries, precision, and accuracy.[131]

D. Acylcarnitines and Acylglycines

The oxidation of fat plays a major role in energy metabolism especially during fasting periods.[37,141] Fatty acids with carbon chain lengths of primarily 18 carbons or less are metabolized in the mitochondria by a process known as β-oxidation (Figure 13). They are transported through the cellular membrane into the cell cytosol and are translocated across the outer mitrochondrial membrane to form fatty acyl-CoA thioesters.[37] The fatty acyl group is transferred to carnitine and transported into the inner mitochondrial matrix, where it is transferred back to CoA to reform a fatty acyl-CoA plus free unesterified carnitine. The fatty acyl-CoA metabolites undergo oxidation by a complex of membrane-bound and matrix-soluble enzymes (that are "size"-specific), producing acetyl-CoA and fatty acyl-CoA thioesters. Each round of metabolism produces a single molecule of acetyl-CoA and a fatty acyl-CoA that is two carbons shorter. Acetyl-CoA is converted in the liver to other substrates (ketone bodies) that are utilized by other tissues for energy metabolism. Very long-chain fatty acids, primarily 20−26 carbons, are metabolized by peroxisomal β-oxidation to a chain length that is within the range for mitochondrial β-oxidation.[38,69] Intermediates of mitochondrial β-oxidation or peroxisomal β-oxidation are important clinical diagnostic markers for many disorders of fatty acid and organic acid metabolism. Knowledge of these pathways helps in understanding disease process as well as assisting in the interpretation of complex metabolic profiles. For example, in the inherited metabolic disorder, MCAD (medium-chain acyl-CoA dehydrogenase) deficiency, the metabolism of medium-chain length fatty acids is impaired. As shown in Figure 13, this disorder primarily produces an increase in mitochondrial 8-carbon fatty acids as well as significant concentration of 6- and 10-carbon saturated and unsaturated fatty acylcarnitines. These mitochondrial fatty acids can be found in blood and plasma and detected using methods such as MS/MS. Knowledge of β-oxidation assists in the recognition that an impairment of medium-chain length fatty acids would lead to an accumulation of these fatty acylcarnitines. Likewise, very long- or short-chain acylcarnitines and numerous other disorders would likely produce disease-specific metabolic patterns.[37,141]

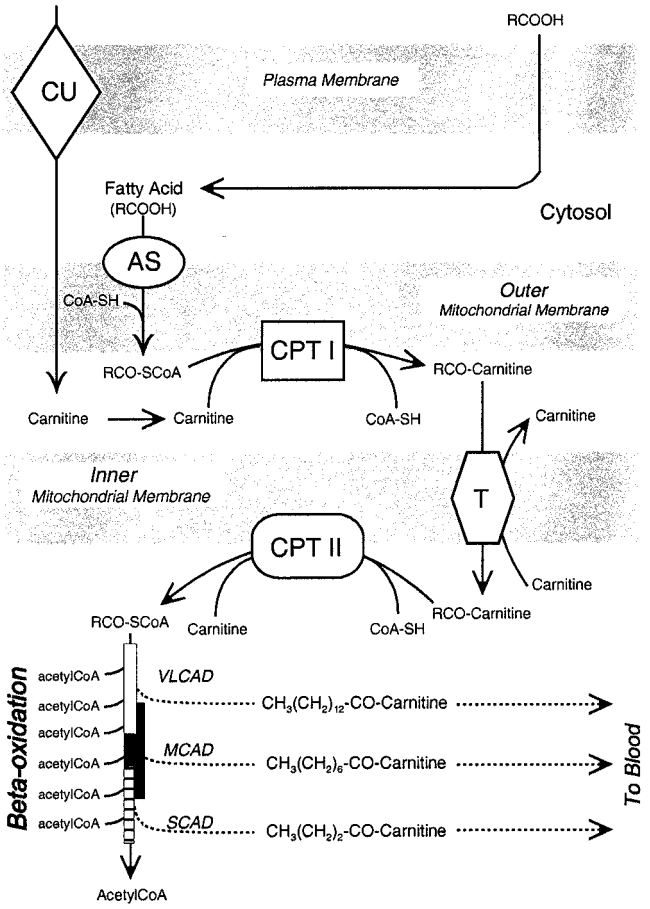

Figure 13. Schematic of β-oxidation of fatty acids.[435] CU, carnitine uptake transporter; AS, acyl-CoA synthase; CPT I and II, carnitine palmitoyl transferase I and II; T, carnitine, acylcarnitine translocase; VLCAD, MCAD, and SCAD, very long-chain, medium-chain, and short-chain acyl-CoA dehyrdogenases, respectively.

1. Inherited Disorders of Fatty Acid and Organic Acid Metabolism

Inherited disorders of fatty acid oxidation are an important class of metabolic diseases; between 20 and 30 disorders have been characterized.[38,141] These inherited metabolic diseases can produce hypoglycemia, vomiting, liver disease, cardiomyopathy, developmental delay, hypotonia, seizures, coma, and premature sudden death.[37] Symptoms can occur early in the newborn period through adult life in varying degrees of severity. Alternatively, these diseases may be asymptomatic until a life-threatening episode. Exacerbation of the fatty acid oxidation disorders occurs especially during fast or inadequate caloric intake whereas organic acidemias are exacerbated by high protein intake. Several disorders in mitochondrial β-oxidation (Figure 13) have been characterized and include very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, short-chain acyl-CoA dehydrogenase (SCAD) deficiency, multiple acyl-CoA dehydrogenase deficiency (MADD, GA-II), carnitine transporter defects, long- and short-chain hydroxyacyl-CoA dehydrogenase (LCHAD and SCHAD, respectively) deficiencies, carnitine-palmitoyl transferase type I and II deficiencies (CPT-I and CPT-II), HMG CoA-lyase, and 2,4-dienoyl-CoA reductase deficiencies.[37]

Disorders of fat metabolism produce significant elevations of free fatty acids in plasma.[37] These fatty acids are eliminated in urine predominantly as a conjugate with glycine (an acylglycine). Fatty acids in the mitochondria form acylcarnitines and are exported into the cell cytosol and plasma. Acylcarnitines are eliminated in bile and urine. In metabolic disorders, carnitine deficiency results in many cases from the continued formation of fatty acylcarnitine and its subsequent loss in urine and bile. Amelioration of these deficiencies may include enhancing elimination of toxic fatty acids as acylglycines or acylcarntines by administration of glycine and carnitine or reducing metabolism of fatty acids by restoring and maintaining blood glucose at normal levels. In the past decade, MS has played a critical role in the analysis of these important biomarkers and is rapidly becoming the method of choice in newborn and clinical screening.[2,22,23]

2. Acylcarnitines

a. GC/MS. Carnitine is a highly water-soluble and polar quaternary ammonium compound that combines with fatty acids to form acylcarnitines of different carbon chain length. The high polarity makes these compounds particularly suitable for LC-MS analysis. However, early studies relied upon hydrolysis of the fatty acyl group followed by GC/MS or, alternatively, upon specialized derivatization techniques. Bieber first characterized acylcarnitines in 1977 with GC/MS analysis of acyl residues following hydrolysis[142] and later characterized short-chain acylcarnitines.[143] The therapeutic value of measuring these fatty acids released from acylcarnitines was recognized by Roe et al.[144] Kerner characterized acylcarnitines in urine with GC/MS following saponification.[145]

Another approach to analyze acylcarnitines by GC/MS is based on the preparation of volatile lactones via cyclization.[146−149] Isolated acylcarnitines are transformed into acyloxylactones and are analyzed by positive CI-GC/MS, using isobutane as reactant gas. The selected ion monitoring of a common ion at m/z 85 and its molecular ions enabled a selective and sensitive detection of all C2−C18 acylcarnitines. Alternatively, Huang developed a novel approach that uses N-demethylated derivatives.[150,151] This approach involved esterification followed by ion-pair extraction with potassium iodide into chloroform and subsequent on-column N-demethylation of the resulting acylcarnitine propyl ester iodides. The products, acyl demethylcarnitine propyl esters, are volatile and are amenable to CI-GC/MS analysis.

b. LC-MS and HPLC-MS. Although GC/MS offers the advantage of characterizing acylcarnitines by chromatography and MS, these methods are tedious and time-consuming. Because carnitine is a preformed cation, it is readily detected with LC-MS as a positive ion with high sensitivity. Therefore, methods utilizing LC-MS and LC-MS/MS have grown rapidly during the past 10 yr for several reasons that include the following: a relatively simple sample preparation and analysis; multiple compound and chemical class analysis; and an accurate, selective, sensitive, and rapid analysis.

One of the earliest clinical applications for acylcarnitine analysis was reported by Roe et al.,[152,153] who used a high-resolution mass spectrometer to detect propionylcarnitine in urine. This work was followed by research that identified several other acylcarnitines with similar methods.[154−156] Acylcarnitines were analyzed underivatized or as methyl esters. HPLC thermospray MS was used to separate on-column mixtures of acylcarnitines[157] and subsequently used to analyze acylcarnitines in biological fluids using isotope dilution techniques.[158] In another application, desorption chemical ionization was used for the analysis of acylcarnitines.[159] FAB-MS was subsequently applied to the analysis of acylcarnitines,[145,160,161] using a quadrupole MS rather than high-resolution sector mass spectrometers. With the introduction of continuous-flow FAB technology, carnitines were soon analyzed with these techniques in combination with HPLC.[162,163] Recently, separation of acylcarnitines by capillary electrophoresis combined with MS detection has been demonstrated.[164]

c. LC-MS/MS. Substantial improvements in the analysis of acylcarnitines and clinical diagnosis of inherited disorders of fatty acid metabolism occurred with the use of a tandem quadrupole mass spectrometer.[165−169] Several organic acidemias and fatty acid oxidation defects were found in the plasma or urine of patients with these disorders. It was recognized that detection of metabolic disorders in the newborn period, prior to clinical symptoms, could prevent hospitalization and premature death. Therefore, the newborn screening MS/MS applications of acylcarnitines extracted from dried filter paper blood samples was developed, using liquid secondary ionization tandem mass spectrometry (LSI-MS/MS) in manual introduction and dynamic modes.[9,93,96] The LSI MS/

MS methods (including FAB and FIB ionization) were further refined including variations and improvements in derivatization[93] (i.e., methyl and butyl esters, validation of clinical methodology for MCAD deficiency,[170] clinical studies,[171−175] and the combination of amino acid and acylcarnitines in a single assay[9,93]).

