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The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

In this issue

In this issue: Recent Developments in the Clinical Application of Mass Spectrometry

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Foreword of the editor

Editor in Chief: Gábor L. Kovács, MD, PhD, DSc

Dr. Ronda Greaves (Australia) was asked to be the guest editor of this special issue on "Recent Developments in the Clinical Application of Mass Spectrometry". Dr. Ronda Greaves is the Senior Lecturer in Clinical Biochemistry at Royal Melbourne Institute of Technology University and an Honorary Research Fellow at the Murdoch Children's Research Institute in Melbourne Australia. She is an active Member of the Australasian Association of Clinical Biochemists and Australasian Paediatric Endocrine Group and a Founding Fellow of the Royal College of Pathologists of Australasia Faculty of Science. Ronda completed her PhD on "Current Challenges in the Interpretation of Paediatric Clinical Biochemistry Test Results". Her training and continued interest is in ensuring the quality of paediatric clinical biochemistry results. Specifically, she has received two scholarships to undertake training in paediatric steroid analysis at the Children's Hospital in Zurich, Switzerland and has also received training at Sahlgrenska Hospital in Gothenburg, Sweden.

In addition, Ronda is active as a technical adviser to the biochemistry department at the National Hospital of Pediatrics in Hanoi, Vietnam, and contributes to scientific output through peer reviewed publications, referee of manuscripts, and membership of the editorial board for the journals Clinical Biochemistry and Clinical Mass Spectrometry. Ronda currently serves on the following committees: 1) officer of the IFCC as a member of the Committee for Distance Learning; where she is coordinating the development of mass spectrometry distance learning material; 2) chair of the Asia Pacific Federation of Clinical Biochemistry and Laboratory Medicine Mass Spectrometry Harmonisation Working Group; 3) Joint Committee for Traceability in Laboratory Medicine review team member for vitamins and non-peptide hormones; 4) Australasian Paediatric Endocrine Group Subcommittee on Disorders of Sex Differentiation/Development; and 5) European Cooperation in Science and Technology Action BM1303 on Disorders of Sex Differentiation/Development Networking Group for the "Harmonisation of Laboratory Assessment".

As the editor-in-chief of the journal, I am thankful to Dr. Greaves for accepting the responsibility of guest editing this issue.

In this issue: Recent Developments in the Clinical Application of Mass Spectrometry

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

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Recent advances in the clinical application of mass spectrometry

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EDITORIAL

Since the latter half of the 20th century mass spectrometry (MS) applications, associated with gas chromatography (GC) separation (i.e. GC-MS), have been the "gold standard" in specialised clinical laboratories for the quantitation of drugs, organic acids and steroids [1]. This status quo remained unchallenged until just over a decade ago when liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and inductively coupled plasma mass spectrometry (ICP-MS) were introduced into routine clinical chemistry testing. This expansion and integration for many has been disruptive, but overall by and large, clinical chemistry laboratories are embracing MS for many analytes. This is exemplified by its increased presence in external quality assurance (EQA) programs [2,3,4,5]; Table 1 (see following page).

Today many clinical chemistry diagnostic laboratories have embraced MS, with electrospray ionization LC-MS/MS being the primary application. As such, there has been a rapid succession of methods in the peer reviewed literature which attest to their accuracy and precision. Whilst this technology clearly offers a number of significant advantages, including improvements in specificity and sensitivity, there is a dichotomous divide between advocates and detractors of MS based applications [6]; Table 2 (see table on page 269).

Table 1 Mass spectrometry based method principles reported for clinical chemistry analytes in the Royal College of Pathologists of Australasia (RCPA) Quality Assurance Programs (QAP)

Measurands included in the RCPAQAP Chemical Pathology Programs	Matrix	Program	Percentage of participants using MS method principle	Method principle
3-methoxytyramine	Plasma Urine	Plasma Metanephrines Urine Biogenic Amines	100% 58%	LC-MS/MS
4-hydroxy-3-methoxymethamphetamine (HMMA) / Vanillylmandelic Acid (VMA)	Urine	Urine Biogenic Amines	25%	LC-MS/MS
5-hydroxyindoleacetic Acid	Urine	Urine Biogenic Amines	27%	LC-MS/MS
17-hydroxy progesterone	Serum/Plasma	Endocrine	45%	LC-MS/MS
25-hydroxy vitamin D3	Serum/Plasma	Endocrine	10%	LC-MS/MS
Adrenaline	Urine	Urine Biogenic Amines	23%	LC-MS/MS
Aldosterone	Serum/Plasma	Endocrine	11%	LC-MS/MS
Aluminium	Serum Urine	Trace Elements	62% 83%	ICP-MS
Amiodarone	Serum/Plasma	Special Therapeutic Drugs & Antibiotics	25%	LC-MS/MS
Androstenedione	Serum/Plasma	Endocrine	44%	LC-MS/MS
Arsenic	Urine Whole blood	Trace Elements	90% 88%	ICP-MS
Benzodiazapines e.g. Oxazepam	Urine	Urine Toxicology	30%	GC-MS (11%), LC-MS/MS (14%), LC-TOF/MS (5%)
Cadmium	Urine Whole blood	Trace Elements	83% 83%	ICP-MS

Chromium	Serum Urine	Trace Elements	78% 80%	ICP-MS
Clozapine	Serum/Plasma	Special Therapeutic Drugs & Antibiotics	23%	LC-MS/MS
Cobalt	Serum Urine	Trace Elements	100% 90%	ICP-MS
Copper	Serum Urine	Trace Elements	39% 60%	ICP-MS
Cortisol	Saliva Serum/Plasma	Salivary Cortisol Endocrine	19% 2%	LC-MS/MS
Cyclosporin	Serum/Plasma/ whole blood	Special Therapeutic Drugs & Antibiotics	13%	LC-MS/MS
DHEAS	Serum/Plasma	Endocrine	5%	LC-MS/MS
Dihydrotestosterone	Serum/Plasma	Endocrine	63%	GC-MS (13%), LC-MS/MS (50%)
Dopamine	Urine	Urine Biogenic Amines	24%	LC-MS/MS
Homocysteine	Serum/Plasma	Endocrine	2%	LC-MS/MS
Homovanillic acid (HVA)	Urine	Urine Biogenic Amines	23%	LC-MS/MS
IGF-1	Serum/Plasma	IGF-1 / C-peptide	3%	LC-TOF/MS
Iodine	Urine	Trace Elements	89%	ICP-MS
Lead	Urine Whole blood	Trace Elements	77% 48%	ICP-MS
Manganese	Serum Urine Whole blood	Trace Elements	100% 88% 78%	ICP-MS
Mercury	Urine Whole blood	Trace Elements	100% 100%	ICP-MS

Metanephrine	Urine	Urine Biogenic Amines	48%	LC-MS/MS
Mycophenolate	Serum/Plasma	Special Therapeutic Drugs & Antibiotics	33%	LC-MS/MS
Nickle	Urine	Trace Elements	89%	ICP-MS
Noradrenaline	Urine	Urine Biogenic Amines	21%	LC-MS/MS
Normetanephrine	Urine	Urine Biogenic Amines	48%	LC-MS/MS
Oestradiol	Serum/Plasma	Endocrine	1%	LC-MS/MS
Plasma free metanephrine	Plasma	Plasma Metanephrines	93%	LC-MS/MS
Plasma free normetanephrine	Plasma	Plasma Metanephrines	93%	LC-MS/MS
Progesterone	Serum/Plasma	Endocrine	1%	LC-MS/MS
Selenium	Serum	Trace Elements	82%	ICP-MS
	Urine		100%	
	Whole blood		83%	
Serotonin	Urine	Urine Biogenic Amines	50%	LC-MS/MS
Sirolimus	Serum/Plasma/ whole blood	Special Therapeutic Drugs & Antibiotics	38%	LC-MS/MS
Sweat Chloride	Sweat	Sweat Electrolytes	24%	ICP-MS
Tacrolimus	Serum/Plasma/ whole blood	Special Therapeutic Drugs & Antibiotics	17%	LC-MS/MS
Testosterone	Serum/Plasma	Endocrine	9%	LC-MS/MS
Thallium	Urine	Trace Elements	100%	ICP-MS
Tricyclic antidepressant general screen	Serum/Plasma	Special Therapeutic Drugs & Antibiotics	13%	LC-TOF/MS
Vanadium	Urine	Trace Elements	67%	ICP-MS
Vitamin A (retinol)	Serum/Plasma	Vitamins	3%	LC-MS/MS
Vitamin B1 (thiamine pyrophosphate)	Whole blood	Vitamins	4%	LC-MS/MS
Vitamin B6	Serum/Plasma	Vitamins	17%	LC-MS/MS

Zinc	Serum Urine Whole blood	Trace Elements	39% 80% 67%	ICP-MS

The percentage of mass spectrometric methods reported is based on the latest end of cycle or interim reports available on the RCPAQAP website. This data is presented with permission from the RCPAQAP Chemical Pathology Programs

In addition, there is a clear and real problem of finding staff equipped with the dual skills of MS and laboratory quality management. Hence, we need to look for new education and training approaches for emerging and current medical scientists/technologists that accommodate for these prerequisites. This will support the use of MS within a quality framework, enabling us to continue to meet expectations of MS as the "gold standard" method.

In this issue of the *eJournal of the International Federation of Clinical Chemistry and Laboratory Medicine,* there are four articles which highlight the changing landscape of MS based applications [7,8,9,10]. Together these explore changes and advances to instrumentation which paves the way for new approaches. The opening manuscript by Mbughuni and colleagues provides a clear overview of the range of current and emerging MS technologies available; which is driven in part by the significant need for the toxicology laboratory to keep abreast of illicit drugs and challenges of detection and quantitation [7]. Mbughuni further explores the matrices available for drug analysis which includes the use of dried blood spots. Following on from this article a detailed review of the extensive application of dried blood spot MS analysis, for analytes outside of new born screening applications, is provided by Zakaria and colleagues [8]. Then Kam and colleagues explores the emerging applications of peptide quantification by MS, taking a specific look at insulin-like growth factor I (IGF-I) [9]. Finally, in the last article of the special edition, Dias and Koal explore the future of MS in the clinical laboratory through the progress of standardisation in metabolomics and its potential role in laboratory medicine [10].

Together these manuscripts highlight the challenges and importance of quality management principles to achieve results that are fit for their intended clinical purpose. There are five recognised pillars supporting standardisation; certified reference materials (CRM), reference measurement procedures (RMP), reference laboratories, reference intervals or decision points and participation in an external quality assurance program. Information on the first three pillars is provided in the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database [11]; currently some (e.g. serum testosterone) but not all measurands (e.g. dried blood spot analytes) measured by mass spectrometry have complete listings, indicating deficiencies in the traceability chain [11]. As we continue to embrace MS technology, it is important that we also concentrate on developing and implementing these five important pillars to ensure that standardisation with traceability is achieved.

Participation in an EQA program is recognised as the central pillar supporting harmonisation of methods [12]. Such harmonisation is not however necessarily true for these newer applications which do not yet have robust EQA programs available or the critical number of laboratories for this comparison to occur. This is particularly highlighted in the discussion from

Table 2 Five points and counterpoints why laboratories are reticent to introduce LC-MS/MS. Points of detractions are provided from an online social media blog. Counterpoints are provided by the author (RG)

No.	Point of detraction [6]	Counterpoint
1	"Mass Spec is Too Complicated"	Quality Management (QM) is also complicated. A director of a large laboratory said "It is easier to train a diagnostic laboratory scientist in MS, as they understand the background, than to take someone from e.g. a research background with MS experience and train them in pathology" [anonymous personal communication].
2	"Mass Specs Are Too Big"	But many of our automated analysers are also large.
3	"Too Expensive"	Agree MS does seem expensive, but this is because we are use to reagent rental agreements from some immunoassay companies. It is important to create a business case to demonstrate return on investment.
4	"Testing Takes Too Long"	This is currently usually true, but will probably change in the future as MS becomes more automated.
5	"We use GC-MS/MS, and it Works Fine"	There is still an important place for GC-MS or GC-MS/MS in the laboratory, but the advantage of LC-MS/MS is that derivatisation is not mandatory. In addition, GC-MS or MS/MS has a clear role in discovery applications as highlighted by Dias and Koal [10].

Kam and colleagues related to the measurement of peptides by MS. Whilst there are EQA schemes available for IGF-1, participation is currently predominated by immunoassay methods and medians are often used to assess performance [2]. In the absence of a CRM and RMP robust EQA target values cannot be developed to aid the determination of bias for the small number of MS participants. However, there is still some value in participation in an EQA program (such as the RCPAQAP) as imprecision and linearity can be determined statistically and participation encourages other MS users to join to create the critical numbers. When an EQA program is not available sample exchange should be given high priority to support both method validation and on-going harmonisation of MS methods.

Sample exchange and/or EQA participation is often the first step in the recognition of discordance between results. A number of studies have demonstrated that there are factors independent from the choice of calibrator that can cause variation in MS results [13,14,15,16]. Whilst the authors in this special edition have drawn our attention to a number of important considerations, there is little discussion related to the choice of isotope selected for use as the internal standard and how this can influence the quantitation of results [7,8,9,10]. A two deuterated (D) internal standard is generally

not recommended where there are reasonable alternatives, as it is only two additional daltons from the target analyte which may lead to interference at high concentrations due to the presence of 13C2 isotopomers of the target [15,17,18]. A study by Owen and colleagues, comparing three internal standards (D2, D5 and C13) for serum testosterone quantitation by LC-MS/MS, demonstrates the influence of internal standard choice on patient results [16]. In addition, a study by Flynn and colleagues for the quantitation of epi-25 hydroxy vitamin D3 highlights the need for internal standards to co-elute with the compound of interest so they are present in the ion source at the same time. Hence attention is required for the appropriate selection of the internal standard for accurate quantitation of LC-MS/MS measurands and to achieve harmonisation of the current and future methods [17,19].

A contemporary challenge exists in relation to the amount of data generated from the MS. Interpretation of results against a reference interval or clinical decision point is critical to turn the numerical result into a clinically meaningful result. This is the challenge for many current MS assays and also the newer methods discussed in this edition of the journal [7,8,9,10]. In particular, the metabolomics discussion by Dias and Koal illustrates the need to develop an additional skill set of statistical analysis and/or employ statisticians to support the analysis of the magnitude of data generated in these MS discovery applications [10].