The number of samples analyzed per day was limited with manual sample introduction LSI-MS/MS (static FAB or FIB). The analysis of hundreds of samples per day required multiple instruments and adequate staff. Automated sample introduction was achieved with continuous flow LSI-MS/MS (dynamic FAB or FIB) although the method was problematic because of sample retention on the probe tip or clogging at the end of the capillary.[96] The use of electrospray ionization MS/MS methods[98−100,176,177] enabled high throughput analyses of clinical samples, without problems encountered in continuous flow FAB- or FIB-MS/MS, and improved sensitivity for several acylcarnitines. Numerous applications of MS/MS to the analysis of acylcarnitines have been published recently that demonstrate the scope of ESI-MS/MS for clinical and newborn screening in plasma, urine, or filter paper blood samples.[2,101−104,178−183] Recently, acylcarnitine analysis has been applied to cultured cells[184−187] obtained from patients with suspected or known metabolic disorders. Further improvement of acylcarnitine analysis by MS/MS is still occurring. One problem identified by Johnson[188] is the hydrolytic instability of acylcarnitines during storage and sample preparation. Short-chain acylcarnitines are highly susceptible to hydrolysis to fatty acids and carnitine.

d. MS/MS Analyses of Acylcarnitines in a Dried Blood Sample. The principals and fundamentals of MS/MS analyses of acylcarnitines have been relatively unchanged since the first studies were published.[93] Acylcarnitines were extracted with ethanol or methanol directly from plasma or dried filter paper blood samples. Isotopically labeled acylcarnitine internal standards are used for the quantification of individual acylcarnitines. Acylcarnitines contain a quaternary nitrogen that carriers a positive charge. However, carnitine also has a carboxyl group that could also carry a negative charge, resulting in a zwitterion. Esterification of acylcarnitines to methyl or butyl esters will prevent the formation of zwitterions, leaving acylcarnitines with a net positive charge. The prevention of formation of a net negative charge is especially true for dicarboxylic acid acylcarnitines.

The MS/MS analysis of acylcarnitines is characterized by a stable product ion at *m*/*z* 99 or 85 for methyl and butyl esters, respectively. An example of the CID for butyl esters of carnitine and acylcarnitines is shown in Figure 14. Underivatized acylcarnitines also produce an intense product ion at *m*/*z* 85. It is important to ensure complete derivatization if butyl esters are chosen as the derivative because of possible loss of selectivity from interference between butyl esters and underivatized acylcarnitines, which both share a common fragment ion. On the basis of the product ion spectra, all acylcarnitines of the same

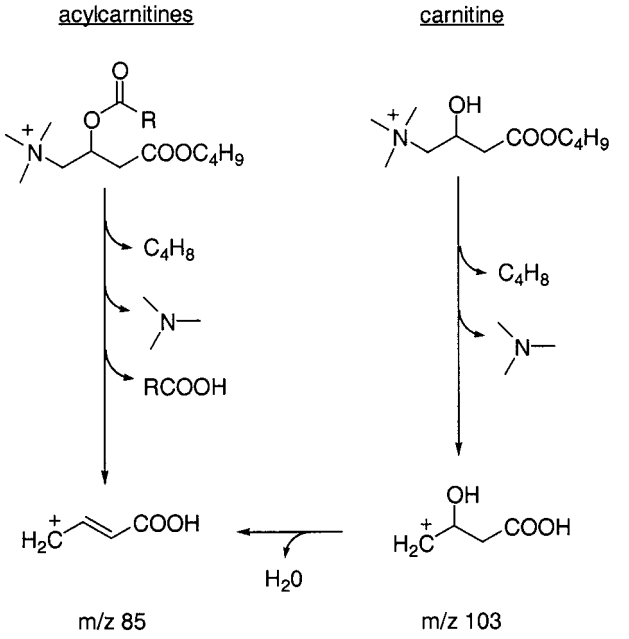

Figure 14. Schematic of collision-induced dissociation (CID) of the protonated butyl esters of carnitine and acylcarnitines. R indicates a fatty acid of 2−20 carbons.

derivatization technique share a common mass for that product ion, e.g., *m*/*z* 99 or 85 for methyl or butyl esters of acylcarnitines, respectively. Precursor ion scans are therefore used to generate a selective MS/MS analysis of acylcarnitines.

One of the most important developments that occurred during the 1990s was multiple metabolite analysis in a single sample injection. For example, both butyl esters of amino acids and acylcarnitines are analyzed concurrently. Furthermore, additional scan functions have been developed for free carnitine and basic amino acids.[2,102] These multiple scan functions have enabled a comprehensive multiple metabolic profile in newborn screening.[103] Disorders of amino acid metabolism, organic acid metabolism, and fatty acid metabolism are measured in a single test. As described previously, the introduction of electrospray ionization led to rapid throughput systems and sample introduction robotics. Automated data reduction and computer-assisted interpretation are commonplace.[98,99,102]

An example of an acylcarnitine analyses, using flow injection ESI-MS/MS from dried filter paper blood samples of a normal newborn and newborn subsequently diagnosed with MCAD deficiency, is shown in Figure 15. The method includes several MS/MS scan modes.[102] There are two full scans (precursors of 85 for acylcarnitines and NL 102 for amino acids) and three specialized MRM analyses (Pre 103 for free carnitine, NL 119 for basic amino acids, and NL 161 for arginine). An example of a the full-scan NL 102 profile for amino acids is shown in Figure 9.

Disorders are indicated when the ratio of diagnostically important metabolites to internal standards is high. In Figure 15, MCAD deficiency was indicated because of the substantially increased concentration (8 μM) of octanoylcarnitine (C8, *m*/*z* 344) and other metabolites (hexanoylcarnitine, C6, *m*/*z* 316, and decenoylcarnitine, C10:1, *m*/*z* 370) as compared to a

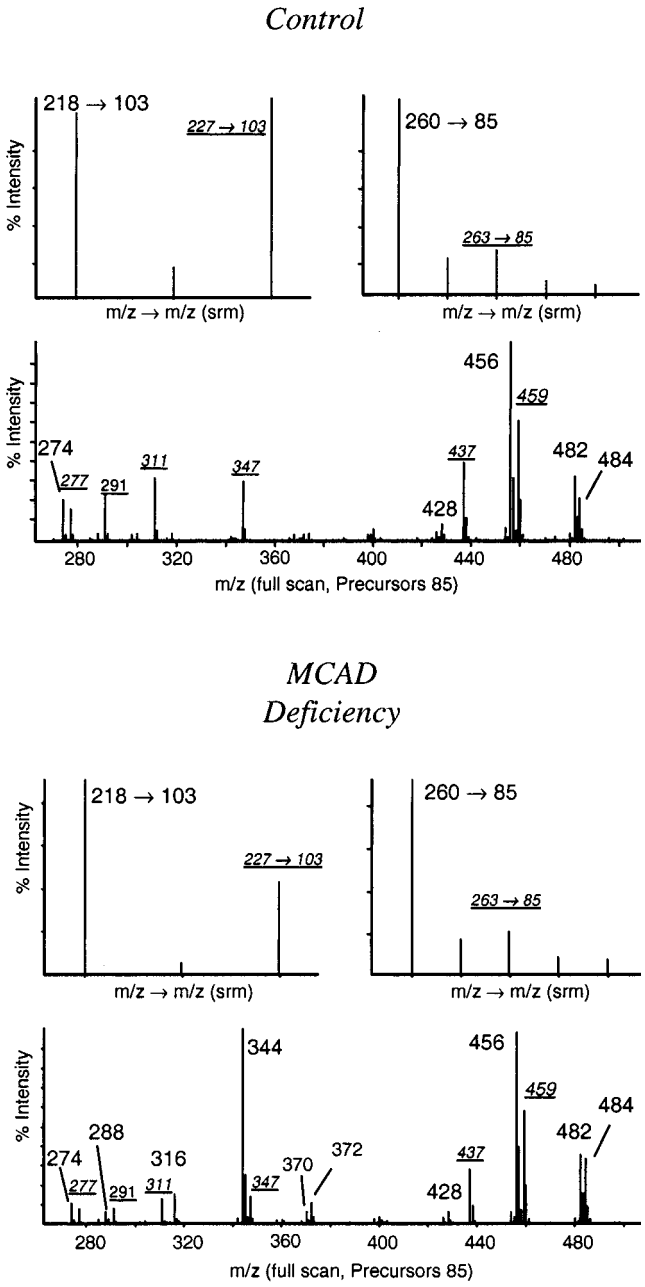

Figure 15. ESI-MS/MS of the butyl esters of carnitine and acylcarnitines from a filter paper blood spot extract of a normal newborn and newborn with MCAD deficiency. MRM transitions are shown (Pre/Pro) for free carnitine (CN) and its internal standard, acetyl (C2), and propionylcarnitine (C3) and internal standards, and a full-scan acylcarnitine profile (Pre 85 Da) of mass range 255−500 Da. Stable isotope internal standards are underlined italic. Laboratory data from D. Chace, J. DiPerna, and E. Naylor.