In conclusion, MS is now firmly established in the clinical space and the range of applications will continue to expand. Whilst MS is not yet applicable for all regions, in the future just like the manual immunoassays of old, MS throughput and user friendliness will improve. As we embrace MS our current and future scientists ideally should have the combined skills to 1) validate and run the current and new clinical MS applications, 2) work within a quality framework and 3) apply appropriate statistical analysis for the interpretation of the data. Developing scientists with these combined skills will support the robustness of methods, goals of harmonisation and eventual standardisation with traceability of MS methods.

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Mass spectrometry applications for toxicology

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ABSTRACT

Toxicology is a multidisciplinary study of poisons, aimed to correlate the quantitative and qualitative relationships between poisons and their physiological and behavioural effects in living systems. Other key aspects of toxicology focus on elucidation of the mechanisms of action of poisons and development of remedies and treatment plans for associated toxic effects. In these endeavours, Mass spectrometry (MS) has become a powerful analytical technique with a wide range of application used in the Toxicological analysis of drugs, poisons, and metabolites of both. To date, MS applications have permeated all fields of toxicology which include; environmental, clinical, and forensic toxicology. While many different analytical applications are used in these fields, MS and its hyphenated applications such as; gas chromatography MS (GC-MS), liquid chromatography MS (LC-MS), inductively coupled plasma ionization MS (ICP-MS), tandem mass spectrometry (MS/MS and MS^n) have emerged as powerful tools used in toxicology laboratories. This review will focus on these hyphenated MS technologies and their applications for toxicology.

Abbreviations (in alphabetical order)

***ADME**: absorption, distribution, metabolism, and elimination*
***APCI**: atmospheric pressure chemical ionization*
***API**: atmospheric pressure ionization techniques*
***CI**: chemical ionization*
***CID**: collision induced dissociation*
***DOA**: drugs of abuse*
***DRC**: dynamic reaction center*
***EI**: electron ionization*
***ESI**: electrospray ionization*
***FDA**: food and drug administration*
***FS**: full scan*
***FT-ICR**: fourier transform ion cyclotron resonance*
***FT-IT**: fourier transform ion trap*
***FWHM**: full width at half height*
***GC**: gas chromatography*
***GC-MS**: gas chromatography mass spectrometry*
***GLC**: gas-liquid chromatography*
***HR**: high resolution*
***IA**: immunoassays*
***ICP-MS**: inductively coupled mass spectrometry*
***IT**: ion trap*
***LC**: liquid chromatography*
***LC-MS**: liquid chromatography mass spectrometry*
***m/z:** mass to charge ratio*
***MALDI**: matrix assisted laser desorption ionization*
***MRM**: multiple reaction monitoring*
***MS**: mass spectrometry*
***MS/MS** and **MS^n**: tandem mass spectrometry*
***MW:** molecular weight*
***PAH**: polycyclic aromatic hydrocarbons*
***PK/PD**: pharmacokinetic/pharmacodynamics*
***Q1**: first quadrupole in MS instrument*
***Q2**: second quadrupole in MS instrument*
***Q3**: third quadrupole in MS instrument*
***QE** or **Q Exactive**: hydrid qudrupole-orbitrap mass spectrometer*
***QIT**: quadrupole ion traps*
***QTOF**: hybrid quadrupole time-of-flight mass spectrometer*
***RF**: radion frequency*
***SRM**: single reaction monitoring*
***TDM**: therapeutic drug monitoring*
***TOF**: time of flight*
***TQ-MS/MS**: triple quadrupole tandem mass spectrometer*
***WD**: waldenstrom's disease*
***2D**: two dimension*
***3D**: three dimension*

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INTRODUCTION

Toxicology can be thought of as the study of poisons, how poisonous encounters occur, how individuals respond to these encounters, and how to develop strategies for the clinical management of toxic exposures[1]. Poisons can be broadly defined as biologically active substances causing toxic effects in living systems. In essence, any biologically active molecule capable of altering normal physiology within a living system becomes a poison upon accumulation to quantities sufficient for a toxic effect[1]. For this reason, even therapeutic remedies can become poisons and toxic effects depend not only on the dose, but also on the overall pharmacokinetic and pharmacodynamic effects[2].

Since we are constantly surrounded by various chemicals, exposure can occur at home, work, or from the environment. The sheer complexity of possible poisons requires the use of sophisticated analytical tools and techniques to evaluate toxic exposures[3-6]. Toxic evaluations usually begin with qualitative or quantitative assessment in order to identify and/or quantify a toxic substance that could account for observed toxic syndromes (toxidromes) which are characteristic of different classes of poisons[7]. In addition, identification of the source for toxic

exposures is equally important. However, the overall role of laboratory testing is to identify and confirm the presence of a suspected poison and also to provide prognostic information when test results are able to predict clinical outcomes and/or help guide patient management.

In toxicology, the general analytical scheme for assessment of poisons in various matrices involves; 1) extraction, 2) purification 3) detection and 4) quantification (Scheme 1, A)[8]. The rise of modern analytical tools used by toxicology laboratories seems to have coincided with the chemical/industrial revolution (roughly 1850's to 1950's). A time which saw development of new liquid-liquid and solid-phase extraction methods along with qualitative or quantitative methods of detecting poisons based on their physical characteristics[8,9]. By the early twentieth century, chromatographic techniques using differential migration processes for separation of target molecules were developed by Mikhail Tsvet[9] and with the first versions of modern separation techniques such as liquid chromatography (LC) and gas-liquid chromatography (GLC or simply gas chromatography, GC) became routine in both analytical and preparative applications by mid-20th century[1,10,11]. At this time, labs also started to see the development of the first versions of modern mass spectrometers being used primarily for analysis of relatively pure materials[11-12].

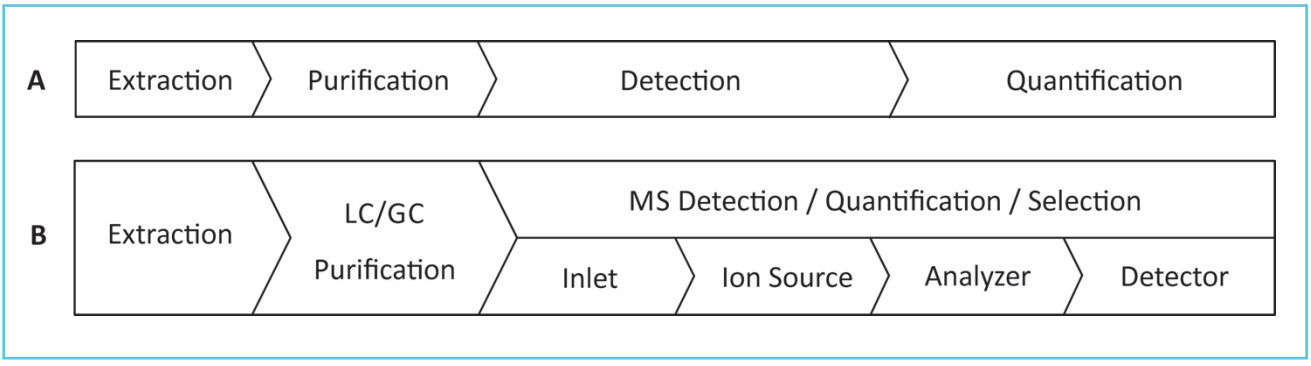
As MS, GC and LC technologies continued to advance in the second half of the 20th century, the more sophisticated methods used in modern toxicology laboratories started to emerge as amalgamations of separation and detection modes, creating new powerful analytical applications.

These included; high pressure liquid chromatography (HPLC), GC-MS, LC-MS, MS/MS and MS^n. These new technologies were initially used by research laboratories and later adopted into clinical laboratories[11,13]. To date, many of the modern analytical applications such as GC-MS and LC-MS still incorporate the same analytical scheme used by the earliest toxicology laboratories. But they are more stream-lined by combining multiple steps in the process with potential for automation (Scheme 1, B). This review will highlight current MS applications for Toxicology.

Mass spectrometry

Mass spectrometry is a quantitative technique which determines the mass-to-charge (m/z) ratio. In general, a mass spectrometer can be divided into four main components (Scheme 1, B): 1) a sample inlet, 2) an ion source, 3) a mass

Scheme 1 The analytical process for toxic compound evaluation in toxicology



**A) Steps involved in toxic compound isolation, identification and quantitation.*
B) GC-MS and LC-MS amalgamation of steps in the analytical process for toxic compound detection and quantitation.

analyzer, and 4) a detector. The sample inlet is where the sample enters the instrument before reaching the ion source. Ion sources are generally distinguished based on their underlining ionization technique[11,12]. The ionization technique used will determine the type of sample (e.g solid, liquid, vs gaseous samples) that can be analyzed in a given instrument and therefore also the type of chromatographic separation technique that should be coupled to the MS. Furthermore, the efficiency of sample ionization also determines in part the instrument's analytical sensitivity[11,12]. MS instruments in toxicology laboratories generally have LC or GC front ends, feeding into the instrument inlet either a liquid or gaseous sample for downstream ionization, analysis, detection, and quantitation (Figure 1, A-C)[3,4].

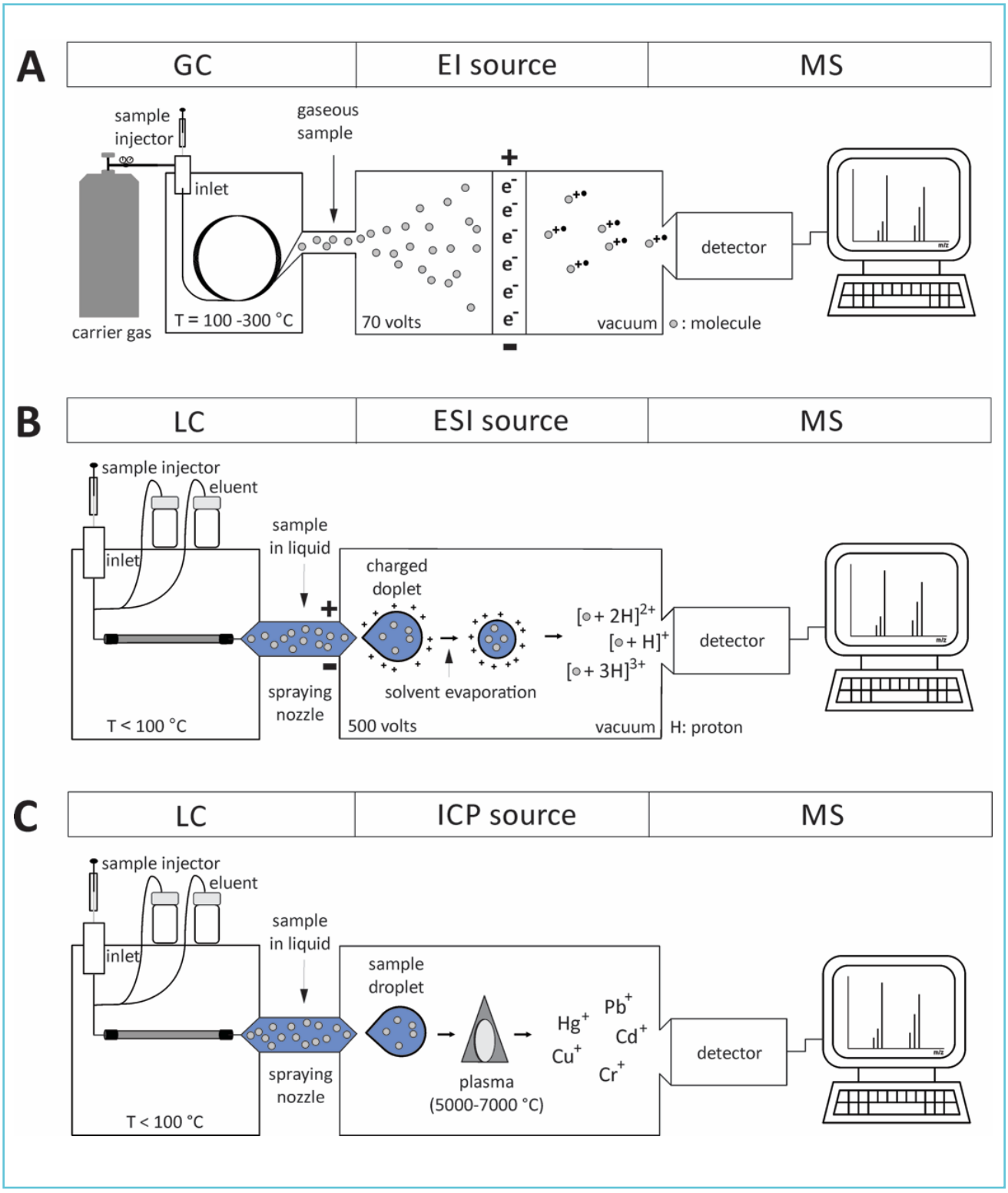
Common ionization techniques used by GC-MS include; electron ionization (EI) and chemical ionization (CI) for analysis of volatile and heat stable compounds (Figure 1A, GC-MS)[11]. For LC-MS, Atmospheric pressure ionization techniques (API) such as; electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are used for non-volatile and heat labile compounds (Figure 1B, LC-MS). Inductively coupled plasma ionization (ICP) is another ionization method used for elemental analysis usually for metals determination using ICP-MS (Figure 1C, ICP-MS) and matrix assisted laser desorption ionization (MALDI) for ionization of solid samples for MS analysis. Since MALDI techniques are not commonly used in toxicology applications, these won't be discussed in much detail here. Furthermore, the focus will be on the more prevalent EI, ESI and ICP ionization techniques used for toxicology applications despite the fact that modern GC-MS and LC-MS instruments can usually switch between EI/CI and ESI/APCI ionization mechanisms, respectively[4,5,11].

Mass analyzers and MS performance

From the ion source, sample ions enter the mass analyzer. Mass analyzers are the heart of the instrument and determine key performance characteristics such as the instrument's mass resolution, accuracy, and range. The mass range is the analytical mass range of the instrument. The resolution determines the ability of the analyzer to resolve two adjacent masses on the mass spectrum and is defined by the full width of the mass peak at half height of the peak maximum (FWHM). For a given *m/z* value, the resolution can be expressed as a ratio of *m/z* to FWHM such that for an ion with *m/z* 1000 and peak width of 0.65 atomic mass unit (amu) at FWHM the resolution is 1538. Low resolution instruments have FWHM > 0.65 amu and high resolution instruments reaching FWHM < 0.1 amu. The mass accuracy of MS instrument refers to the error associated with a particular *m/z* measurement. High mass accuracy gives the ability to measure the true mass of an ion to more decimal points. For example if the true mass of target ion is 1000 *m/z* and the measured mass from the instrument is 1000.002 *m/z*. The mass accuracy can be expressed in parts per million based on the ratio of the difference between the true mass and the measured mass to that of the true mass. So a ratio of 0.002/1000 which equals 0.000002 or a mass accuracy of 2 ppm in this example.