control samples, where the mean octanoylcarnitine concentration is less than 0.15 μM. Molecular methods were also used to support this presumptive diagnosis of MCAD by mutation analysis for the most common genetic mutation, A985G.[189]

3. Acylglycines and Other Fatty Acyl Conjugates

Another method to diagnose patients with metabolic disorders is to measure by GC/MS glycine conjugates of diagnostic fatty acids and organic acids in urine.[169,190−197] An LC-MS method for acylglycines was described by Rinaldo et al.[198] In addition to measuring acylglycines, investigators have developed methods to detect acyl-CoA thioester intermediates of fatty acid β-oxidation as the *N*-acylglycines by negative-ion chemical ionization GC/MS[199] and acylglucuronides as TMS derivatives by GC/MS.[200]

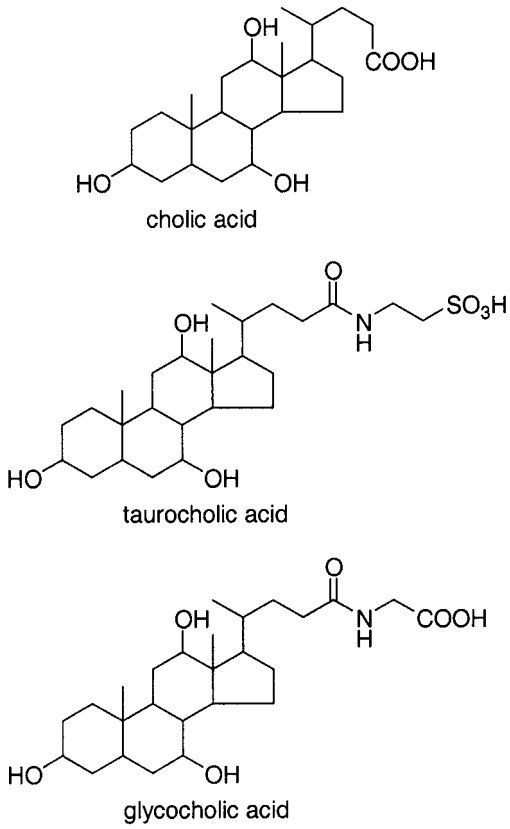

Figure 16. Examples of the bile acid, choline, and its taurine and glycine conjugates.

E. Bile Acids

Bile acids serve important roles in cholesterol metabolism and digestion of lipids.[201] Produced by the liver and secreted in the intestine, bile acids solubilize lipids to facilitate their absorption. Bile acids also serve as the major mechanism for cholesterol homeostasis. The major route of elimination of cholesterol is by excretion of bile acids into the intestine. Cholesterol elimination/conservation is regulated in part by the extent of reabsorption of bile acids in enterohepatic circulation. Therefore, bile acids serve as a major mechanism to regulate cholesterol levels in blood. Most disorders of bile acid metabolism are reflected in an abnormal liver function. There are several metabolic disorders characterized by defective bile acid synthesis or diseases that produce elevated concentrations of bile acids, i.e., certain peroxisomal defects.

There are two primary bile acids, cholic and chenodeoxycholic acid. These acids are conjugated with the amino acids taurine and glycine in the liver to form taurocholic and glycocholic acids, respectively. Figure 16 provides the chemical structures of cholic acid and its taurine and glycine conjugates. Bile acids are synthesized from cholesterol and are produced in the liver primarily as four different bile acid conjugates. These acids contain polar and nonpolar groups that

together serve to solubilize biliary lipids via their detergent action. Conjugation with amino acids increases the water solubility and further augments the detergent properties of bile acids. Several assays have been developed to quantify bile acids in blood.[202] MS has been important in the identification and quantification of individual bile acids in body fluids such as plasma and serum.[203] Methods include GC/MS, LC-MS, and MS/MS. Arguments have been made to include bile acid analyses in newborn screening panels.[204]

1. GC/MS

Bile salts are complex biomolecules of which there are three major classes: (i) unconjugated bile acids, (ii) glycine- or taurine-amidated conjugates, and (iii) sulfate, oxo, and glucuronide conjugates. GC/MS analysis of biles acids provides unequivocal identification through retention time and mass spectra and forms the basis for the claim that GC/MS is a reference method, especially for the determination of stereochemistry.[203] Setchell developed the first applications of MS to the analysis of bile acids.[205−207] The technique uses solid−liquid/liquid gel extractions, ion-exchange chromatography, and bile acid analysis by capillary GC/MS. The techniques demonstrate increased sensitivity as compared with other conventional methods. Several papers demonstrate the applicability of clinical bile acid analysis and disease detection with GC/MS.[208−220]

2. LC-MS

The number and scope of LC-MS techniques for the analysis of bile acids has increased substantially due to improvements in sensitivity, simplicity, and speed. The earliest LC-MS methods for bile acid and bile acid conjugate analyses were published by Setchell et al. using either FAB[208] or thermospray[221] MS. Other applications of thermospray MS have been described.[222,223] In addition, tandem mass spectrometric methods have been used to further characterize bile acids.[224,225] Continuous-flow FAB-MS with flow injection sample introduction was developed for high-throughput applications.[226] More efficient ionization and sample analysis is achieved with spray ionization techniques, i.e., ESI-MS/MS[227,228] and ion spray MS.[229] These methods have been applied to the diagnosis of cholestatic hepatobiliary disease[230] as well as Smith−Lemli−Opitz (SLO) syndrome[231] and other disorders of bile acid metabolism.[232,233]

a. Clinical Application of Bile Acid Analysis Using ESI-MS/MS. The conjugated bile acids glycocholic (GC), taurocholic (TC), glycochenodeoxycholic (GCC), and taurochenodeoxycholic (TCC) were analyzed in dried filter paper blood specimens in infants with cholestatic hepatobiliary disorders with ESI-MS/MS.[228,230] Bile acids were eluted with methanol that contained deuterium-labeled standards of the bile acid conjugates named above. Negative ion ESI-MS/MS was performed, using 50:50 acetonitrile:water as the mobile phase. Glycine conjugates of bile acids produce an intense product ion at *m/z* 74, whereas taurine conjugates produce a product ion at *m/z* 80. MRM analyses of bile acid conjugates and their internal standards extracted from dried blood spots of a normal neonate, a neonate with idiopathic neonatal hepatitis, and extrahepatic biliary atresia (EHBA) are shown in Figure 17. The method is simple, sensitive, and accurate. However, the use of this method in newborn screening is limited by the probability of high false-positive rates estimated at 10%.[230] Introducing a confirmatory test in addition to bile acid screening would enable the consideration of newborn screening for bile acid disorders.

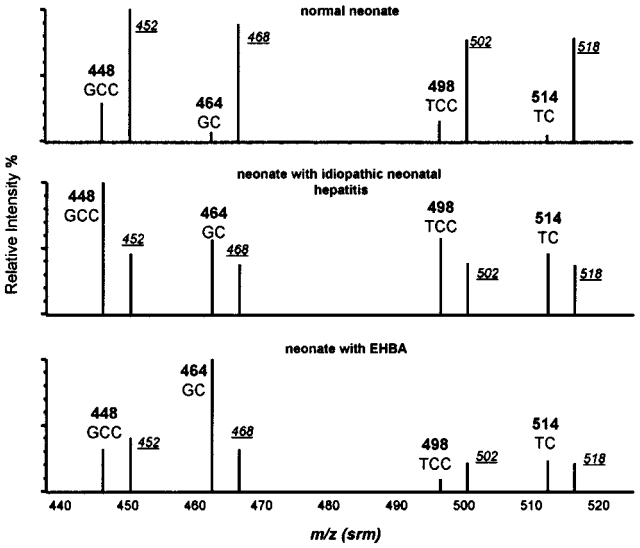

Figure 17. ESI-MS/MS analysis of bile acid conjugates extracted from a filter paper blood spot obtained from a normal neonate (top), a newborn with idiopathic neonatal hepatitis (middle), and a newborn with extrahepatic biliary atresia (EHBA) (bottom). MRM transitions are shown for GC and GCC (Pro 74) and TC and TCC (Pro 80). See text for abbreviations. Stable isotope internal standards are underlined italic. Laboratory data from M. Morris.

F. Cholesterol and Steroids

Steroid hormones are synthesized from cholesterol in the adrenal glands and gonads. Structures of cholesterol and other clinically relevant steroids are shown in Figure 18. The type and quantity of individual steroids produced in plasma depends on which gonads (testes or ovaries) are present and on the activity of the adrenal gland.[234] Steroids are biosynthesized by a complex group of enzymes that includes hydroxylases, lyases, isomerases, and dehydrogenases. Catabolism of steroids occurs primarily in the liver where the potency of steroid hormones is reduced by addition of hydroxyl groups, dehydrogenation, reduction, and conjugation with sulfuric or glucuronic acid. Most steroids are excreted as water-soluble sulfate and glucuronide conjugates.