Mass analyzers typically used in toxicology include; quadrupole, ion traps, time of flight (TOF) and sector[4,11,15,16]. Quadrupole analyzers use four parallel metal rods to create a variable electromagnetic field which allows ions within a particular *m/z* range to reach the detector in order to record the mass spectrum. Quadrupole analyzers are cheap and robust, but can typically only achieve resolution around 1000 and mass accuracies of 100 ppm[16].

Figure 1 **Simple representation of A) GC-MS; B) LC-MS; and C) ICP-MS instruments and the ionization process for EI, ESI, and ICP occurring prior to mass analysis and detection in the mass spectrometer**



Ion trap (IT) instruments include quadrupole ion traps (QIT), Fourier Transform Ion Cyclotron Resonance (FT-ICR) and orbitraps. QIT use 2D or 3D quadrupole fields to trap target ions in a confined space and the mass spectrum is acquired by scanning the radion frequency (RF) and direct current (DC) fields to eject selected ions for detection[11,12]. Resolution for QIT is about 1000 – 10,000 with mass accuracy > 50 ppm[16]. FT-ICR are ion trap that keep ions in cyclotron motion within the trap. *m/z* detection occurs through measurement of induced currents from changes in ion orbits when an RF field is applied. This, allows calculation of *m/z* values with high accuracy (resolution > 200,000 and accuracy 2-5 ppm)[11,12,16]. Orbitraps use a metal barrel to create an electrostatic field for trapping ions in cyclical motion. The detection method is similar to that use in FT-ICR traps but with lower resolution < 150,000 but similar mass accuracy to FT-ICR[16].

TOF mass analyzers use a fixed potential to accelerate ions through a drift tube. Since all ions in a given pulse will attain the same kinetic energy, ions accelerate according to their *m/z* value and the mass spectrum is collected based on the time it takes individual ions to strike the detector. TOF analyzers generally have a higher mass range than quadrupole and IT instruments with relatively high resolution (1000 - 40,000) and mass accuracy (> 5 ppm)[16].

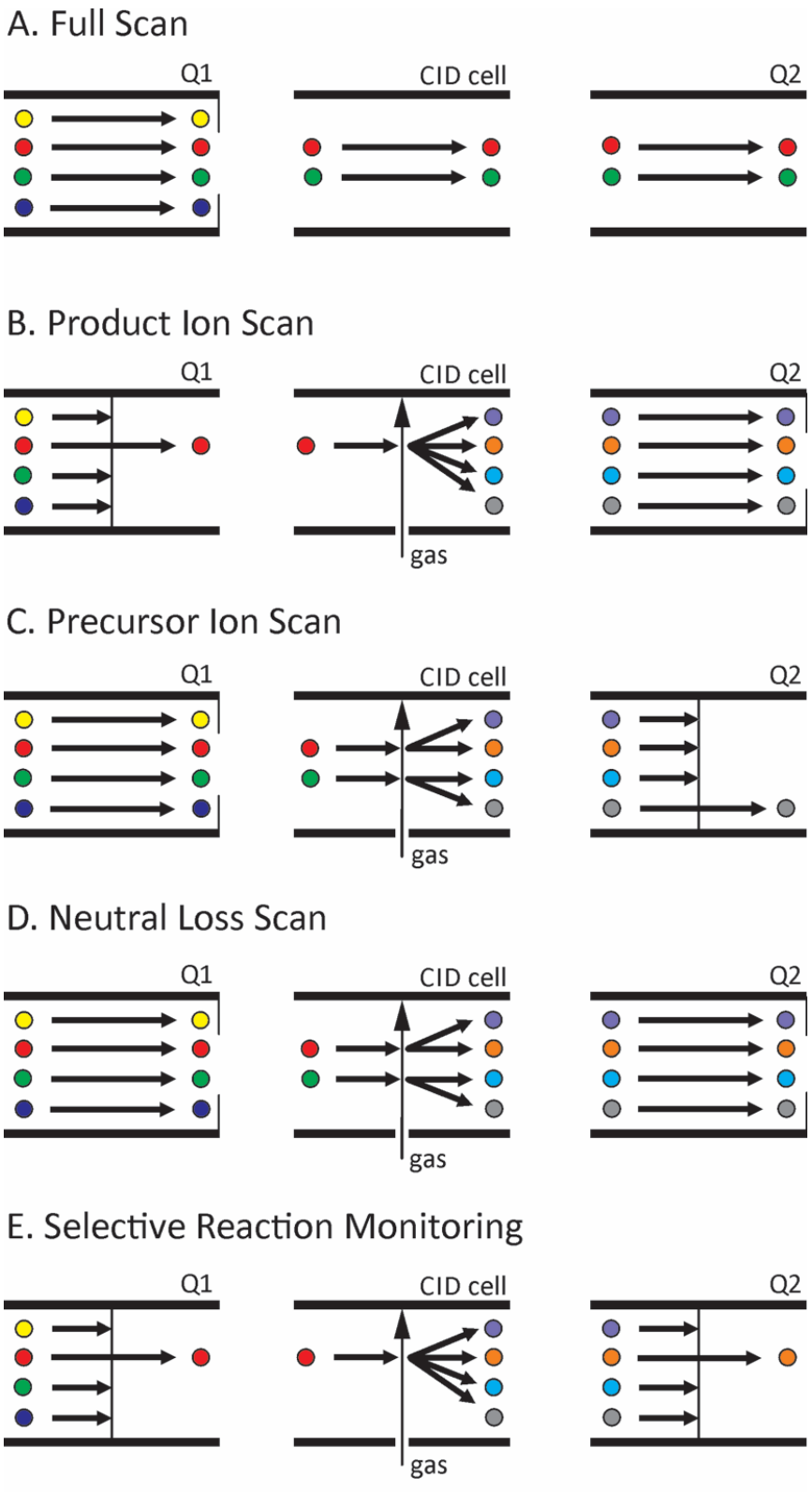
Sector analyzers are either magnetic sectors or double focusing (magnetic and electric) sectors. Similar to a TOF analyzer, magnetic sectors use a fixed potential to accelerate ions coming from the source such that ions attain the same kinetic energy but different momentum according to their *m/z*[16]. Accelerated ions are then passed through a magnetic field which guides ions through an arched path in order to strike the detector according to their momentum to charge ratio. By scanning the magnetic field strength, ions with different *m/z* are selected for detection. In magnetic sectors, resolution is limited by minor kinetic energy dispersions (ion velocities). A double focusing sector analyzer adds a electric field before or after the magnetic field to also focus ions according to their kinetic energy to charge ratios. Focusing ions of different velocities to the same point. This gives double focusing magnetic sectors relatively high resolution (100,000) and high mass accuracy (<1 ppm)[16].

In summary, the ion source, mass analyzer, and detector for a particular instrument all play a role in defining the instrument's analytical capabilities. It is also important to note that even though the basic design of MS instruments has stayed relatively unchanged over time, the performance capabilities of MS sources, analyzers, and detectors have continued to improve over time[4,11,13,15]. The strength of MS for Toxicology is the combined sensitivity and specificity that is needed to identify and quantify the toxic agents.

MS instruments

The versatility of MS analytical applications comes from the ability to couple different separation techniques in the front-end (i.e. GC or LC) and various analyzers either in tandem or hybrid configurations[4,5,11,12,15]. The type and arrangement in a given instrument not only determines its resolution, mass accuracy, and analytical range, but also the type of experiment(s) possible for analytical applications (Figure 2, A-E)[4,11,13,15]. In clinical applications, the MS instrument with most versatile capabilities is perhaps the triple quadrupole tandem mass spectrometer or TQ-MS/MS with three quadrupole analyzers arranged in tandem for MS/MS experiments[13]. The first quadrupole (Q1) selects ions that will enter the second quadrupole (Q2), a collision cell able to carry out collision induced dissociation (CID) of selected ions. From the collision cell, product ions enter the third quadrupole (Q3) which can guide selected ions into the detector. TQ-MS/MS instruments are capable of

Figure 2 **Analytical experiments possible with a TQ-MS/MS instrument**



performing full MS scans (FS, Figure 2A), multiple reaction monitoring (MRM, Figure 2, B-E), or single reaction monitoring (SRM, not shown) for analyte detection[13,3].

The MS/MS experiment involves selected fragmentation of target ions using CID followed by analysis of the products (Figure 2B, product ion scan)[13]. The target ion is often referred to as parent ion and CID fragments are referred to as product ions. In MS/MS experiments, MRM will follow the conversion of one parent ion to one product ion via CID (indicated as parent *m/z* > product *m/z*) or any experimentally feasible combination of parent and product ions given analytical capabilities of the instrument. MRM and SRM usually increases sensitivity based on improved signal to noise ratio, and the MS/MS offers increased specificity at the cost of decreased sensitivity since signal is lost at each round of fragmentation. Specificity improves when unique fragmentation patterns are able to distinguish co-eluting ions with identical exact mass as targeted molecule, but different chemical composition. In addition, MS/MS can also be used for structural determinations. A key advantage of the TQ-MS/MS instrument is the ability to do precursor ion scan (PI, Figure 3C) or neutral loss (NL, Figure 3D) reaction scans over a wide *m/z* range[4,11,13,15]. This application can use a single sample injection for rapid scanning of the full *m/z* spectrum in order to identify compounds with known functional groups that dissociate as detectable ions or neutral masses following CID.

Due to the tandem arrangement of quadrupole analyzers in the TQ-MS/MS, MS/MS is done sequentially in space between different analyzers. In IT instruments (QIT, Fourier transform ion trap or FT-IT, and orbitrap), MS/MS experiments are done in sequence over time based on the ability of the trap to retain selected ions following each round of CID[4,11]. MS/MS also occurs with high efficiency in IT instruments but one key limitation is the capacity to retain ions and *m/z* scanning speed[4,11]. 2D ion traps were designed to overcome the ion capacity problem and have a higher analytical range giving FS, SRM, and MRM capabilities over a wider *m/z* range compared to 3D ion traps[4,11]. The in-time MS/MS application of IT instruments means PI and NL screening experiments are not possible. However, MS^n experiments for structural determination of larger molecules are possible, usually with no more than three rounds of fragmentation due to loss of signal following each consecutive round of CID[4].

Over time, MS instruments have continued to improve in selectivity, mass accuracy, and resolution, along with formation of hybrid instruments with enhanced capabilities often designed to overcome limitations of available instrumentation. For example, one key limitation of TQ-MS/MS instruments is that the PI/NL scans cannot be performed in a single injection along with MS/MS acquisitions for targeted structural determination. The QTRAP is a hybrid TQ-IT instrument where the third quadruple is a linear IT, making possible the acquisition of PI, NL, and MS^n experiments in a single injection[4,11]. Other hybrid instruments are designed to couple more accurate mass determination with MS/MS or MS^n capabilities like the hybrid quadrupole time-of-flight (QTOF) instrument or quadrupole-orbitrap hybrid (QE or Q Exactive).

MS APPLICATIONS FOR TOXICOLOGY

To date, MS and its hyphenated applications (GC/LC/ICP-MS) have emerged as a powerful analytical tool for toxicology applications. GC-MS is generally used for analysis of volatile and heat stabile compounds, LC-MS for analysis of non-volatile and heat labile compounds, and ICP-MS for elemental analysis usually in metals determination[4,5,11,13,14,17]. Owing to the analytical versatility of MS methods with exceptional specificity,

sensitivity, dynamic range, and the ability to screen large numbers of unrelated compounds, MS applications are central for toxicological analysis of drugs and poisons. Current use includes drug analysis for targeted applications (e.g. in TDM and pain management), screening applications (e.g. in drugs of abuse (DOA), forensic toxicology, environmental toxicology, and clinical toxicology), and in pharmacokinetic/pharmacodynamics (PK/PD) research[5,11,14,15,17,18]. Here, we will focus on GC-MS, LC-MS, ICP-MS, and MS/MS capabilities and respective applications for toxicology.

Overcoming limitations of Immunoassays (IA) in TDM and drug screens

Since MS applications emerged at a time where IAs were already established in clinical laboratories, one driving force for the expansion of GC and LC-MS application in Toxicology has been efforts to overcome the limitations of IAs in drug analysis[13,19-22]. One limitation is IA are usually developed by manufacturers who seek FDA test approval based on commercial interests, with the end user having little control over this process. Another limitation is poor analytical specificity and analytical interferences[13,19-22]. The specificity of IA's developed for small drugs is usually limited to the detection of drug classes, but not necessarily individual drugs within a given drug class. This limitation could stem from the fact that antibodies generally recognize epitopes on large biomolecules, making the specificity of IAs poor for recognizing specific small molecules[13,22]. Currently, IA's are often used in first line screening for Toxicology since they can quickly identify a potentially negative sample, and are useful in identifying drug classes or specific drugs (i.e. benzodiazepines, opiates, amphetamines, cannabinoids, methadone, fentanyl, and phencyclidine), but suffer from high rates of false positive and false negative results due to a lack of specificity, cross reactivity, or interferences[4,21]. Since immunoassays are generally available as FDA approved tests on large automated analyzers, the common approach is to screen using an immunoassay first and then confirm positive results using GC-MS or LC-MS techniques which have superior sensitivity and specificity to identify specific molecules[4,21].