Disorders of steroid biosynthesis and metabolism that occur in the adrenal cortex are recognized by depressed or excess hormone production. Adrenal hypofunction is a result of adrenal insufficiency of key steroids (Addison's disease), whereas adrenal hyperfunction is characterized by the excess of production of glucocorticoids, mineralcorticoids, and androgens (Cushing's syndrome). The genetically

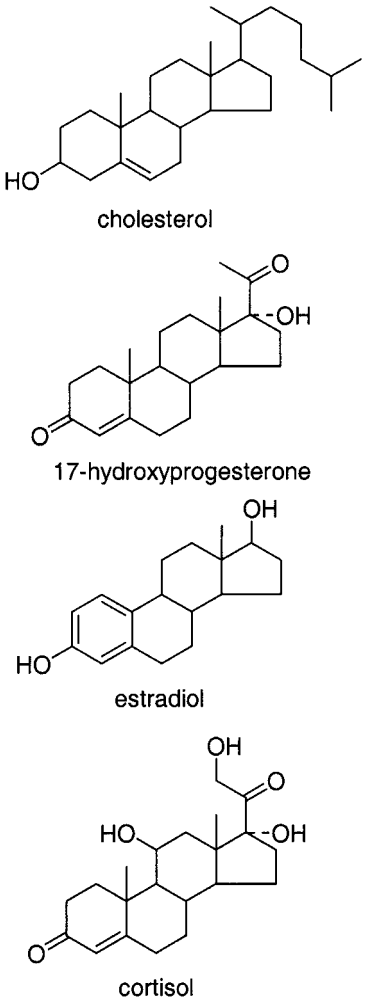

Figure 18. Steroids commonly measured in the clinical laboratory.

inherited disorder, congenital adrenal hyperplasia (CAH), is routinely assayed in many newborn screening laboratories. This disorder produces an excess of 17OH-progesterone as a result of a deficiency of 21-hydroxylase enzyme. The SLO syndrome is a disorder of cholesterol metabolism that is characterized by low blood cholesterol and a marked increase in 7-dehydrocholesterol. The analysis of steroid hormones has been traditionally performed with GC/MS. Diagnosis of these disorders has often involved GC/MS analyses of steroids.[235−237]

1. GC/MS

Isotope dilution GC/MS has been shown to be a reliable, important method for the quantitation of steroids in serum.[14] There have been several publications that describe steroid-specific applications that have been available for decades. For example, GC/MS techniques have been used to quantify progesterone since the early 1980s.[236,238,239] Several recent clinical GC/MS papers have been published that focus on mineralcorticoid analysis and on its relationship to hypertension,[240] whereas other studies have described methods for disease detection such as the SLO syndrome[235,241] and others.[242] Steroid analysis has been used to screen infants with suspected disorders of the metabolism of these compounds.[237]

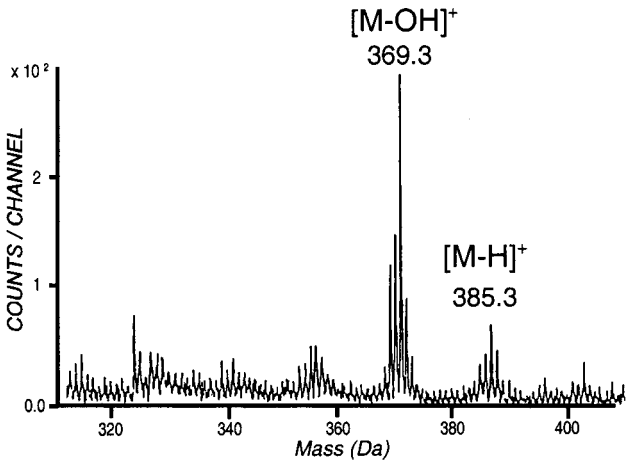

Figure 19. ToF spectrum of cholesterol (*m*/*z* analyzed directly from a filter paper blood sample used as the target matrix). The $[M - H]^+$ and $[M - OH]^+$ ions of cholesterol are observed at *m*/*z* 385.35 and 369.35, respectively. Laboratory data from D. Hercules and P. Zimmerman.

Many other studies have focused on the analysis of androgenic steroids[243−246] as well as testosterone.[247] In addition to the use of quadrupole mass analyzers, ion trap GC/MS methods have now been used in the detection of urinary anabolic agents.[248]

2. LC-MS

The LC-MS analysis of steroids was introduced by Shackleton in the early 1980s using FAB ionization.[249,250] HPLC and thermospray ionization was used in the MS analysis of corticosteroids.[251,252] Comparison of GC/MS analysis with thermospray MS[253] demonstrated that LC-MS was a satisfactory and simple analysis but was not nearly as precise as GC/MS for cortisol measurements. Plasmaspray (discharge-assisted thermospray) has been used to quantify 17OH-progesterone present in samples from the saliva of patients diagnosed with CAH.[254] Substantial quantitative improvements were observed when electrospray ionization[240] was coupled to microbore HPLC. The analysis of anabolic steroids using atmospheric pressure chemical ionization (APCI) LC-MS, has been described by Joos et al.[255] Several methods that use HPLC-MS[256−259] and HPLC APCI-MS/MS[260] have been described.

3. Time-of-Flight MS

The use of time-of-flight MS analysis (ToF-MS) in the analysis of proteins and oligonucleotides is undergoing a rapid expansion. The number of applications for ToF-MS of metabolomes are relatively few. One important application that has been described is the ToF-MS analysis of cholesterol and metabolites for the diagnosis of the SLO syndrome.[261,262] Cholesterol and 7-dehyrocholesterol standard are both measured in this assay. An example of a ToF spectrum for cholesterol directly analyzed from a filter paper blood spot (no sample preparation was used) is shown in Figure 19. (Note also that the filter paper blood spot was used as the target.) The $[M - H]^+$ and $[M - OH]^+$ ions of cholesterol are detected a *m*/*z* 385.35 and 369.35, respectively. In the SLO syndrome, the relative ratio of 7-dehydrocholesterol to

cholesterol is significantly elevated relative to control and is diagnostic for that disorder.[261]

G. Biogenic Amines

Catecholamines serve an important role as neurotransmitters for a wide range of physiological processes.[263] The three naturally occurring catecholamines are epinephrine, norepinephrine, and dopamine. The major sites of synthesis of catecholamines are the adrenal medulla and the sympathetic nervous system. Epinephrine is the most important amine produced by the adrenal medulla and, like other catecholamines, is rapidly metabolized ($T_{1/2} = 2$ min) by catechol O-methylation and oxidative deamination. Most biogenic amines and metabolites are excreted in urine as conjugates of sulfuric and glucuronic acid.

Disorders of catecholamine overproduction produce the following clinical sequellae: increased stress, decreased blood pressure, decreased blood volume, thyroid hormone deficiency, congestive heart failure, and arrhythmias. Low levels of these neurotransmitters can result in postural hypotension. Pheochromocytomas and neuroblastomas are tumors that produce excess catecholamines. Elevated concentrations of catecholamines following quantitative plasma or urine analysis provide clinical data that may be used to diagnose these disorders. In addition, measurements of catecholamine concentrations in blood or urine may provide information that is useful in the diagnosis of orthostatic hypotension and some psychiatric disorders.

Pheochromocytomas arise from neurochromaffin cells of the autonomic nervous system or the adrenal medulla and produce excess catecholamines and metabolites. The classical symptoms of this disorder are sustained hypertension, weight loss, headache, palpitations, and anxiety. Ninety percent of these rare tumors occur in the adrenal medulla. Neuroblastomas are one of the most common malignant tumors in pediatric patients. Approximately 80% are found in children under 5 years of age. More than 90% of these tumors produce excess norepinephrine, dopamine, and vanillylmandelic acid (VMA). The important analytes that are measured with MS in the clinical laboratory for these diseases are described.[264,265]

GC/MS is the most commonly used mass spectrometric method for catecholamine and catecholamine metabolite analysis.[266−269] Pheochromocytomas have been diagnosed with these methods.[270] Furthermore, applications of GC/MS has also been used for the diagnosis of neuroblastomas.[271,272] Most methods incorporate isotope dilution techniques in their mass spectrometric analysis, with special attention to quality assurance.[273] Analyte-specific methods have been developed for specific disorders. For example, GC/MS has been used to analyze dopamine[274,275] in research and clinical investigations of Parkinson's disease. Recently, LC-MS methods have been used to analyze neurogenic amines and include thermospray[276] and electrospray MS[277,278] applications.

H. Other Classes of Small Biomarkers

1. Lipids

Lipids are characterized by their high solubility in organic solvent and near insolubility in water. They yield fatty acids on hydrolysis or are esters of fatty acids with complex alcohols.[279] Clinically important lipids include sterols (Section II.F), fatty acids (Section II.B.), glycerol esters, ceramides,[280] phospholipids, sphingosine derivatives, and terpenes. MS is commonly used for the analysis of triglycerides and other lipids,[281] especially by isotope dilution GC/MS.[282,283] LC-MS applications for triglyceride analysis include ESI-MS[284] and MALDI-ToF-MS.[285] Many other applications of GC/MS, LC-MS, and MS/MS have been developed for prostaglandins and thromboxanes. An extensive series has been published by Murphy et al.[286] and other investigators.[287−293]

2. Carbohydrates

Carbohydrates are aldehyde or ketone derivatives of polyhydroxy alcohols or are compounds that when subjected to hydrolysis produce these derivatives. Monosaccharides are simple sugars, e.g., glucose, and consist of a single polyhyddroxy aldehyde or ketone unit that cannot be hydrolyzed. Disaccharides are two monosaccharides joined covalently by an O-glycosidic bond.[294] Polysaccharides are multiple monosaccharide units linked together via glycosidic bonds. The metabolism of carbohydrates produces glucose, the primary energy source in humans. In addition, carbohydrates serve important functions in glycoproteins such as antibody recognition. Carbohydrates are also incorporated in cell membranes and participate in cell surface recognition. GC/MS and ESI-MS have both been used to measure carbohydrates in blood.[295−300] Carbohydrate analyses by MS has recently been reviewed.[301]

Glucose is routinely measured with a wide variety of methods. Rapid glucose measurements are important in disease monitoring, especially for diabetic patients. Although MS will not likely be used to routinely measure a single carbohydrate such as glucose, it is more likely that glucose will be part of a multi-analyte test of several markers for diabetes. The identification of unusual carbohydrates produced by bacteria has been performed by MS in several applications of infectious disease screening. GC/MS has been used to analyze carbohydrates unique to bacteria for the identification of the microbes responsible for infection.[302−306] Other techniques for carbohydrate analysis that use a mass spectrometer have been developed.[307,308]

3. Trace Elements

Trace elements are essential elements that are present in milligram per kilogram amounts or less, and if deficiencies occur, then physiological impairment results.[309] Important trace elements in biochemistry include iron, zinc, copper, iodine, cobalt, molybdenum, and selenium. The quantification of trace elements is important for diagnosing disorders produced by their deficiencies. One of the key aspects of the clinical analyses of trace elements is sample

handling. Important considerations are given to preventing contamination of environmental sources of these trace elements. Two methods are most often used in the clinical laboratory, atomic absorption spectrophotometry (AA) and inductively coupled plasma emission spectrometry (ICP-ES). However, inductively coupled plasma *mass* spectrometry (ICP-MS) is replacing these other methods because of its high specificity and accuracy. The analysis of several trace elements in biological samples has been developed.[310−316]

III. Diagnostic Proteins and Glycoproteins

A. Proteome Analyses

The important role of MS in the identification of proteins and their correlation to the genes that encode them is discussed in the paper "Mass Spectrometry in the Age of the Proteome" by Yates.[317] With near completion of Human Genome Project, emphasis will shift from genomics to proteomics. MS will have an important role in the characterization of expressed proteins.[6,318−320] Gene expression involves the production of mRNA that translates DNA-based information into protein synthesis.[35,321] Abnormal proteins are produced from errors in gene sequences provided that these sequences are used in the translation of information from DNA to RNA. In addition, the "predicted" abnormal sequences may be altered or removed by post-translational modifications.[322] Proteins with altered amino acid composition may or may not have clinical consequence, depending on which amino acids are altered, whether these altered amino acids change substrate specificity, or other changes that affect protein activity, specificity, or function.

MS is important in protein identification and characterization.[34,318,319,323−326] In clinical research, mass information and structure elucidation is often used to understand the function of a protein and can be used to correlate the protein composition with a gene sequence. It is likely that, on completion of the human genome project, research dollars will be redirected to the investigation and characterization of proteins coded by human genes. MS will play a dominant role in this area, especially with regard to post-translational modifications.[327] As proteins are identified as important biomarkers of disease, the use of MS to screen proteins that are diagnostic for these disorders will increase. The MS analysis of hemoglobin[328] is an example of the potential role of protein analyses in a clinical screen.

Normal adult hemoglobin (hemoglobin A, HbA) is a tetrameric protein that is composed of two sets of polypeptide chains, two α and two β chains. Hemoglobin exists in various forms that are characterized by the composition of the globin chains. Some hemoglobin variants exists normally and, in fact, are predominant at defined stages of development, i.e., hemoglobin F is the dominant hemoglobin in the fetus. Other variants of the globin chain produce serious disease, i.e., hemoglobin S is the dominant hemoglobin in sickle cell disease. MS has played a role in identifying many more variants that may or may not have adverse consequences. With the power of μs analysis for analyzing intact hemoglobin in hemoglobinapathies, the question remains: why is it not used routinely in the clinical laboratory? One answer may be the lack of experience by clinical chemists in performing these assays or the difficulty of integrating them in routine clinical screening. Second, other assays perform these analysis equally well and at lower cost. Third, no laboratory has taken the lead in developing an MS-based hemoglobin assay that is robust, is validated, and can be applied to hundreds of sample analyses per day. The final answer may be the most important. Currently, intact hemoglobin MS methods cannot detect several important variant hemoglobins. Altered amino acids of many variant hemoglobin associated with disease (HbC, HbE, and HbO) alter the mass by only 1 Da.[5,328] The resolution of mass spectrometers are not sufficiently adequate to separate the globins by mass and, as a result, cannot detect some hemoglobinapathies.

Further advances in MS will permit more rapid amino acid sequencing and more accurate, higher resolution analyses of intact large biomolecules.[34] Ionization techniques are playing key roles in this area and include ESI and MALDI.[329] New developments in mass analyzers (including quadrupole MS and MS/MS systems, ion trap MS, Fourier transform MS, and ToF-MS[1]) and analyzer configurations, e.g., Q-ToF, will demonstrate improved sensitivity and resolution at higher masses. This section of this review will emphasize MS analyses of hemoglobin and glycohemoglobin as an example of protein analysis via MS in the clinical laboratory.

B. Hemoglobin

HbA comprises 96% of normal adult hemoglobin whereas hemoglobin A_2 (HbA_2) accounts for 3%. HbA_2 is composed of two α and two β chains. During fetal development, the dominant hemoglobin is fetal hemoglobin (hemoglobin F, HbF). Fetal hemoglobin is composed of two α and two γ globin chains. After birth, the amount of HbF diminishes to levels of less than 1% in adults.[330]

Thalassemias are inherited disorders characterized by decreased synthesis of either α or β globin chains. α-Thalassemias are the most common genetic abnormalities of hemoglobin in humans. Symptoms of α- and β-thalasemias are categorized as minor to severe with the latter including lethal anemia, growth retardation, and bone malformation. In contrast, hemoglobinopathies are disorders characterized by structural alternations in either one or more globin chains. Over 700 structural variants of hemoglobin have been found, with the majority having no clinical or hematological manifestations.[328] Some hemoglobinopathies, however, have significant clinical sequellae. Hemoglobin S (HbS) is a form of hemoglobin that forms long rope-like polymers with other HbS molecules. These hemoglobins aggregate and distort the shape of red blood cells to form a characteristic "sickle" shape. Clinical symptoms of sickle cell anemia include joint pain and organ damage in patients that are homozygous for this disease.[330] Heterozy-

gotes (carriers of one copy of the altered gene) are generally unaffected unless another hemoglobin variant is present, i.e., HbC, HbO-Arab. These affected patients are classified as compound heterozygotes for a sickle cell hemoglobinapathy and often exhibit symptoms of sickle cell disease. A combination of HbS with a thalassemia may produce a sickle cell disorder. This is because HbS becomes the dominant hemoglobin in the absence of HbA. Due to the extremely large number of hemoglobin variants discovered, a classification system was developed.[328] Variants are classified by the globin chain affected and the sites of substitution. In addition, a characterization of the mutation is often included in the notation. HbS, for example, is denoted as β_6Glu→Val.

Traditionally, hemoglobins have been analyzed with electrophoresis, isoelectric focusing, and ion-exchange HPLC. With the advent of LC-MS techniques such as electrospray and more recently MALDI-ToF, MS has been used to identify specific hemoglobin variants. It has also been used as a confirmatory method. With further improvements, MS may become a primary screening tool to analyze blood for hemoglobinopathies and thalassemias. However, it should be noted, that simple and accurate non-MS-based methods exist for hemoglobinopathies, that the comprehensiveness of MS results are not always needed, and that the expertise and resources needed to perform these analyses are high. Therefore, implementation of MS analysis of proteins such as hemoglobins may require the consideration of new models for laboratory services before MS testing can be widely implemented.

Several excellent reviews on the subject of hemoglobin analysis are available.[5,331,332] The analysis of hemoglobin can be achieved in two ways: (i) proteolytic fragment analysis and (ii) intact hemoglobin analysis. It is worth noting that a comprehensive analysis of hemoglobin includes proteolytic fragment and intact hemoglobin analysis. Because certain limitations exist in both methods, a comprehensive hemoglobin analysis will use both methods to provide the most accurate clinical information.

1. Proteolytic Fragment Analysis

The study of proteolytic fragments of hemoglobin were describe by Wada et al.[333] and others,[334−336] using desorption ionization techniques. In addition, FAB-MS[337−343] and more recently, electrospray[344−346] and MALDI-ToF MS[347] have been used to rapidly analyze protein digests. In general, the proteolytic MS analysis of hemoglobin is comprised of a few important steps. First, preparative LC techniques are used to separate globin chains. Second, derivatization of some amino acids are required to prevent proteolysis, i.e., alkylation of cysteine residues. Third, various proteolytic enzymes, e.g. trypsin are used to hydrolyze proteins at specified locations to form defined peptide fragments. Trypsin is the most commonly used enzyme because it produces peptide fragments in a mass range that is most suitable for MS analyses. This enzyme hydrolyzes the peptide bonds of basic amino acids (i.e., lysine and arginine). Other enzymes hydrolyze proteins at different sites and may provide further information for protein identification. Fourth, these peptic digests are analyzed with HPLC-MS, HPLC-MS/MS, or MS/MS without chromatography.

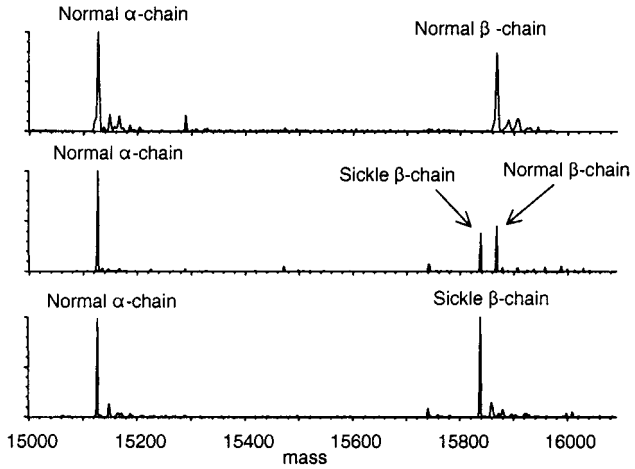

Figure 20. ESI-MS spectra of extracted hemoglobin from a dried filter paper blood sample for a normal newborn and a newborn categorized as a carrier (heterozygote) or homozygous for sickle cell disease. Laboratory data from M. Morris.