Drug analysis by GC-MS

Coupling of GC to MS provided an opportunity for development of routine applications with the specificity and sensitivity of MS (Figure 1A)[11,14,17,23]. GC is an analytical separation technique using a liquid or polymer stationary phase along with a gas mobile phase for separation of molecules based on partitioning between the stationary and gas phase. The process usually requires high temperature or temperature gradients (up to 350°C) in order to facilitate compound elution into the mobile gas phase (Figure 2A). The analytes are separated based on their column retention time, entering the MS in the gas phase for ionization usually with EI sources to facilitate MS detection. EI ionization uses the kinetic energy from a stream of high energy electrons (usually 70 eV) to strip electrons from analyte molecules at high temperatures, a process that produces a reproducible fragmentation patter from organic compounds (Figure 2A)[11]. For this reason, EI-GC-MS data is conducive to inter-laboratory spectral comparisons and extensive EI-GC-MS libraries have been generated for spectral matching based identification[11,23,24]. These libraries supplement "in-house" generated libraries and greatly increasing the ability to identify unknown compounds using GC-MS. This analytical advantage has made EI-GC-MS a premier tool for untargeted detection and quantitation of small molecules with MS specificity. EI-GC-MS is still used for general unknown screening applications using nearly any sample type[17,21,25]. Additionally, GC-MS is commonly used to confirm IA positive results in drug screens in

clinical toxicology[4,18,22,23]. One key limitation of GC-MS is the need to have volatile and heat stabile analytes, this means that some analytes require chemical derivatization in order to make the drugs sufficiently volatile for GC-MS analysis[23,25]. This limits GC-MS expansion to analysis of many drugs and adds additional steps and cost during sample preparation.

GC-MS applications for toxicology

GC-MS does have several advantages compared to its LC-MS/MS counterpart that include: efficient GC separation with higher chromatographic resolution and peak capacity, a homogeneous gas mobile phase (usually helium or hydrogen), optimization of separation conditions with precise electronic controls such as temperature programming, and the ability to search EI-MS databased for library based toxic compound identification[11,24]. Taken together with good MS sensitivity (1-10 µg/L) and specificity, a leading application of GC-MS is the general screening of unknown drugs or toxic compounds in doping control, environmental analysis, and clinical and forensic toxicology[24].

Therefore, in clinical toxicology, GC-MS is commonly used for screening blood and urine for acute overdose of prescription and over the counter medications in emergency room settings. This is specifically useful for drugs with toxic effects and known antidotes or therapies that can be initiated to treat the toxic effect[1,17,25]. It is also commonly used to perform drug screens for identification and/or quantitation of poisons in the clinical evaluation of toxindromes or in forensic investigations. Drugs commonly quantitated by GC-MS include; barbiturates, narcotics, stimulants, anesthetics, anticonvulsants, antihistamines, anti-epileptic drugs, sedative hypnotics, and antihistamines[24]. In environmental toxicology, GC-MS is used for the convenient screening of a wide range of toxic compounds such as; chloro-phenols in water and soil or polycyclic aromatic hydrocarbons (PAH), dioxins, dibenzofurans, organo-chlorine pesticides, herbicides, phenols, halogenated pesticides, and sulphur analysis in air[24]. One thing to mentions is most toxicology laboratories which can afford it are slowly replacing GC-MS with LC-MS as the method of choice for targeted drug screens for clinical and forensic toxicology applications[4,14,23]. Lastly, the higher specificity of MS detection compared to enzymatic spectrophotometric assays, GC-MS is sometimes used for identification and quantitation volatile substances (e.g. ethanol, methanol, acetone, isopropanol, and ethylene glycol) in body fluids such as blood and urine.

LC-MS applications for drug analysis

Due to the limitation of GC-MS for analysis of volatile and heat stable compounds, LC-MS applications have expanded MS applications to the direct analysis of non-volatile and heat labile molecules in toxicology laboratories (Figure 2B)[4,11,13,21,22,26]. The coupling of MS to LC was first possible when API-ESI sources became available in the 1990s, making it possible to ionize samples in the condensed phase and inject ions directly for MS analysis[11,12]. In contrast to EI used in GC, ESI is a soft ionization technique which does not induce fragmentation, instead, singly or multiply charged ions form from intact molecules due to proton transfer events (Figure 2B)[11,12]. ESI uses a capillary tube to flow solvent through a voltage potential before the solvent is sprayed into the MS vacuum as an aerosol[12]. Under vacuum, a heated gas (e.g. N_2) is used to dry the droplets and release gas phase ions for MS detection. The exact mechanism of ion formation by ESI is not fully understood, but the aerosol droplets are either negative or positively charged depending on the voltage applied and protonation/deprotonation events giving intact $[M+H]^+$ or $[M-H]^-$ ions for MS analysis (Figure 2B)[11,12]. To date, there seems to be no limit to

the size of molecule which can be ionized by ESI in biological samples[12]. Multiple protonation/deprotonation events also means ESI can yield more than one *m/z* peak from a single compound, a phenomenon that can either complicate the MS analysis or facilite measurements which improve precision or allow observation of *m/z* from targets with MW above the instrument range[12]. One inherent limitation of the ESI process, and therefore LC-MS, is the mass spectra of a given compound can vary depending on instrument conditions, including the capillary diameter, sample flow rate, and voltage applied[4,23]. The consequence is ESI mass spectra are instrument dependent, requiring the development of in-house derived spectral libraries for compound analysis[23,26]. Regardless, by overcoming key limitations of GC-MS, LC-MS has significantly expanded MS applications to targeted drug analysis of non-volatile and heat labile compounds such as drug metabolites[11,13-15,26].

The switch form GC-MS to LC-MS for analysis of toxin and drug metabolites in toxicology is notable[11,18,27-29]. One reason for this is that most drugs or toxicants entering the body undergo biotransformation by phase I (functionalization) and phase II (conjugation with hydrophilic endogenous molecules) metabolic reactions in order to facilitate elimination from the body[11,30]. The transformations often result with structurally diverse hydrophilic and heat labile metabolites with biological activities ranging from no pharmacological activity, to pharmacologically activity, to toxicity[15,23,29,30]. The nature of these drug metabolites, especially phase II metabolites, gives LC-MS a unique advantage for analysis of drugs and their metabolites using LC-MS, MS/MS and MS^n applications for identification, structural determination, and mapping PK/PD interactions during ADME [30]. To date, numerous studies have demonstrated that combined analysis of drug and metabolites greatly increases the ability to positively identify drug use using blood or urine samples[25]. Furthermore, urine has a much wider window of detection for detecting drug use, but extensive drug metabolism for urine excretion makes metabolite analysis more important for interpretation of results of urine drug analysis in pain management or DOA screening[18,25]. Lastly, LC-MS is also routinely used for targeted drug analysis in TDM, forensic toxicology, PK/PD pharmaceutical analysis, or in confirmation of compounds that do not work with GC-MS[4,18,25,31].

ICP-MS applications for analysis of toxic metals

ICP-MS was introduced for clinical use in 1980's for individual or multi-elemental metals analysis in toxicology[5,32]. The ICP source is designed for sample atomization and elemental analysis. Usually a peristaltic pump is used to inject aerosolized liquid samples into an argon plasma discharge at (5000-7000°C), but an LC can also be used for the separation of elements that require speciation (Figure 2C)[33]. The plasma vaporizes, atomizes, and effectively ionizes the sample for elemental analysis by MS. Advantages of LC-ICP-MS include the ability for metal speciation, multiple element measurements, and a wide dynamic range with accurate and precise trace metal measurements[34,35]. Detection limits for ICP-MS are commonly in the low ng/L range, giving an advantage in quantification of low levels of trace elements or toxic metals[5,35].

A key limitation of ICP-MS applications for metals analysis is polyatomic interferences[5,32,34]. These are interferences that result from the combination of two (or more) atomic ions from the sample matrix to form molecules which have the same *m/z* with analytical targets. One example is the combination of the argon plasma gas (40 Da) with a chloride ion (35 Da) or carbon (12 Da) from the biological matrix to produce ArCl (75 Da) and ArC (52 Da) ions. ArCl and ArC have the same *m/z* as arsenic and chromium,

two metals commonly incorporated into toxic metal surveys by ICP-MS[5]. To date, several ICP-MS applications have been developed in order to overcome isobaric or polyatomic interferences to improve specificity using collision/reactions cell applications. A dynamic reaction cell (DRC) uses a reactive gas in quadrupole ICP-MS instruments to overcome isobaric interferences from the plasma by reacting the gas with either the analyte (ion) of interest or isobaric compound (ion) in order to distinguish the two[5]. Equally, the quadrupole can act as a collision cell where a inert gas is introduced and will preferentially interact with polyatomic ions with larger radii, reducing their kinetic energy to allow resolution of polyatomic interferances from the analyte of interest through kinetic energy discrimination (KED). Lastly, collision induced dissociation (CID) in a triple quadrupole ICP-MS/MS can be used to break up polyatomic interferences prior to MS detection or a higher resolution instrument (e.g. double focusing sector ICP-MS) can be used to resolve polyatomic inteferences through accurate mass determination[5]. Owing to the high specificity, sensitivity, and reproducibility in elemental analysis by ICP-MS, this technique is now used in clinical laboratories for toxic metal and trace elements quantitation in a wide variety of samples, these include; whole blood, serum, plasma, urine and dry spots of these liquid samples (using laser ablation with ICP-MS). Sample collections in metal-free tubes are required for accurate determinations[5,34,35]. Other sample types used in forensic toxicology include; urine, hair, nail, tissue, and or other forensic materials.

Toxic metals and metal exposures

Metals represent some of the oldest toxicants known, with records of toxic metal exposures dating back to ancient times[1]. Nonetheless, many metals are also essential or trace elements with vital functions for life (i.e. cobalt, copper, iron, magnesium, selenium or zinc), but will become toxic with increased levels or pathologic metabolism like Cu in Wilson Disease (WD)[5]. Others like; thallium, arsenic, mercury, and lead, are poisons with no well-established physiological function. Other potentially toxic metals include: chromium, cadmium, platinum, nickel, aluminum, and gadolinium[5]. Metals exert their toxic effects through redox chemistry with biological targets, a process that might change the oxidation state of the metal and lead to formation of characteristic organometallic compounds[5,36]. Each metal has a specific mechanism of toxicity with different metal species varying in toxic effects. For this reason, metal speciation is an important aspect of clinical evaluations of toxic metal exposures[36]. Speciation involves identification and quantitation of different forms of a given chemical species. For example, chromium^{VI} (Cr^{VI}) is a powerful toxic oxidant whereas Cr^{III} is less toxic and plays a role in metabolism[5,36,]. Elemental mercury (Hg^{o}) has a lower toxicity than methyl mercury (MeHg), and arsenic is present in seafood as innocuous arsenocholine and arsenobetaine, but elemental arsenic is highly reactive and toxic to humans[5,36]. The different metal species can be distinguished through distinct; isotopic composition, oxidation state, or over-all molecular structure with speciationbeing essential in theevaluation of some toxic metal exposures[34-36]. Speciation with LC-ICP-MS effectively relies on LC separation of various metal species followed by MS detection. To date, methods have been developed for speciation of Hg, Arsenic, Cr and other[36].

Furthermore, isotopic fractionation by high resolution ICP-MS (HR-ICP-MS) or Q-ICP-MS can function as another method of metal identification. For example, lead isotopic ratios (^{206}Pb, ^{207}Pb, ^{208}Pb) may be useful to confirm the source of metal exposure in clinical toxicology or in forensic toxicology[5]. Studies have also shown $^{65}Cu/^{63}Cu$ isotopes ratios in dried urine

spots or serum can be used to classify treated and untreated Waldenstrom's disease (WD) patients when isotopically enriched sampes are administered[36-38]. For this reason, ICP-MS is a powerful tool for evaluation of metal exposures in forensic and clinical investigations with the ability to also use isotopic analysis to confirm the source of lead contamination. These distinctions are important since anthropogenic activities have introduced toxic metals such as lead (from gasoline) into the environment (air, water, and soil), the workplace, and consumer products such as food and pharmaceuticals[5,34-36]. Furthermore, metals are also used in implants for joint replacement (e.g cobalt, chromium, and titanium) and may leach-out during wear of the prosthetic device leading to the endogenous accumulation with potentially toxic consequences[36,39]. For these reasons, ICP-MS screening and speciation assays for toxic metals are commonly developed in order to evaluate toxic exposures in clinical toxicology, lethal exposures in forensic toxicology, and investigate environmental sources of metal exposure.

ICP-MS applications in clinical toxicology

ICP-MS is extensively used in multi-analyte toxic metal screens in whole blood, plasma serum and urine[5]. Blood and urine analysis is generally useful in assessing acute and chronic metal exposure with reference values available to aid with result interpretation from several geographical locations around the world[36]. Newer applications using dried blood or urine spots along with laser ablation for multi analyte metal analysis have also been described[38,40]. The multi-analyte ICP-MS metal panels can include up to dozens of targets including; lead, mercury, arsenic, cobalt, chromium, manganese, molybdenum, nickel, titanium, aluminum, and silver[5,36]. Lead is commonly evaluated in children due to its adverse effects on development[41]. Exposures can also occur from buildings with old lead water pipes, lead containing paint, or exposure from environment accumulation due to historic use of gasoline with tetraethyl lead[5,41]. Mercury exposure can occur from eating carnivorous fish which tend to contain high MeHg content as it accumulates up the food chain from environmental contamination. Exposures to mineral mercury leaching from dental amalgams has also been described[42]. Mineral mercury is usually measured in plasma and MeHg in whole blood to distinguish exposures from seafood and dental amalgams[5,36,42]. Arsenic is a substance that has been used in intentional poisonings, but accidental exposure can also occur through contaminated ground water[5,43]. Toxic levels of cobalt, chromium, manganese, molybdenum, nickel and titanium have been shown in people with various metal replacement joints or dental implants[5,39]. Aluminum is routinely quantified in plasma to monitor hemodialysis patients and it is also the subject of toxicological controversies associated with adverse effects from vaccines[5]. Historically, silver has been used as an effective bactericide but when taken in excess, exposures can result with development of argyria along with neurologic, hematologic, renal, or hepatic involvement with blood silver toxic levels as reported from cases of argyria[44-46].

ICP-MS applications in forensic toxicology

Deaths due to metal toxicity are uncommon and often unexpected, as a result, all unexplained deaths often prompt blood analysis for traditional metal poisons (e.g arsenic, thallium) toxic heavy metals (e.g arsenic, lead, cadmium, mercury) and other toxic metals (e.g aluminum, chromium, cobalt, molybdenum, nickel, vanadium or tungsten) or drugs (e.g contrast media). One advantage of forensic metals analysis by ICP-MS is the ability to use other sample types in addition to blood or urine[5]. For example, the use of laser ablation coupled with ICP-MS detection can allow the analysis of various samples

such as nail and hair in clinical or forensic toxicology analysis[5,40,47]. Blood and urine usually reflect exposure in the last days or hours[5]. Hair is a cumulative biomarker for longer term exposure compared to blood or urine. Each centimeter of hair represents one-month of exposure and can therefore be used to check for a longer window of exposure in clinical and forensic toxicology investigations. Hair can be used in conjunction with blood or urine results to differentiate a single exposure from chronic exposure by comparison with hair samples from a given growth period[5]. Alternatively, nails are another biomarker for forensic metals analysis by ICP-MS. Nails incorporate elements from blood during linear growth and thickening, providing a window of detection spanning 3 to 5 month for toxic metal exposure[5]. In clinical toxicology, nail collections are also considered non-invasive and contain more disulfide groups which help incorporate higher metal content, making it a preferred matrix for metals analysis for a longer window of detection when hair is not available due to balding or other reasons (e.g. religious reason)[5]. Lastly, tissue and biopsies for metals analysis by ICP-MS becomes important when blood and urine are not available and hair and nails are affected by external contamination, or when specific organs biopsies need to be checked for metal accumulation[5].

CONCLUSIONS

In summary, mass spectrometry (MS) is a powerful analytical technique able to distinguish ionizable chemical compounds or elements based on their *m/z* ratio in the gas phase. With exceptional sensitivity, accuracy, precision, and dynamic range, MS has emerged as an important tool in analytical determinations of poisons and their metabolites in clinical, forensic, and environmental toxicological evaluations. GC-MS is commonly used for general unknown screen (GUS) of poisons, drugs and their metabolites based on the capacity to identify a vast majority of chemical compounds using inter-laboratory EI-MS libraries. The limitation of GC-MS is that compounds need to be volatile or heat stable for compatibility with GC separation. This restriction often requires derivatization of non-volatile compounds for compatibility with GC separation and limits analysis of heat labile compounds which often includes drugs and their metabolites. LC-MS overcomes these limitations by using ESI to introduce ions from liquid samples into the MS for analysis of non-volatile and heat labile compounds. As such, LC-MS is slowly replacing GC-MS for the analysis of poisons, drugs, and their metabolites. Disadvantages of LC-MS include high cost and the inability to use inter-laboratory spectra for compound identification. To date, both GC/LC-MS are used in advanced laboratories along with MS/MS and MS^n applications for increased specificity in drug identification, drug metabolite analysis, and structural determination. Lastly, ICP-MS is commonly used for trace and toxic metal analysis in toxicology laboratories. A key advantage of ICP-MS is the ability to do multi-element panels in toxicological analysis along with the use of MS/MS, HR-MS, and DRC applications for resolving interfering compounds. Overall, MS is a versatile analytical tool with many useful applications and has the potential for automation. In general, trends for adopting MS applications for toxicology relies on the ability to multiplex quantitative and qualitative compound evaluations and hyphenated MS applications with higher mass resolution for increased analytical specificity.

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In this issue: Recent Developments in the Clinical Application of Mass Spectrometry

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Advantages and challenges of dried blood spot analysis by mass spectrometry across the total testing process

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ABSTRACT

Introduction

Through the introduction of advanced analytical techniques and improved throughput, the scope of dried blood spot testing utilising mass spectrometric methods, has broadly expanded. Clinicians and researchers have become very enthusiastic about the potential applications of dried blood spot based mass spectrometric applications. Analysts on the other hand face challenges of sensitivity, reproducibility and overall accuracy of dried blood spot quantification. In this review, we aim to bring together these two facets to discuss the advantages and current challenges of non-newborn screening applications of dried blood spot quantification by mass spectrometry.

Methods

To address these aims we performed a key word search of the PubMed and MEDLINE online databases in conjunction with individual manual searches to gather information. Keywords for the initial search included; "blood spot" and "mass spectrometry"; while excluding "newborn"; and "neonate". In addition, databases

were restricted to English language and human specific. There was no time period limit applied.

Results

As a result of these selection criteria, 194 references were identified for review. For presentation, this information is divided into: 1) clinical applications; and 2) analytical considerations across the total testing process; being pre-analytical, analytical and post-analytical considerations.

Conclusions

DBS analysis using MS applications is now broadly applied, with drug monitoring for both therapeutic and toxicological analysis being the most extensively reported. Several parameters can affect the accuracy of DBS measurement and further bridge experiments are required to develop adjustment rules for comparability between dried blood spot measures and the equivalent serum/plasma values. Likewise, the establishment of independent reference intervals for dried blood spot sample matrix is required.

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INTRODUCTION

A century ago, for the very first time, *Ivar Bang* described a dried blood matrix as an unconventional sampling method (1). Later, *Robert Guthrie* in 1963 introduced the dried blood spot technique for screening. Guthrie's application of the dried blood spot, and his personal crusade to utilise this approach to screen intellectually disabled children, heralded the introduction of newborn screening. Although the particular assay is now defunct, the term "Guthrie card" remains to colloquially describe the dried blood spot collection technique which still underpins today's newborn screening programs worldwide (2).

The original semi-quantitative bacterial inhibition test developed by Guthrie to screen for phenylketonuria was highly sensitive but had a low analytical throughput (3). Through the introduction of advanced analytical techniques, that have expanded testing options and improved throughput, the scope of newborn screening blood spot sample applications were extended; this included screening applications for congenital hypothyroidism and cystic fibrosis in many centres (4). Further to this expansion was the introduction of tandem mass spectrometry for newborn screening dried blood spot analysis in the 1990s (5).

Mass spectrometry is now the most common technique reported in the literature for dried blood spot analysis (6-9). Dried blood spot analysis offers the advantage of collecting a small sample volume, which is easily transported. However, this also means that the concentration of the target analyte is potentially quite low (*e.g.* less than 1 ng/L), requiring a sensitive and specific assay for detection and quantification. These considerations, coupled with the expansion of mass spectrometry into clinical laboratories, have led to a surge in the utilisation of this sampling method outside of the scope of newborn screening in the published literature.

Clinicians and researchers have become optimistic about the potential applications of dried blood spot based mass spectrometric applications and it has been used for a range of clinical utilities including drug toxicology and sports doping screening. Scientists and technical analysts on the other hand face challenges regarding how to ensure optimal sensitivity, reproducibility and overall accuracy of dried blood spot quantification. In this review, we aim to bring together the clinical and analytical facets to discuss the advantages and current challenges of non-newborn screening applications of dried blood spot quantification by mass spectrometry.

To address these aims, we performed a key word search of PubMed and MEDLINE online databases in conjunction with individual manual searches to gather information. Keywords for the initial search included; "blood spot" and "mass spectrometry"; while excluding "newborn"; and "neonate". In addition, databases were restricted to English language and human specific. No time period limit was applied. As a result of this selection criteria, 194 references were identified for review. For presentation, this information is divided into clinical applications and analytical considerations across the total testing process. It is not our intention in this review to highlight all analytical aspects related to quality mass spectrometric analyses (as this has been covered extensively elsewhere) but rather to address the analytical aspects pertinent to the dried blood spot matrix.

CLINICAL APPLICATIONS

The first application of mass spectrometry (MS) to dried blood spot (DBS) analysis was reported 40 years ago (in 1976) for fatty acid determination by direct chemical ionisation (10). By the mid-1980s, when gas chromatography (GC) was the technique of choice for separation and analysis of volatile small molecules, derivatized fatty acids were measured from DBS samples using GC-MS (11). In the 1990s, when electrospray ionization became commercially available, liquid chromatography - tandem mass spectrometry (LC-MS/MS) began to be incorporated into the analytical tools for newborn screening laboratories, leading to the significant expansion of screening applications with phenylalanine and tyrosine being two of the early markers (5, 12). Today dried blood spot based mass spectrometric (DBS-MS) applications are the workhorse for many newborn screening (NBS) laboratories worldwide; and additional tests are continually being added to the repertoire. Outside of NBS, an epidemiological study analysing benzoylecgonine was the first reported DBS-MS application (13).

Now, many potentially reliable and compatible MS detection methods are available across a wide range of disciplines (14). Our literature search identified 97 references encompassing 121 distinct biomarkers determined from DBS samples utilising MS technology beyond its application in NBS. Notably, the role of DBS analysis by MS now encompasses translational research and clinical diagnostic analytes in the areas of therapeutic drug monitoring (TDM); pharmakinetics); toxicokinetics; forensic; endocrinology and metabolism; and other areas of bio-analysis. Table 1 provides a list of these biomarkers.

Therapeutic and toxicological drug analyses are the most extensively reported DBS-MS applications in the literature (Table 1). These DBS-MS applications, (encompassing both LC-MS/MS and GC-MS techniques) are particularly fit for population-based studies of multiple biomarkers (15, 16). Similarly, DBS-MS applications are now applied in sport related doping tests for the detection of anabolic, ergogenic and masking agents (17-19).

Irrespective of the clinical application, there are specific analytical considerations. Several parameters can impact on the accuracy of DBS measurement (10). The following sections of this review will focus on important considerations for DBS-MS quantification in the pre-analytical, analytical and post-analytical phases.

PRE-ANALYTICAL

The pre-analytical phase of testing incorporates the following processes: 1) blood collection from the patient; 2) its application onto the filter paper; 3) drying; and 4) transport and storage of the DBS sample. In addition to the pre-analytical variables identified for routine blood collection, DBS faces additional challenges, including the quality of the DBS sample (which is subject to

sample collection and spotting variations), choice of collection card, collection (bleeding and blotting), transport and storage. Moreover, biological factors such as sample viscosity, haematocrit level and the nature of the target analyte, may lead to variation in sample quality.

Abbreviations for Table 1 (in alphabetical order)

2D: *two dimensional*
AnalTech: *analytical technique*
APTDCI-MS: *atmospheric pressure thermal desorption chemical ionization mass spectrometry*
CE: *chemical exposure*
D: *day*
DAA: *drug abuse athletics*
Dab: *drug abuse*
Dad: *drug adherence*
ESI: *electrospray ionisation*
FIA-ESI-MS/MS: *flow Injection analysis-electrospray ionisation tandem mass spectrometry*
FT-ICR-MS: *fourier transform ion cyclotron resonance mass spectrometry*
GC: *gas chromatography*
GC-HRMS: *gas chromatography–high resolution mass spectrometry*
HILIC-MS/MS: *hydrophilic Interaction chromatography tandem mass spectrometry*
ID: *illicit drug*
IDES-MS/MS: *isotope-dilution electrospray tandem mass spectrometry*
LC: *liquid chromatography*
LC-HRMS: *Liquid chromatography–high resolution mass spectrometry*
LLOQ: *lower limit of quantitation*
M: *month*
MetV: *method validation*
MI: *metabolic intermediate*
MS: *mass spectrometry*
NR: *not reported*
PD: *pharmaceutical drugs*
PS-MS: *paper spray mass spectrometry*
PK: *pharmakinetics*
Ref: *reference*
RepU: *reporting unit*
Stab: *stability*
TDM: *therapeutic drug monitoring*
TK: *toxcicokinetics*
TLC-MS: *thin-layer chromatography mass spectrometry*
W: *week*
Y: *yes*

Table 1 The list of biomarkers determined from dried blood spot samples utilising mass spectrometry technology, beyond its application in newborn screening studies

No	Analyte	AnalTech	LLOQ	RepU	Stab	MetV	Ref
Metabolic intermediate							
1	Acylcarnitines	APTDCI-MS/MS	NR	µmol/L	NR	NR	(110)
		ESI-MS/MS	NR	µmol/L	NR	NR	(111)
2	Adrenal steroids	LC-ESI-MS/MS	0.75-6.3	nmol/L	NR	Y	(112)
3	Amino acids profile	LC-MS/MS	0.25	µmol/L	NR	Y	(113)
4	Bile Acids	ESI-MS/MS	1	µmol/L	10d	Y	(114)
5	Carnitine	IDES-MS/MS	1	µmol/L	NR	Y	(115)
		ESI-MS/MS	NR	µmol/L	NR	Y	(111, 116)
6	Creatine	FIA-ESI-MS/MS	0.25-3.57	µmol/L	NR	Y	(117)
7	Creatinine	LC-MS/MS	116	µmol/L	7d	Y	(118-120)
8	CYP450enzymes	LC-MS/MS	0.1	ng/ml	4w	Y	(121)
9	Cystathionine	LC-MS/MS	0.1-2.5	µmol/L	14d	Y	(122)
10	F2-isoprostanes	LC-MS/MS	6	pg/mL	NR	Y	(123)
11	Gamma-butyrobetaine	ESI-MS/MS	NR	µmol/L	NR	NR	(116)
12	Glucosylceramide	LC-MS/MS	NR	µg/mL	NR	Y	(124)
13	Guanidinoacetate	FIA-ESI-MS/MS	0.25-3.57	µmol/L	NR	Y	(117)
14	Haemoglobin peptides	LC-MS/MS	NR	Ratio	NR	NR	(125)
15	Haemoglobin variants	MS	NR	NR	NR	NR	(64)
16	Haemoglobins α- and β-chains	FT-ICR-MS	NR	%	NR	NR	(126)

17	Homocysteine	LC-MS/MS	1	µmol/L	3m	Y	(127)
		LC-MS/MS	0.1-2.5	µmol/L	14d	Y	(122)
18	Insulin-like growth factor-1	LC-MS/MS	50	ng/ml	8d	Y	(19)
19	Methotrexate polyglutamates	LC-MS/MS	5	nmol/L	NR	Y	(128)
20	Methylmalonic acid	LC-MS/MS	10	nmol/L	8w	Y	(129)
21	Methylmalonyl-carnitine	LC-MS/MS	0.025	µmol/L	NR	Y	(130)
22	Nucleoside profile	LC-MS/MS	NR	µmol/L	NR	Y	(131)
23	Orotic acid	HILIC-MS/MS	0.18	µmol/L	NR	Y	(132)
24	Protein profile	LC-MS/MS	NR	NR	NR	NR	(133)
25	Proteomics	LC-MS/MS	NR	µmol/L	NR	NR	(134)
26	Peptide profile	LC-MS/MS	NR	µg/ml	10d	NR	(135)
27	Stroles	APTDCI-MS	NR	Ratio	NR	NR	(110, 136)
28	Succinylacetone	LC-MS/MS	0.67	µmol/L	NR	Y	(137)
		LC-MS/MS	0.25	µmol/L	NR	Y	(113)
29	Succinyl-carnitine	LC-MS/MS	0.025	µmol/L	NR	Y	(130)
30	Sulfatides	LC-MS/MS	NR	µg/mL	NR	NR	(6)
31	Vitamin D	LC-MS/MS	4.8	nmol/L	>20y	Y	(38, 138-141)
Pharmaceutical drugs							
32	Acetaminophen (Paracetamol)	LC-MS/MS	50	ng/ml	NR	NR	(142)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
		TLC-MS	5.0-50	ng/ml	NA	NR	(143)
33	Amitriptyline	PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
		LC-MS/MS	20	µg/L	1m	Y	(144)