2. Intact Globin Analysis

Electrospray MS has been used to characterize intact hemoglobin.[331,348−350] The mass spectrum generated by electrospray ionization is characterized by a series of molecular ions of different charge states, ranging from approximately 11 to 21 under the most common operating conditions.[5] Adjacent peaks differ by 1 charge in the "molecular envelope" of hemoglobin multiply protonated molecules. Software transforms these data to a unique mass that characterizes the average molecular mass of hemoglobin. An example of transformed mass spectra for the α and β chains of hemoglobin, using a quadrupole mass spectrometer, is shown in Figure 20. Wada describes the use of a high-resolution sector instrument for this analysis.[351] TNBA (trinitrobenzenesulfonic acid) and TNB (5,5′-dithiobis(2-nitrobenzoic acid)) conjugates of hemoglobin have also been analyzed.[352]

In an alternative approach to proteolysis, MS/MS with electrospray ionization has been used to analyze hemoglobin fragments produced by CID of the hemoglobin chains.[353−355] Recent progress has led to the development of nano-ESI-MS/MS analysis.[346] The potential use of ToF analysis of hemoglobin has been recently investigated.[356] The use of MS analysis for screening of hemoglobinopathies has been described.[357−359]

a. Clinical Screening of Hemoglobinopathies. Although MS shows great promise in newborn screening, its use has been limited to confirmation of known variants or the identification of new variants. An ESI-MS spectra of filter paper-extracted hemoglobin from a control patient and patients that are heterozygote and homozygote for sickle cell disease are shown in Figure 20. HbS is easily identified in both the homozygote and heterozygote in this rapid analysis. Heterozygotes are differentiated from homozygotes by the presence of a sickle β chain and a normal β

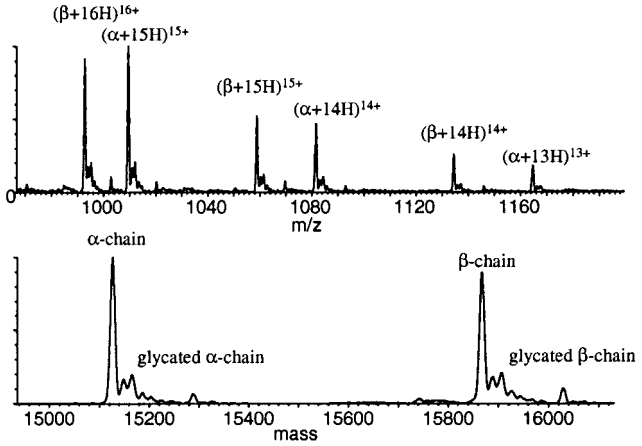

Figure 21. ESI-MS spectra of glycated hemoglobin from a patient with diabetes. Laboratory data from M. Morris.

chain. The method is simple because hemoglobins are extracted from a dried blood sample and followed by flow injection ESI-MS analyses. The methods have limitations in newborn screening. MS cannot detect mutations that differ by only a few mass units due to inadequate mass resolution. For example, HbC, HbE, HbO-Arab, and D-Punjab may be not differentiated because of a mass difference of 1 Da.[5] However, with extreme care, some recent work suggests that it is possible to differentiate heterozygotes from homozygotes with a 1-Da mass change even though the individual components are not resolved (M. Morris, personal communication).

3. Glycohemoglobins

Glycation of hemoglobin primarily occurs in diabetic patients.[330] The degree of glycation may serve as an index of disease control during treatment, i.e., increased glycation indicates poorer control when compared to previous measurements. It has been shown that low concentrations of glycated Hb are associated with fewer complications from the disease.[330] The ability of MS to measure glycated and nonglycated normal hemoglobin offers an improvement over other methods that require an external reference standard to quantify glycohemoglobin. In addition, problems with sample quality (i.e., specimen degradation or the effect of therapeutic interventions that interfere with other glycohemoglobin assays) are reduced by using MS-based analyses.[360−362]

Glycohemoglobin (GHb) is the term used to denote the binding of glucose to hemoglobin as a ketoamine. Primarily, glucose condenses predominantly with the N-terminal valine of the β-chains and to a lesser extent with α-chains and other residues such as lysine. MS can detect the number of glycations in a given hemoglobin by examining its mass shift but cannot identify the actual site of binding. Mass spectrometric analysis with electrospray ionization methods has been performed.[360,361] Recently, MALDI-ToF has been used to identify glycated hemoglobin.[363−365] An example of the application of LC-MS to the analysis of glycated hemoglobins is shown in Figure 21. This analytical approach is similar to that used to detect hemoglobinopathies. Recently, Niwa et al.[366] demonstrated that another hemoglobin adduct, glutathionyl hemoglobin, is also useful as an index of oxidative stress often found in diabetes and other hyperlipidemias. However, others have observed increases in glutathionylated β-chain on prolonged storage of liquid blood (M. Morris, personal communication).

C. Specific Disease Diagnostics Using MS

MS has been used in several other clinical applications for the diagnosis of disease. In the area of cancer diagnostics, MALDI-ToF has been used to characterize glycoproteins and tumor markers.[367−369] Other areas include peptide analysis of insulin[370] or neuropeptides.[371,372] The use of MS in the characterization of proteins in cerebrospinal fluid has also been described.[373−375] There are numerous other applications in clinical research and protein analysis that are more appropriate for other reviews.[5,34,329]

1. Protein Profiling

MALDI-ToF is a technique that is amenable to direct analysis of proteins or peptides from cells, cell lysates, protein digests, etc.[323] This ease and versatility has led to the development of protein profiling, which is essentially a qualitative technique that uses MS to identify the mass of hundreds of proteins and peptides from a specimens in a single analysis.[324,325,329,376−378] This profile is analogous to a fingerprint for the identification of cells or cellular preparations. Examples of emerging applications include identification of bacteria[379−383] and tumor markers.[384,385]

a. Identification of Baterial Isolates. An example of protein profiling that is emerging in proteomics and MS is shown in Figure 22. Bacteria are isolated from patients with infections, grown overnight on agar plates, and washed. A 500-nL sample of a single colony is washed and diluted. Then 500 ml of the dilute solution was mixed with an equal volume of sinapinic acid matrix and placed on a stainless steel target and analyzed by MALDI-ToF. The top panel shows a spectrum of *Escherichia coli* while the bottom panel shows a spectrum of *Staphylococcus aureus*. *S. aureus* was digested with lysozyme (identified in the spectrum as the large peak at 14000 Da).

Although, most individual proteins/peptides are not identified in these spectra, the profiles for each bacterial isolate are unique and hence suggest the ability of MS to identify bacteria on the basis of a unique peptide pattern or fingerprint. Data has been shown to be reproducible, and much research and development is being devoted to the development of industrial high-throughput methods and fingerprint (interpretation) design.[386,387] Efforts to standardize protocols are being coordinated by analytical chemists at the FDA.

b. Identification of Tumor Markers. Another example of protein profiling that has potential in the clinical laboratory is characterization and identification of tumor markers. An example of a procedure for identifying normal tissue from malignant tissue is shown in Figure 23. These MALDI-ToF mass spectra were directly acquired from cells that are

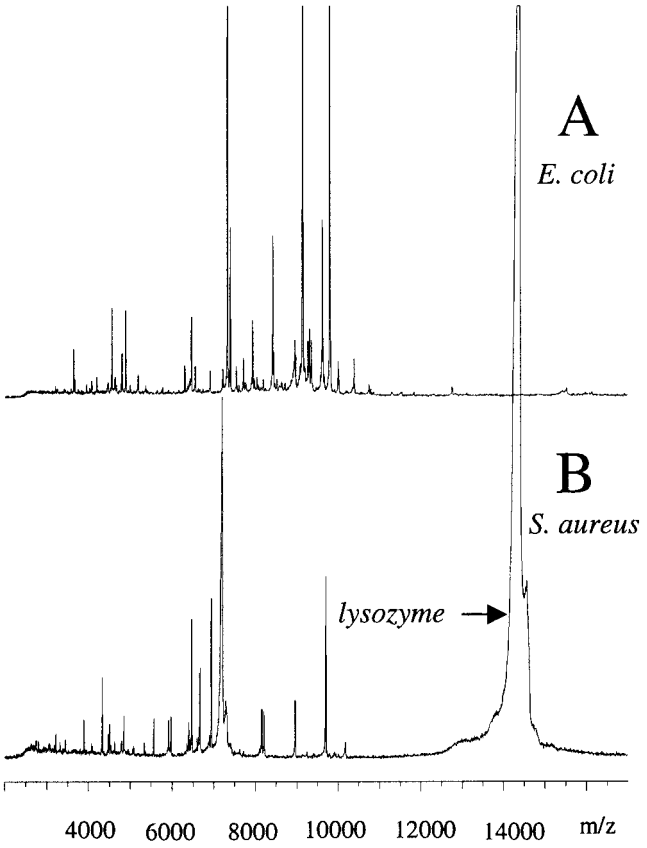

Figure 22. Representative spectra from obtained from freshly grown bacteria analyzed by MALDI-ToF MS. Bacteria were obtained from overnight growth on agar plates and washed to remove interfering components such as salts. Gram-negative organisms such as *E. coli* (A) were spotted immediately while Gram-negative organisms such as *S. aureus* (B) were incubated with lysozyme prior to spotting and subsequent MALDI analysis. Each spectrum has 50−100 individual peaks in a unique pattern. Laboratory data from S. C. Smole, L. A. King, and P. E. Leopold.

laser capture microdissected[384] from a single patient's frozen tissue: normal breast epithelium, invasive ductal carcinoma of breast, and metastatic ductal carcinoma. The spectra obtained from tumor cells is clearly different from normal. This "fingerprint" serves as a mechanism of identifying various types of tissue in a rapid manner. It may be especially useful in the identification of the site of origin for cancers that have metastasized.