34	Amprenavir	LC–MS	11.7	ng/ml	3m	Y	(145)
35	Paroxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
		LC–MS	11.7	ng/ml	3m	Y	(145)
36	Atazanavir	LC-MS/MS	0.1	mg/L	7d	Y	(146)
37	Atenolol	LC-HRMS	25	ng/ml	2m	Y	(147)
38	Benzodiazepines	LC-MS/MS	NR	ng/mL	NR	NR	(148)
39	Benzethonium chloride	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
40	Bisoprolol	LC-HRMS	0.5-5.0	ng/ml	12w	Y	(149)
41	Bosentan	LC-MS/MS	2	ng/ml	5w	Y	(150)
42	Busulfan	LC-MS/MS	50	ng/ml	NR	Y	(151)
43	Citalopram	PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
44	Clarithromycin	LC-MS/MS	0.05-0.15	mg/L	2m	Y	(152)
45	Clomipramine	LC-MS/MS	20	µg/L	1m	Y	(144)
46	Cyclosporin A	LC-MS/MS	116	µmol/L	7d	Y	(118, 120, 153-155)
47	Darunavir	LC-MS/MS	0.1	mg/L	7d	Y	(156)
		LC–MS	11.7	ng/ml	3m	Y	(145)
48	Dasatinib	LC-MS/MS	2.5-50	µg/L	28d	Y	(157)
49	Dexamethasone	LC-MS	15	ng/ml	28d	Y	(158)
50	Efavirenz	LC-MS/MS	41-102	ng/ml	NR	N	(145)
		LC-MS/MS	0.1	mg/L	7d	Y	(156)
51	Emtricitabine	LC-MS/MS	2.5	ng/ml	6 d	Y	(159)
52	Endoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
53	Ertapenem	LC-MS/MS	0.2	mg/L	30d	Y	(54)

54	Etravirine	LC-MS/MS	50	ng/ml	7d	Y	(160)
		LC–MS	11.7	ng/ml	3m	Y	(145)
55	Everolimus	LC-MS/MS	116	µmol/L	7d	Y	(118, 120, 153, 155, 161)
56	Fluoxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
57	Flurbiprofen	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
58	Gamma-hydroxybutyric acid	GC-MS	1	µg/ml	15d	Y	(67, 163)
59	HIV protease inhibitors	LC-MS/MS	0.025-10	µg/ml	7d	Y	(164)
60	Ibuprofen	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
61	Imatinib	LC-MS/MS	20.5-50	µg/L	28d	Y	(157)
62	Imipramine	LC-MS/MS	20	µg/L	1m	Y	(144)
63	Linezolid	LC-MS/MS	0.4	mg/L	1m	Y	(165)
64	Loratadine	LC-MS/MS	0.2	ng/ml	271d	Y	(166)
65	lopinavir	LC–MS/MS	0.1	mg/L	7d	Y	(156)
		LC-MS	11.7	ng/ml	3m	Y	(145)
66	Losartan	LC-MS/MS	1	ng/ml	30d	Y	(167)
67	Mefloquine	LC-MS/MS	2.5	nmol/L	3w	Y	(168)
68	Midazolam	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
69	MK-1775 (Wee1 inhibitor)	HILIC-MS/MS	2	ng/ml	14m	Y	(169)
70	N-desmethyltamoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
71	Nevirapine	LC-MS/MS	41.102	ng/ml	NR	N	(156)
		LC-MS/MS	0.1	mg/L	7d	Y	(53)
72	Nilotinib	LC-MS/MS	2.5-50	µg/L	28d	Y	(157)
73	NIM811 (cyclophilin inhibitor)	LC-MS/MS	10	ng/ml	24h	Y	(170)

74	Nitisinone	LC-MS/MS	0.1	µmol/L	NR	Y	(171)
		LC-MS/MS	0.25	µmol/L	NR	Y	(113)
75	Norfluoxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
76	Nortriptyline	LC-MS/MS	20	µg/L	1m	Y	(144)
77	O-desmethylvenlafaxine	LC-MS/MS	20	µg/L	NR	Y	(172)
78	Omeprazole	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
79	Oseltamivir	LC-MS/MS	5	ng/mL	7d	Y	(173)
80	Paclitaxel	LC-MS/MS	0.2	ng/ml	45d	Y	(174)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
81	Paroxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
		LC–MS	11.7	ng/ml	3m	Y	(145)
82	Phenobarbital	LC-MS/MS	1	mg/L	10d	Y	(175)
83	Phenytoin	LC-MS/MS	0.3	mg/L	30d	Y	(176)
84	Posaconazole	LC-MS/MS	5	ng/ml	13d	Y	(177)
85	Proguanil	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
86	Propranolol	LC-MS/MS	2.5	µg/L	30d	Y	(178)
87	Raltegravir	LC-MS/MS	0.125	µg/L	7d	Y	(179)
88	Ramipril	LC-HRMS	0.5-5.0	ng/ml	12w	Y	(149)
89	Reboxetine	GC-MS/MS	1.0-20	ng/ml	30d	Y	(70)
90	Ribavirin	LC-MS/MS	0.05	ng/ml	140d	Y	(180)
91	Rifampicin	LC-MS/MS	0.05-0.15	mg/L	2m	Y	(152)
92	Rifapentine	LC-MS/MS	51	ng/ml	11w	Y	(181)
93	Rifaximin	LC-MS	0.1	ng/ml	30d	Y	(182)
94	Ritonavir	LC-MS	11.7	ng/ml	3m	Y	(145)

95	Rosiglitazone	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
96	Saquinavir	LC–MS	11.7	ng/ml	3m	Y	(145)
97	Simvastatin	LC-HRMS	0.5-5.0	ng/ml	12w	Y	(149)
		TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
98	Sirolimus	LC-MS/MS	116	µmol/L	7d	Y	(118, 120, 153, 155)
99	Sitamaquine	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
		PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
100	Sunitinib	PS-MS	0.25-0.75	ng/ml	NR	NR	(7)
101	Tacrolimus	LC-MS/MS	1	µg/L	20d	Y	(8, 60, 118-120, 153, 155, 183)
102	Tamoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
103	Tenofovir	LC-MS/MS	2.5	ng/ml	6d	Y	(159)
104	Topiramate	LC-MS/MS	10	µg/ml	194d	Y	(184)
105	Valproic acid	LC-MS	10	µmol/L	42d	Y	(185)
		GC-MS	5	µmol/L	21d	Y	(69)
106	Vemurafenib	LC-MS/MS	1	µg/ml	NR	Y	(186)
107	Venlafaxine	LC-MS/MS	20	µg/L	NR	Y	(172)
108	4-nitrophthalic acid	TLC-MS	5.0-50	ng/ml	NR	NR	(143)
109	4-hydroxytamoxifen	LC-MS/MS	0.5	ng/ml	20d	Y	(9)
Illicit drugs							
110	Amphetamines	ESI-MS/MS & GC-MS	2.3-11	ng/ml	NR	Y	(187)
		2D-LC-MS/MS	5	ng/ml	6 m	Y	(188)

111	Caffeine	LC-MS/MS	0.35-250	ng/ml	5d	Y	(162)
112	Cocainics	ESI-MS/MS & GC-MS	2.3-11	ng/ml	NR	Y	(187)
		2D-LC-MS/MS	5	ng/ml	6 m	Y	(188)
113	Novel psychoactive substances	LC-ESI-MS/MS	1.0-10	ng/ml	1w	Y	(189)
114	Opiates	2D-LC-MS/MS	5	ng/ml	6m	Y	(188)
		ESI-MS/MS & GC-MS	2.3-11	ng/ml	NR	Y	(187)
115	Δ9-tetrahydrocannabinol	LC-MS/MS	100	ng/ml	3m	Y	(190)
Drug abuse athletics							
116	Anabolic steroid esters	LC-MS/MS	0.1	ng/ml	28d	Y	(18)
117	Centchroman metabolites	LC-MS/MS	1.5-4.5	ng/ml	3m	Y	(191)
118	Testosterone glucuronides	GC-MS	NR	pg/mL	NR	NR	(17)
119	Therapeutic proteins	LC-MS	NR	ng/mL	2w	NR	(192)
Chemical exposure							
120	Benzene oxide-Hb	GC-MS	NR	pmol/g	NR	NR	(193)
121	Polybrominated diphenylethers	GC-HRMS	0.05	ng/ml	30d	Y	(194)

To support the quality management of the pre-analytical phase, there are defined recommendations for positive patient identification (20), sample collection of capillary blood (21), choice of filter paper (22), application of the sample onto the filter paper (23) and shipment of the DBS sample (24). An example of a standardised protocol for the DBS pre-analytical process is provided in Table 2.

Sample collection

The sample collection technique is important for accurate analysis of the DBS. Capillary blood collection is a common approach, as it usually requires less sample volume and is more patient friendly, compared to venepuncture. Both the World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) certify the quality of DBS samples and maintain healthcare workers safety through the provision of guidance manuals and standard operating procedures for DBS sample collection (25, 26). Due to the likelihood of significant sources of artefact formation, specific DBS collection training points are highlighted, including the choice of prick point, lancet type/size, prick depth, pressure rate during blood drop collection, dropping size/speed

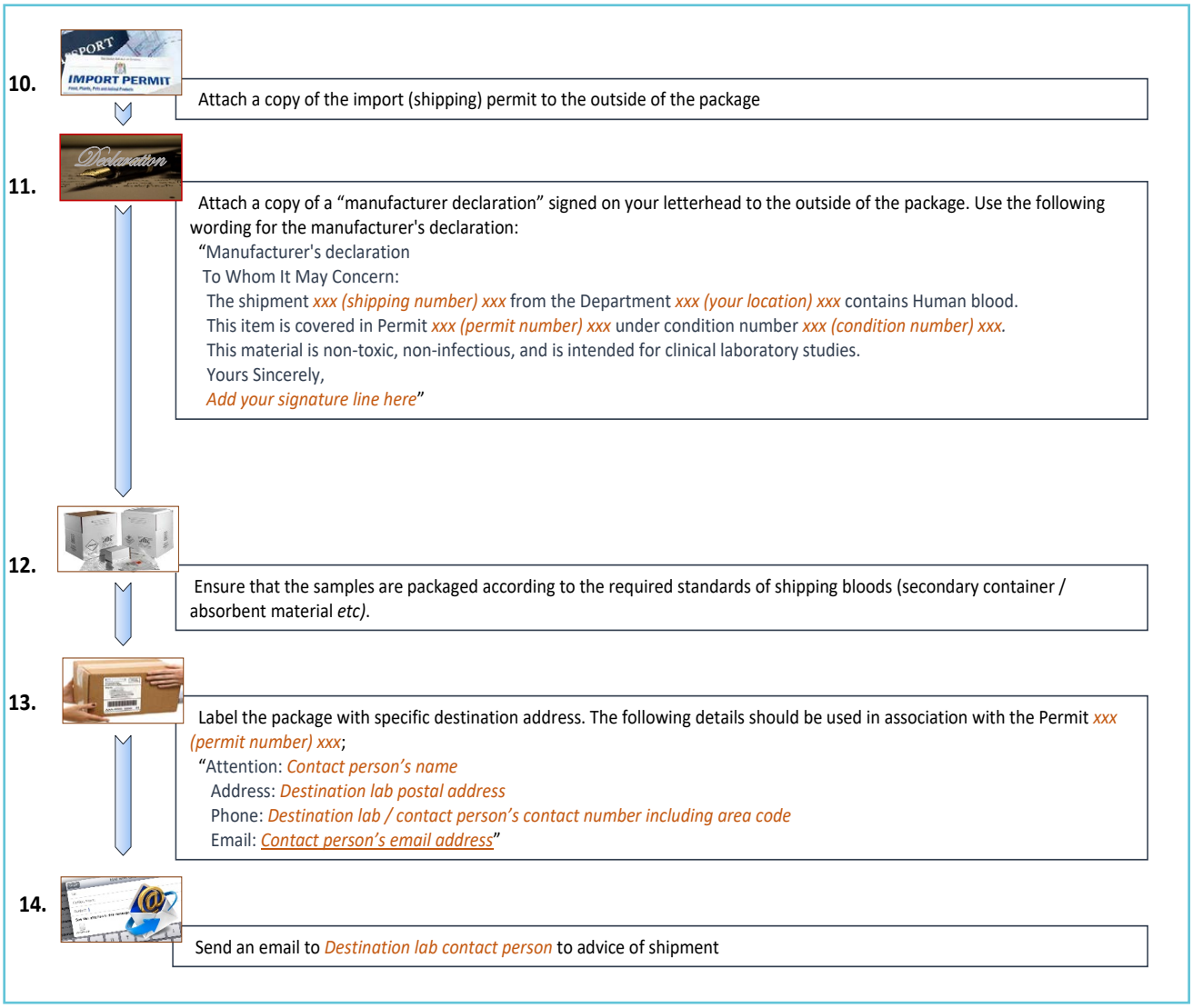
consistency, uniformity of sample diffusion onto the collection card and DBS contamination due to extended air or light exposure during the drying process. Importantly, WHO highlight that "working with DBS, whole blood or plasma requires the same biohazard safety precautions as whole blood" (26).

Selection of filter paper

Collection of DBS samples is onto one of two types of untreated solid support: pure cotton filter paper and glass microfiber paper. The choice of paper, including its thickness and density, influences the rate of adsorption and dispersion. As an example, the rate of membrane non-specific analyte adsorption is reduced in glass microfibre paper (27). Accordingly, dissimilarities in these solid supports may induce variations in the DBS sample attributes leading to potential differences in analyte stability, commutability, volume per area, and analytical effects (covered later in this review) (28). These DBS specific pre-analytical variables require standardisation and the Clinical and Laboratory Standards Institute (CLSI) offer a guideline, NBS01-6, to support DBS collection (22).

Table 2 Example dried blood spot sample collection and transport protocol

1.		Positive patient identification
2.		From a capillary collection, allow one drop of blood to penetrate each position of the filter paper. This should be one large drop of blood approx. 20µl
3.		Repeat for all positions
4.		Record at least two (prefer 3) identifier on the card (*eg.* Name, date of birth, study number, Health record number)
5.		Record sample collection date and time
6.		Record the following information in the logbook; a) Participants first name and surname, b) Participants date of birth, c) Study number, d) Card bar code number, e) Date of collection, f) Any other relevant information
7.		Allow samples to air-dry for a minimum of 2 hours (longer may needed in humid or cold environment)
8.		Store in Biohazard / Specimen Transport Bag in dry place at room temperature until shipment to the destination lab
9.		Send dried blood spot sample cards following the protocol to the destination lab with a copy of the logbook entries



The CLSI guideline recommends the use of two specific collection cards: the Whatman 903 and Ahlstrom 226. Both filter papers are approved by the Food and Drug Administration (FDA), Newborn Screening Quality Assurance Program (NSQAP) and also the Centers for Disease Control and Prevention (CDC) (23).

Sample application to filter paper

Both the Whatman 903 and Ahlstrom 226 cards have a target collection area printed on the paper to indicate and ensure the 50-75 µl sample volume limits (23). The whole blood drop should be spotted onto the mid-point of the collection area to allow for radial dispersion to the designated edge of the spot. This lateral distribution is uneven; as clearly evaluated by autoradiography (29). Usually, the concentration of biomarkers is decreased along the edge of the DBS, while the middle is affected by the volcano effect (paper chromatography effect) and occasionally has a speckled pattern (30). The physical characteristics of the DBS sample are also potentially affected by the patient's haemoglobin and haematocrit level; which also influences the spreading area of the blotted blood.

Variations in haematocrit will affect the relative plasma percentage of the spot. This is important

for analytes predominately found in serum/plasma, as the relative amount of plasma in a disk punched from different spots can vary; and particularly exaggerated when the haematocrit is extremely high or low (31). Whole blood samples with a higher haematocrit tend to distribute to a lesser extent across the filter paper (*i.e.* smaller blood spot diameter), and consequently the target analyte diffusion distance is shorter. The inverse applies for lower haematocrits. As a result, the determined concentration compared with the "normal" haematocrit sample would be over or under estimated due to the change in the analyte's distribution and infusion pattern across the blood spot (32, 33). Hence, for accurate quantitation, ideally the patient's haematocrit needs to be determined, by either a separate capillary drop/sample collected at the time of the DBS collection or directly from the DBS card. Figure 1 provides a visual demonstration of the effect of haematocrit on diffusion.

Analytes stability (storage and transport condition)

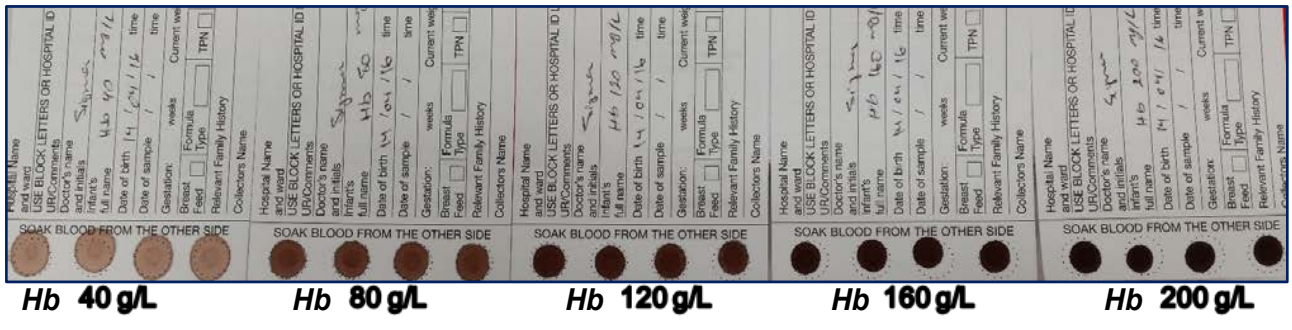
DBS samples should be allowed to completely dry before transport and/or storage. It has been demonstrated that rapid drying and storage in low humidity conditions improves the stability of DBS samples (16). The length of time required for air-drying will depend on the local environmental conditions such as air conditioning, room temperature and humidity. DBS drying usually takes from 90 minutes to approximately 4 hours and ideally, the DBS sample should not be left exposed to direct strong sunlight during this period.

The appropriately dried sample can then be placed in an envelope or similar container for the logistically simple and cost effective transport process (16). As the low volume of the dried specimen significantly reduces the risk of infection transmission compared to the other biological samples (34, 35), it can be transported in small lightweight packages that do not require temperature regulation. This negates the often cumbersome and expensive processes associated with transport of liquid biological samples (16).

Once the DBS samples are received at the destination, the size and properties of the DBS samples make storage relatively easy as minimal space is required; and they can often be stored at room temperature.

The stability of the DBS sample does require consideration, as the relevant stability for different analytes on DBS is quite variable. The stability of numerous blood biomarkers on blotting paper at

Figure 1 **Whole blood samples with different levels of haemoglobin/haematocrit (Hb/Hct) do not diffuse similarly on the collection card**



For a fixed punch size, a high level of Hb/Hct results a higher amount of blood on the punch thus the measured target analyte is falsely elevated. This figure is a visual demonstration of the effect of haematocrit on diffusion.

room temperature has been confirmed for a minimum of 7 days (27), several months (pharmaceutical drugs, opiates and nucleic acids) (36, 37), and even more than 20 years (vitamin D) (38). Freezing at -20°C or -80°C has been shown to further increase the stability of DBS samples; often extending the stability from days/months to years (39-41). Further, some reports show that some metabolites have better stability in the form of card-blotted whole blood compared to plasma (42).

Despite these exciting reports of long-term stability, there are many recognized potential influences, in addition to storage time, which could affect stability; including the type of filter paper, light exposure, temperature, humidity as well as the nature of the target analyte. Examples of changes to the collection process to improve stability include: 1) rapidly lowering the pH of the spotted blood sample to extend the stability time of some structurally unstable drugs which degrade during the drying process; and 2) use of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant to preserve and stabilise DBS samples for enzyme activity determination (43, 44). As a result of variations in stability, analyte specific protocols need to be administered for the collection and storage of DBS samples. Table 1 includes a guide to the stability of DBS samples for different analytes found in the literature.

ANALYTICAL CONSIDERATIONS

The solid form of the DBS sample is not compatible with most analytical techniques and requires elution of the sample from the filter paper. Accordingly, the testing process commonly includes three main stages: primary sample preparation, sample pre-treatment and sample analysis. Analysis can be further divided into chromatographic separation and mass spectrometric filtration and detection. The overall quality of this analytical process needs consideration. In this section of the review, we will discuss each of these aspects in turn. Figure 2 provides a general summary of the DBS-MS process.

Primary sample preparation

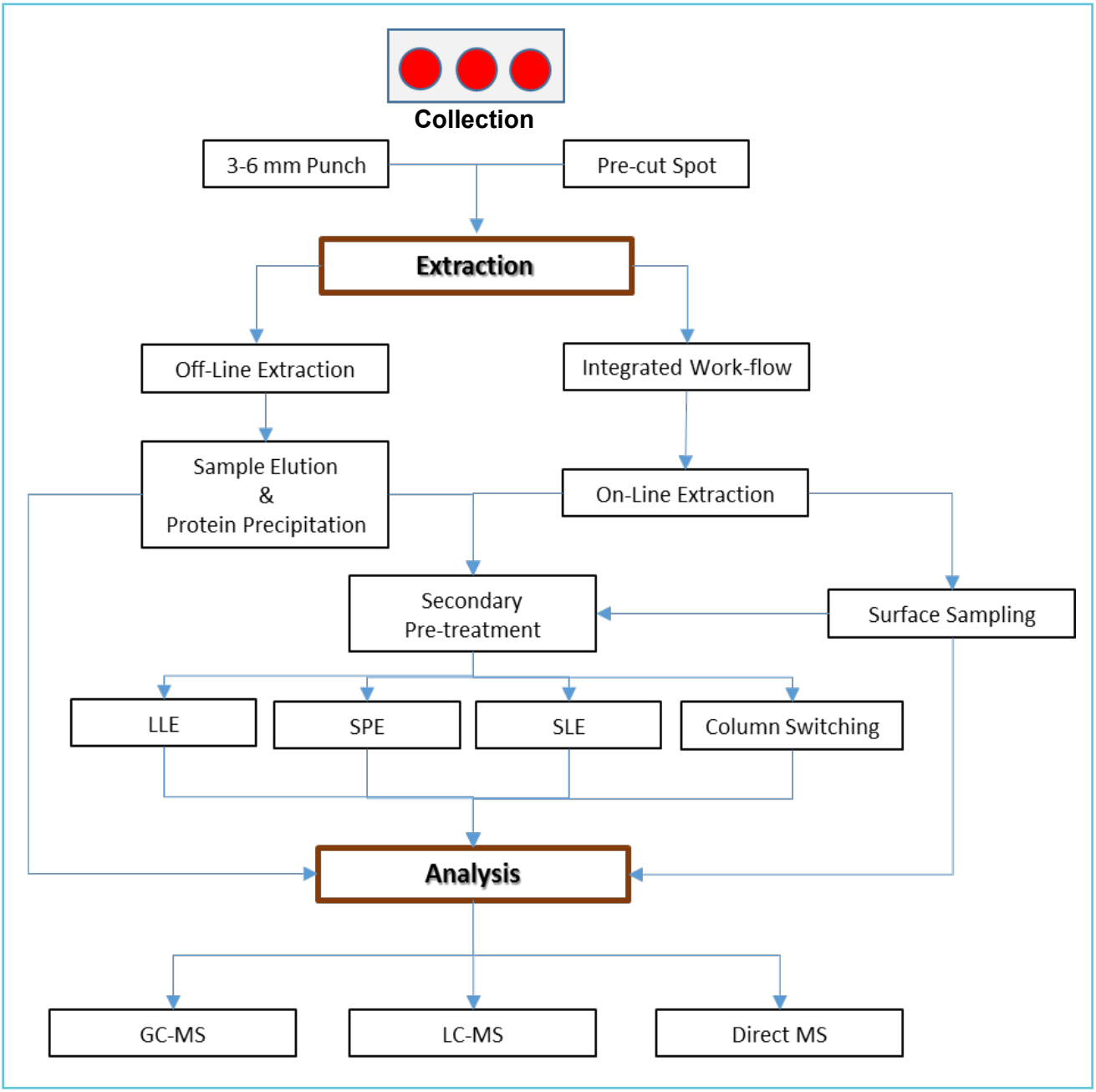
The punch

Sample preparation usually starts with deportation of a segment of the DBS from the blotter using a manual or automated puncher. Commonly, to minimise the assay bias due to punch location, it is recommended to consistently take the DBS punch either from the centre or close to the outer edge (45). The punch size may vary from 3 – 6 mm to the whole spot, depending on the method.

Techniques have been developed to overcome the variations in haematocrit and also minimize the labour associated with the sample preparation process. Strategies to overcome the haematocrit effect include:

1. Pre-cutting or perforating the filter paper as part of the DBS handling procedure to recover the haematocrit effect and eliminate the chance of carry-over between the punches;
2. Blotting of less whole blood volume (*e.g.* 10 µl) on the smaller pre-cut disk (3 or 6 mm) and analysis of the whole disk to disregard the haematocrit effect and improve the assay bias, (46-49);
3. A two-layered polymeric membrane to form a separated secondary dried plasma spot from the whole blood sample to be analysed following solid phase extraction (50);
4. Development of a novel collection card for DBS sampling, which generates a volumetric plasma sample (2.5 or 5.0 µL) from a non-volumetric application of whole blood sample. The purported advantages of this collection matrix includes enhanced assay reproducibility and selectivity, with a simplified sample extraction procedure and elimination of the haematocrit effect (51).

Figure 2 Dried blood spot sample analysis process flowchart for mass spectrometric analysis



Elution

For analysis, the analyte of interest firstly requires elution out of the filter paper along with the whole blood matrix by using appropriate extractor buffers. The efficient elution of analytes from the DBS is challenging and there is always a chance of analyte loss due to ineffective extraction; poor sample elution is due to either incomplete extraction or analyte degradation. Hence, the choice of optimal extractor materials may vary from one compound to the other. As an example, pure methanol is considered a

generic solvent for drugs of abuse extraction of the blood spot sample (52). Water on the other hand impairs the interaction between cellulose and the target analyte's hydroxyl groups and the partial addition of water prior to the organic extraction advances the efficiency in certain cases (*eg.* antivirals) (53). To achieve effective analyte recovery with maximum extraction efficiency, the extraction parameters, including extractor solution mix, duration, temperature and application of additional solvation energy (sonication), need to be optimised for each individual target metabolite (31, 54).

Sample pre-treatment

A variety of sample preparation approaches have been suggested, with selection depending on the molecular characterisation of the target compound. Incorporation of sample pre-treatment methods, either in combination with each other or in isolation, include the classic sample preparation process of: 1) protein precipitation (PPT); 2) liquid-liquid extraction (LLE); 3) solid phase extraction (SPE); 4) supported liquid extraction (SLE); and/or 5) derivatization.

Extraction and derivatization procedures applied manually (or offline) are considerably time consuming and laborious. Whilst derivatization is not required for many plasma based analytes using LC-MS/MS, it is required for many DBS analysis to improve the sensitivity; offsetting the small sample volume. However, as the derivatization process prolongs the overall analysis time it is considered to be a limiting factor and has been a driver for the development of on-line extraction techniques to facilitate the DBS sample pre-analytical treatment.

Automation of sample preparation directly coupled with the LC-MS/MS system has been introduced to improve turn-around time and run cost. PPT is a simple and popular method for automation that has been utilised for TDM (55). However, following a single PPT procedure, salts and other endogenous analytes are still present which may cause ion suppression in the MS process. SPE-LC-MS/MS set-up is designed to facilitate online sample desorption and is a time and cost effective method for DBS analysis (37, 56-59). Compared to PPT, SPE presents an improved sample clean-up (60). There are specific challenges with on-line extraction approaches, in comparison with the off-line extraction methods which may be a significant source of assay bias, including; non-homogenous mixture of internal standard (ISTD) with the analyte in the extract; sample dilution then band broadening in chromatography separation; and/or inadequate focusing of the extract onto the analytical column (10). Accordingly, as part of the method development process, certain strategies are required to eliminate these issues.