IV. Diagnostic Biopolymers

The basis for an inherited metabolic disease can be found in an abnormal human gene.[388] The abnormal gene produces an abnormal protein (either an enzyme, structural, or functional protein). If the protein is an enzyme and its activity is impaired, then abnormal concentrations of metabolites will occur (Figure 1). The clinical laboratory provides data for the diagnosis of disease by measuring the concentration of metabolites, detecting abnormal functional and structural proteins associated with disease, or identifying abnormal gene products known to be associated with specific diseases. Which method is used for disease diagnosis will primarily depend on whether a specific biomarker strongly correlates with a disorder and whether technology is available to accurately measure that biomarker. Laboratory data will be combined with clinical observations for the final assessment by a physician. For diseases that are not solely genetically based such as infection, the use of molecular, protein, and metabolite analysis of foreign DNA, proteins, and metabolites will be used. Molecular, protein, and metabolite analysis will also be important analytical tools for risk assessment of many disorders such as cancer, heart disease, and diabetes.

A. Genome Analyses

The fundamental components of DNA and RNA are nucleic acids. Nucleic acids form nucleosides with the addition of ribose and deoxyribose sugars. Nucleotides are subsequently formed by addition of a phosphate residue.[54] Nucleic acids are either purine analogues (adenine (A) and guanine (G)) or pyrimidine analogues (cytosine (C), thymine (T), and uracil (U)). DNA is composed of deoxyribose sugars and the four bases A, G, C, and T, whereas RNA is composed of a ribose sugar and G, C, T, and U. Nucleotides are joined together by phosphodiester bonds and become either a DNA or an RNA polymer. DNA exists as a double-stranded polymer in its native form while

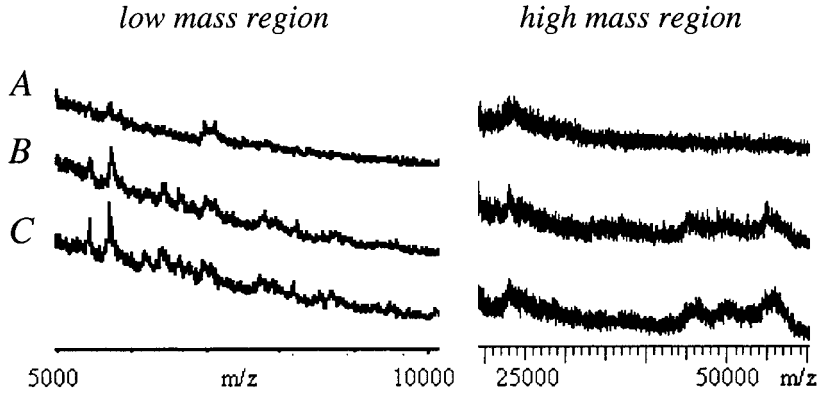

Figure 23. MALDI-ToF mass spectra directly acquired from cells that are laser capture microdissected from a single patient's frozen tissue: normal breast epithelium (top), invasive ductal carcinoma of breast (middle), and metastatic ductal carcinoma (bottom). The low mass region (5000−10 000 *m*/*z*) is shown at a lower magnification than the high mass region (20 000−60 000 *m*/*z*). Note the features at 6100−6900, 7700−7900, and 45 000−58 000 that distinguish the normal cell profiles from those of the carcinomas. Laboratory data from D. E. Palmer-Toy.

RNA is a much shorter single-stranded polymer. Short segments of DNA are called oligonucleotides; with the average size of these polymers (oligomer) ranging between 15 and 45 bases. A common term that is often used to denote the number of bases in an oligonucleotide is the suffix "mer". The number of bases followed by mer in addition to the abbreviations ss and ds for single- and double-stranded DNA is used. Therefore a 15-base double-stranded DNA is denoted 15-mer ds-DNA and is composed of deoxy-A, G, C, and T.

The role of MS in DNA-based analysis and screening is currently being defined.[389−394] Potential routine applications of MS include detection of DNA modifications or mutations and their sequencing. Recently, the use of MS to analyze short DNA fragments for single nucleotide polymorphisms (SNPs)[395] has led to major advances in the automated analysis of DNA via MS. The mass range of mass spectrometric analysis of oligonucleotides increases each year in part due to advances in MS technology and also due to methods for analysis and sample preparation. Mass resolution and the instability of long DNA polymers that are easily fragmented limit the size of the oligonucleotide that can be analyzed.

New methods in DNA array technology[396] are rapidly expanding mutation analysis capabilities for genetic diseases by inclusion of multiple genetic probes for a disease and as a consequence reducing the false-negative rate.[397] However, these methods will identify many carriers of disease (unaffected heterozygotes). The implications of this technology require discussion before implementation. Questions such as "Will carriers (heterozygotes) be reported?" must be answered. The major contribution that MS will have in the DNA diagnostics area may be its capacity to analyze multiple genetic probes or gene fragments in a single test. Further into the future, improvements in MS may enable direct analysis of gene fragments without the need for amplification or excessive sample preparation. The question that must be answered is whether a genetic mutation in question correlates with a disease and if so then how. Most likely, MS applications will be primarily focused on the gene expression. In the near term, the use of DNA as the primary tool for diagnosing disease states will largely depend on the availability and accuracy of other methods that analyze proteins or metabolites. For diseases that are characterized by few mutations and that cannot be screened by conventional methods, DNA-based assays will provide a solution. DNA-based analysis will clearly be used as a confirmation method as well as prenatal diagnosis and genetic counseling. More importantly, DNA analysis will play a critical role in risk assessment of disorders such as cancer, neurological disorders, and heart disease.

Advances in MALDI -ToF analysis has been the major contributor to the growth of MS applications in genomics.[398,399] Most MALDI-ToF applications have been used to analyze DNA, including fragments as small as a 15-mer for a short oligonucleotide sequence (~1500 Da) to greater than a 500-mer (>100 000 Da) in most recent studies. Currently, the practical mass range for analysis of oligonucleotides is based on the ability of the mass spectrometer to resolve an A to T transversion (change of A to T). The mass difference of A and T is 9 Da. A resolving power of 1 in 1000 is necessary to distinguish these two bases. The analyses therefore has a practical, working mass range of 9000 Da or less.

Advances in the next few years will include the use of chip technology (micro-arrays),[396] especially in the area of clinical screening. A DNA chip is a small device that holds a regular array of DNA molecules that are chemically attached to the surface. These DNA molecules generally contain known probe sequences that will hybridize with their target complementary sequences. These arrays are analyzed by a variety of techniques, including MS.[395,397] Chip technology is amenable to automated sample analyses and high-throughput screening. MS may be advantageous in analyzing multiple DNA probes on a single chip because of its high selectivity and specificity. Multi-probe analyses is as important for screening known genetic mutations in a single analysis in a similar manner that MS/MS has been used to screen for multiple metabolites.

Most MS articles related to DNA analysis have been in the areas of biomedical and clinical research, whereas the use of DNA analysis with MS detection in the clinical lab is quite rare. During this decade, it is likely that screening for single nucleotide polymorphisms for risk assessment of cancer and heart disease will be more commonplace.[400] As molecular methods enter routine screening, so will high-throughput MS applications.

B. Current MS Applications

Clearly, MS is an ideal tool for the analysis of modified or unusual nucleic acids, nucleosides, or nucleotides. Electrospray ionization is especially suited for the analysis of these DNA components. Several methods for the measurement of nucleic acids[401−404] have been published, especially in the area of urinalysis of tumor markers.[5,405−407] With regard to oligonucleotide analyses, the analysis of these small DNA fragments are highly indicative of mutations that cause disease. New assays have been developed for clinical disorders,[408] including Tay-Sachs disease,[409] cardiovascular disorders,[410,411] cystic fibrosis,[391,412] cancer,[413] and pathogens.[400] There are several other genomic applications of MS in clinical research that are more appropriately and comprehensively presented in other reviews.[414]

V. Quantitative Analysis and Quality Assurance

A. Quantification in Clinical Chemistry

The importance of quantification in clinical chemistry was described by Yergey at the 11th Sanibel Conference on Mass Spectrometry.[4] At this conference, clinical analyses were described as either qualitative or quantitative. Results from qualitative assays are characterized either as positive and negative or as black and white. Quantitative analyses are characterized by results that measure the degree of

a response such as the intensity of light emission, height of a peak plotted on a graph, or the number of events (i.e., counts in a defined period).[54] A "semiquantitative" analysis is a category used commonly to define an assay that measures a response but is not a highly precise term.[415]

Assays often characterized as "qualitative" are *some* drugs of abuse or metabolite screening tests that are based on immunoassays or other assay that are based on biological responses such as bacterial inhibition assays.[23] In these assays, a positive or negative result is most often reported. However, these assays still have some basis in accurate quantification, i.e., the concentration of the analyte above or below the cutoff but sufficiently accurate in order for the test to be useful. What may be less stringent in qualitative assays is the linearity of the concentrations over a large range of concentrations. It is clear that there is some difficulty in distinguishing a qualitative, semiquantitative, and quantitative assay. A more clear example for qualitative analysis is the application of MS in hemoglobin analyses. The presence or absence of one or two sickle globin chains (HbS) is indicative of a carrier or sickle cell disease. Quantitative analyses are used to determine the concentration of substrates in biological fluids (i.e., glucose, phenylalanine, homocysteine) in blood, urine, CSF, or tissues.[25] Quantitative data are generated by a measurement of a response.[416] The reliability of this response is based on statistical confidence parameters such as precision. Semiquantitative analyses are actually quantitative assays that do not have sufficient statistical power to generate high confidence in the precision of the result because of analytical limitations. This technique is most used in rapid metabolite screening or therapeutic drug monitoring, where the desired results do not need to be highly precise but rather to answer the question of whether a metabolite falls within a certain range.

Standardization is required for all quantitative assays. Concentration measurements are based on the relationship of a measured response of a compound relative to the response of a standard or reference. This relationship of unknown to standard, often expressed as an intensity ratio, is compared to the relationship of a known amount of analyte to the standard. The plot of concentration of known analytes versus standard is defined as the standard curve. Results from an unknown are obtained by interpolation from this standard curve.

Standards are either external or internal. External standards[417] are measured separately and are used to calibrate the signal of an instrument relative to known concentrations. Internal standards[417] are present in the same matrix as the unknown and are co-analyzed. Internal standards are typically homologues or analogues of the analyte being assayed. Standards that differ only by their isotopic content are the most ideal standards for use in MS applications because they differ only by their isotopic content rather than chemical properties in most instances. MS applications that use stable isotopes in quantitation are defined as IDMS techniques.[135,295,415,416,418−424]

In clinical chemistry, the quantitative limits for analytical accuracy must be also be defined.[425] Confidence in an analytical result is highest if the result is within the upper and lower range of standards.[426] Methods that use ratios of ratios (a ratio of an analyte to its internal standard versus ratio of reference material to its internal standard) provide the best statistics. The standard error of the estimates should be used in linearity assessments rather than correlation coefficients. Data that have nonlinear responses should not be used; rather, alternative assays should be developed.

B. Quality Assurance

The other major requirement of clinical analyses is quality assurance.[427] Quality assurance distinguishes methods used in clinical diagnostics from clinical research.[54,428] Quality assurance ensures that the result obtained in an analysis is reliable and accurate. Furthermore, it must detect methodological errors and other problems that may occur at any point between the handling of a specimen and reporting of a result. For example, errors can occur during specimen collection, delivery, storage, sample preparation, sample analysis, data reduction, result interpretation, communication of results to physicians, and follow up. Quality assurance is necessary because undetected errors can result in a false result that may harm a patient. The medicolegal implications or a laboratory error can be serious.

Examples of quality assurance systems developed for mass spectrometric assays have been described for applications that involve newborn screening.[107,108,273,429] It is noteworthy that, to ensure implementation of a quality assurance program in a laboratory that performs clinical analysis, the laboratory must meet Federal laboratory accreditation requirements as defined by CLIA (Clinical and Laboratory Improvement Act) of 1988. Research assays must be clearly delineated from routine clinical assays and cannot be used to report patient results. Several investigators have addressed quality assurance and the use of mass spectrometric based assays by developing standards, reference materials, isotope dilution techniques, laboratory comparisons, and external assessments.[107,108,273,427,429−434]

Two important applications of MS are (i) its use as a confirmatory test for other assays or (ii) its use as part of a quality assurance methods examining the production and quality of reagents or comparison of results from non-MS-based assays such as immunoassays and molecular assays. MS methods developed for these applications are critically important because its serves as a means for identification, characterization, and confirmation of analytes or materials that are used or found in less specific assays.

VI. Conclusions

As new technology is developed by MS manufacturers working in conjunction with private industry and academic institutions, opportunities for innovative research become available. The development of

novel methods enables the discovery of new of biochemical and physiological processes. With a better understanding of these processes, knowledge and insight into the basis and mechanism of disease arise. New biomarkers for disease are soon discovered, and methods are subsequently developed to measure these compounds in blood, plasma, or urine. The methods used in the discovery of disease processes are the methods that are adapted to their laboratory diagnosis. It is clear therefore that advancements in technology directly impact clinical analysis, albeit delayed. Delays in method implementation are a result of stringent method validation that includes statistical assessments, method comparisons, pilot screening studies, and QA/QC implementation. Furthermore, new methods must be integrated into laboratory protocols and information systems. Advances made during the past decade are just now being applied to laboratory diagnostic medicine. These new applications are evidenced by the adaptation and integration of MS/MS methodology used in newborn screening.[2] Developed nearly 10 years ago and originally applied to a few dozen plasma samples per day, metabolic profiling using MS/MS for the analysis of dried blood samples is just now being implemented. Over 1 000 000 infants/year will be screened worldwide with MS/MS in 2000, and this growth is expected to continue.[2]

In addition to the benefits of MS in the clinical lab, there are some limitations. First, mass spectrometric methods are generally not turn-key. Nevertheless, progress will be made in this area as the demands for MS-based solutions increase. Second, there is limited availability of scientists that have expertise in laboratory medicine and in analytical chemistry/MS. Third, the number of users and methods will increase as MS technology becomes more accessible. With the development of slightly different variations of a particular test used by different laboratories, there is a greater likelihood of different results. The chance increases for a miscommunication or misdiagnosis between health care provider and laboratory. Required harmonization of MS methods will ensure that if a physician orders test A, then the results will always include the concentration of compounds X, Y, and Z. Harmonization begins with the development of an independent quality assessment program.[107,108] Does harmonization mean that a single MS instrument or system be used to perform a particular assay? The answer is no. Mass spectrometric methods allow many variations of sample preparation and analyses and yet produce comparable end points, i.e., the concentration of phenylalanine in blood. In fact, recent work in newborn screening has indicated that acylcarnitines may be analyzed either as a butyl ester or directly without esterification The advantages of the removal of the esterification step in the procedure is simplification and sample preparation time. The disadvantages may be loss of poorly ionizable metabolites, unexpected interferences, and inability to co-analyze some amino acids. Therefore, MS allows creativity in method applications while still achieving the same end points, e.g., obtaining the accurate quantification of a metabolite that is used to diagnose a disease. Other important issues in harmonization and quality assurance include clinical interpretation, result reporting, physician education, and clinical and genetic follow up; those areas where mass spectrometrists are generally not experts. These are issues that will require attention as MS expands the clinical lab.

A. Outlook of Clinical MS

It is clear that MS has served an important role in the clinical laboratory, especially in areas of metabolome analyses. However, most of these analyses are still being performed in specialized reference laboratories rather than hospital-based clinical laboratories. This trend is likely to continue in part because MS and the associated specimen preparation and result interpretation requires a high degree expertise not found routinely among clinical chemists. Nevertheless, there are efforts to automate and enable turn-key mass spectrometric bases systems that do not require the extensive experience in MS. The success of turn-key systems in MS as total solutions to a clinical analysis will be more apparent in this decade.

The heart of the mass spectrometer is the mass analyzer, whether that analyzer is a quadrupole, ion trap, or ToF. This past decade has shown that electrospray and MALDI are rugged, reproducible, efficient, and versatile ionization systems. The dramatic changes in mass spectrometer analyzer and source configurations that occurred in the previous two decades will shift to equally important developments in specimen handling, sample preparation, analyte isolation and purification, high-throughput automation, data processing, library searching, and computer-assisted interpretation. Methods will be available to analyze a whole host of metabolites. The question remains as to whether these developments will make mass spectrometric methods cost-effective and as inexpensive as immunoassays, which are most often applied in the “one disease/one analyte” clinical assay. Clearly, mass spectrometric methods, that include multiple analytes as observed in the area of newborn screening of amino acids and acylcarnitines, will continue to grow and become a more common clinical tool.

We are now embarking on the age of proteomics. With the Human Genome initiative essentially complete, the next major endeavor will be research in gene expression. Clearly, specialty analyses will be developed to identify specific proteins that are markers of disease. An example is hemoglobin and sickle cell disorders. Will MS replace robust, well-established techniques such as isoelectric focusing or HPLC? Improvements in resolution, mass accuracy, and sensitivity will be required if MS is to become competitive on a routine basis for such assays. However, new approaches to identification of cellular profiles through protein fingerprinting may rapidly enter the clinical diagnostic field due to simplicity and potential cost-effectiveness. Methods that can qualitatively identify microorganisms or characterize tumorogenic tissue or other diseased tissues may become routine if methodology for specimen prepara-

tion is standardized. The key to the success of these methods will be quality assurance and control programs, which has not been addressed at this time. QA/QC is the "gate- keeper" between research methods and clinical methods.

With regard to genomics, MS applications will likely be applied to the investigation of single nucleotide polymorphisms (SNP) and the correlation with disease risk assessment. As treatment strategies for many genetic disorders become available, SNP analyses using MS will become part of screening programs for risk assessment of disabling diseases. Clearly, the use of MS in providing analytical solutions for clinical laboratory problems will continue to grow.

Finally, MS will provide the most accurate and precise results in this decade and will continue to improve access to cost-effective solutions and comprehensive analyses of complex mixtures by rapid high-throughput analyses. In addition to its increasing roles as a primary clinical test or a screen of many diseases, MS will provide confirmation of results obtained from other assays. It is clear that the future of MS will be its complete integration into the clinical laboratory. Data systems will integrate all laboratory data, including MS results. These data will provide the most comprehensive information used for the diagnose of common and rare diseases, with a very low number of false-positive and false-negative results. The future of MS in the clinical laboratory is promising and is unquestionably exciting.

VII. Acknowledgments

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