Technology has been developed that allows for the direct sampling of the DBS, without the need for a change to liquid or elution. As it is described by the manufacturer, "Liquid Microjunction Surface Sampling Probes (LMJ-SSP) are self-aspirating devices where liquid is pumped to and aspirated away from a surface of interest to a mass spectrometer for integrated extraction and ionization" (61). By utilising the LMJ-SSP technology, the analyte of interest could be directly extracted from the different surfaces and detected by a mass spectrometer in a short time frame with minimum sample handling (62). The LMJ-SSP device coupled with the MS has been utilised for the determination of proteins in the DBS sample (63), direct tandem mass spectrometer for detection of haemoglobin (64), as well as therapeutic drugs (65, 66). Likewise, novel "on spot" direct derivatization approaches provide a time and cost effective alternative sample preparation procedure; a technique introduced to determine thiorphan drug (67, 68).

Sample analysis

Gas chromatography is known as a cheaper and faster separation technique compare to the LC, utilising long and compact capillary columns that enhance resolution. GC-MS analytical techniques often offer a higher separating power and efficient reproducibility compared with LC-MS. Accordingly, GC-MS is still utilised for the determination of volatile biomarkers with low molecular weight and heat resistance fragments. Applications include, steroids, metabolomics and therapeutic drug monitoring studies using DBS samples (17, 67, 69-71) (Table 1). However, the application of GC is limited to gas soluble, volatile and heat resistance small molecules (often derivatization is required to turn non-volatile molecules to volatile), which are gas soluble (72, 73).

LC is preferred for analysis of heat sensitive analytes, with no molecular size restriction. Additionally unlike GC, target compounds co-interact with both mobile phase and solid phase which results in better selectivity (73). The choice of GC or LC instruments depends on the required sensitivity and target analyte characteristics. Despite the fact that the GC-MS provides selectivity, sensitivity and robustness for many DBS analytes, the literature demonstrates that, it is not as popular as LC-MS/MS (Table 1). This is likely to be due to the improved specificity and sensitivity afforded by LC-MS/MS for blood spot analysis; associated with a significantly faster and usually more cost effective process compared to GC-MS (74, 75).Furthermore, advanced UHPLC technology has boosted the resolution of peak separation (even more effective than GC) (72, 73).

By introduction of the two dimensional chromatography (2D-C) tools (applicable on both GC and LC), the separation efficiency, analytical sensitivity, quantitation accuracy and precision have been improved. This 2D-C process has reduced the DBS matrix and carry-over effects, with reports suggesting improved imprecision and bias (76, 77). With the further addition of on-line extraction joined to the 2D-C system, sensitivity and specificity is maximised when coupled with either a triple-quadrupole tandem mass spectrometer or a high-resolution quadrupole time of flight mass spectrometer (QTOF-MS) (60, 78).

The advances in ion source technology have enhanced sensitivity for both polar and non-polar analytes from DBS samples (79-81). Selective/ multiple reaction monitoring (SRM/MRM) modes in MS/MS detection, focusing on specific transitions, have advanced the assay specificity remarkably along with improving linearity and limits of detection (10, 82).

Negating the pre-analytical clean-up and chromatographic front end separation, direct MS methods and surface sampling techniques coupled with MS have been used for DBS samples (83). Desorption electrospray ionisation (DESI), direct analysis in real time (DART) and direct electro spray ionisation mass spectrometry (ESI-MS) methods have been utilised in order to generate ions from the surface, thus avoiding purification or derivatization processes (83-90). However, elimination of this primary sample purification and separation may result in loss of sensitivity and precision due to the disintegrated metabolite interferences (89).

Thus, the application of non-paper blotting matrices and online SPE in conjunction with the direct MS methods is recommended to enhance the sensitivity and measurement precision (75, 91). Ultimately, the gains of time efficiency and throughput need to be balanced with achieving the desired method performance.

Quality considerations

There are some important considerations in relation to the method validation and acceptance criteria for DBS analysis. Accordingly, the European bio-analysis forum (EBF) has described

the details of the DBS sample analysis methodology to provide specific recommendations for validation of DBS methods (92). The EBF recommendations document includes specific concepts of; collection card variability, sample-to-sample variability, DBS homogeneity, punch point effect, sample stability, blood physical parameters effect, matrix effect, extraction recovery, IS application and internal quality control (IQC) and calibrator preparation. In addition to the detail provided in this document, traceability to the liquid matrix sample, fitness for clinical purpose and reference intervals/decision limits for interpretation require consideration.

Collection cards

To avoid issues of inter-card variability, calibrator and control material should be prepared using identical collection card type/manufacture as the patient samples. If multiple type/manufacturer of cards are used, then a method comparison is required to determine the comparative card stability, extraction recovery and matrix effect (93).

Haematocrit effect

As mentioned earlier, the physical behaviour of blotted whole blood is influenced by different parameters such as; haematocrit level, degree of haemolysis and anticoagulant type (if it is applied). Currently the haematocrit is recognised as the most significant parameter affecting blood spot characteristics (drying time, diffusion and homogeneity) and assay reproducibility. The Haematocrit effect is more substantial when a sub-sample disk punch is analysed, rather than the whole DBS sample. Hence, method validation studies for DBS sample applications also need to include investigations of the impact of haematocrit variation on measurement and assay performance (94).

Application of internal standard

The incorporation of the ISTD to the DBS sample processing is an important step and ideally should occur early in the process. 1) Collection cards pre-treated with the ISTD can be prepared prior to the spotting of the blood. This ensures both the ISTD and nominated compounds have undergone the same matrix and extraction effect. However, this approach logistically might not be practicable when dealing with multiple studies. 2) Commonly manual extraction methods utilise the approach of integrating the ISTD into the DBS elution reagent/extraction solvent. In this method, the ISTD is co-extracted along with the target analyte. 3) Addition of the ISTD into the sample along with extraction/preparation process is another simple alternative. However, as the ISTD is not fully incorporated with the paper matrix, variations in elution recovery are not accounted for. 4) Using on-line DBS sample preparation technology, the ISTD is sprayed on the blood spot before the extraction using the Touch-spray technique (95-97).

Carry-over

Carry-over is a significant issue for DBS-MS analysis. Carry-over may have different sources including: physical card to card contact during storage; spot to spot originated from the puncher head and post-preparation initiated from the instrument (*e.g.* auto-sampler and analytical column) (92). As the puncher head is re-used, contamination and sample carry-over are notable concerns. To overcome this issue, either a clean-up step or a blank-card punch in between the samples is recommended (98). To investigate the instrumentation carry-over, two injections of sequential blank DBS extracts should be performed after an injection of a sample with the upper limit of quantitation concentration. The response for the first and second blank matrix should not exceed 20% and 5% respectively of the mean response of the lower limit of detection of the analyte of interest (99).

Internal quality control

IQC spiked samples preparation for DBS analysis requires special considerations compared to liquid phase biologic samples. The main challenge of internal QC is keeping the matrix consistent with that of patient's blood spot sample. Both sample dilution and saturation may occur in spiked sample preparations (93). Ideally fresh non-haemolysed whole blood samples, with a closely matched haematocrit level to the study group, should be chosen for spiking (99). These IQCs should be spotted onto the filter paper and eluted along with the patient samples.

External quality assurance

External quality assurance (EQA) programs are considered essential tools in evaluating the reliability and traceability of the analytical assay as well as monitoring the quality of the laboratory performance. The United Kingdom National External Quality Assessment Service (UK-NEQAS), the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) and the CDC (NSQAP) provide a variety of schemes for DBS-NBS testing. However, there is no further EQA program available to assure the accuracy of DBS analysis outside of NBS. Hence, for most DBS analytes discussed in this review we do not have a peer review process to fully gauge laboratory performance. This represents a gap in harmonisation of analytes measured in this matrix.

Calibration

The preparation of standards for DBS quantitative analysis includes whole blood fortification (replacement of certain amounts of plasma with the artificial plasma containing a known concentration of target analyte) with a set of commercial or in-house calibrator materials before spotting. The percentage of non-aqueous components replaced with plasma needs to be minimised to prevent solvent effects creating inconsistency between spiked samples (calibrators) and patient samples in terms of spot formation (92). Most DBS analytes currently measured (Table 1) are small molecular weight well defined compounds and therefore, in principle, full standardisation with traceability should be achievable. However, in practice the DBS matrix adds an extra level of complexity to the traceability chain and commutability needs to be determined.

POST-ANALYTICAL

Post-analytical concerns of the testing process phase mainly include; result reporting and interpretation, assay total error management and turnaround time (100). The key for the introduction of DBS analysis as a diagnostic tool is the cross validation of the method to a reference plasma/serum based assay. However, it is essential to take into account the fact that the concentration of the biomarkers in whole blood may vary from serum/plasma.

To turn the numerical result generated from the DBS analysis into a clinical meaningful result, a reference interval (RI) or decision point needs to be established. As such DBS specific RI have been developed for many analytes (101-106). For analytes routinely measured in liquid whole blood comparative RI can often be transferred to DBS samples. However, it is often more challenging for DBS analytes that require a comparison to serum for their clinical interpretation.

Some DBS analytes, such as vitamin D, require a conversion to their serum equivalent concentration for interpretation. This requires the development of a robust relationship between the measured analyte in the blotted whole blood and the equivalent serum sample. For this, we need to be able to estimate the equivalent blood volume in the blood spot punch. Two main approaches have been described to

evaluate the corresponding serum volume; the application of chemical tracers and geometric calculation (10).

Chemical tracers, such as ^{125}I-albumin, ^{51}Cr-haemoglubin and ^{125}I-L-thyroxin, have been used to estimate the equivalent serum volume of the blotted whole blood punch. In this process, the serum volume is determined by comparing radiochemical counts of the blood spot punch with the known volume of whole blood samples in the liquid phase (29). A geometric calculation to evaluate the ratio of the punched disk to the known volume of the entire spotted whole blood with the pre-measured haematocrit has been applied (107, 108). However, application of this approach does not fully take into account the chromatographic effect of the blood distribution on the filter paper, and still raises issues of analytical bias for the final measurement (109); this is why it is recommended that the punch is taken from the centre of the spot. Considering the fact that these common methods are not applicable to archived DBS samples, direct estimation of haemoglobin concentration on the DBS punch is an alternative, but is currently not described in the literature.

CONCLUSIONS

The initial widespread application of dried blood spot was utilised for newborn screening. More recently, it has been applied more broadly and mass spectrometric based applications are the dominant techniques, with liquid chromatography separation being more popular than gas chromatography. Drug monitoring (therapeutic and toxicology) and pharma-toxico-kinetics studies are the major application groups outside of newborn screening. For many analytes, method validation and further bridge experiments are required to develop adjustment rules to convert the results obtained from the dried blood spot analysis to the equivalent serum/plasma values. Likewise, establishment of robust reference intervals or decision limits is essential for dried blood spot analytes. It is envisaged, with the inherent advantages of the alternative dried blood spot sampling technique compared to the classic plasma based strategies, in future micro-sampling based assays will certainly play a substantial role for analysis of biomarkers.

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In this issue: Recent Developments in the Clinical Application of Mass Spectrometry

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Serum insulin-like growth factor I quantitation by mass spectrometry: insights for protein quantitation with this technology

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ABSTRACT

Liquid chromatography mass spectrometry (LC-MS) is a widely used technique in the clinical laboratory, especially for small molecule quantitation in biological specimens, for example, steroid hormones and therapeutic drugs. Analysis of circulating macromolecules, including proteins and peptides, is largely dominated by traditional enzymatic, spectrophotometric, or immunological assays in clinical laboratories. However, these methodologies are known to be subjected to interfering substances, for example heterophilic antibodies, as well as subjected to non-specificity issues.

In recent years, there has been a growing interest in using LC-MS platforms for protein analysis in the clinical setting, due to the superior specificity compared to immunoassay, and the possibility of simultaneous quantitation of multiple proteins. Different analytical approaches are possible using LC-MS-based methodology, including accurate mass measurement of intact molecules, protein digestion followed by detection of proteolytic peptides, and in combination with immunoaffinity purification. Proteins with different complexity, isoforms, variants, or chemical alteration can be simultaneously analysed by LC-MS, either by targeted or non-targeted approaches. While the LC-MS platform offers a more specific determination of

proteins, there remain issues of LC-MS assay harmonization, correlation with current existing platforms, and the potential impact in making clinical decision.

In this review, the clinical utility, historical aspect, and challenges in using LC-MS for protein analysis in the clinical setting will be discussed, using insulin-like growth factor (IGF) as an example.

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Abbreviations (in alphabetical order):

***EQA**: External Quality Assurance*
***GH**: Growth hormone*
***IGF**: Insulin-like growth factor*
***LC-MS**: Liquid chromatography mass spectrometry*
***m/z:** Mass-to-charge ratio*
***MALDI:** Matrix-assisted Laser Desorption/Ionization*
***MRM**: Multiple Reaction Monitoring*
***MSIA**: Mass spectrometric immunoassay*
***RCPA**: The Royal College of Pathologists of Australasia*
***SPE**: Solid-phase extraction*
***SRM**: Single Reaction Monitoring*
***TOF**: Time-of-flight*
***US FDA**: United States Food and Drug Administration*

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INTRODUCTION

Liquid chromatography mass spectrometry (LC-MS) is an analytical technique in which analytes are separated by their physical properties on a chromatographic stationary phase, followed by detection based on their specific molecular mass to charge ratio (m/z). Separation of the analytes from sample matrix interferences greatly enhances the robustness and sensitivity of the LC-MS assay. Specificity is provided by the characteristic retention times on a chromatographic column, the exact mass to charge (m/z*)* values of the parent ion, and m/z values of the fragment ions.

The advantage of specific detection of analytes is valuable in laboratory medicine, especially in the determination of structurally similar compounds such as steroid hormones, for therapeutic drug monitoring, and for toxicology screening (1–3). Also, immunoassay may be subjected to the heterophilic antibody interference. Indeed, thyroglobulin quantitation by immunoassay was known to be subjected to the presence of anti-thyroglobulin autoantibodies which is commonly found in patients with thyroid cancer (4). LC-MS-based assays provide a specific and alternative platform for the detection of thyroglobulin. Chromatographic separation of analytes also allows simultaneous determination of multiple molecules in a single analytical run, thereby reduces both sample consumption and turnaround time.

Although powerful in small molecule analysis, applications of the LC-MS technology for protein quantitation in clinical laboratory was until recently relatively lacking. The limitations were due to a lack of LC-MS assay harmonization and standardization, expensive LC-MS platform instrumentation and method development. Due to the availability of automated instrumentation in the clinical laboratory, quantitation of proteins has been dominated by enzymatic, spectrophotometric, and immunological assays, which also defined the reference interval of circulating proteins for clinical management. Most of the LC-MS assays in clinical laboratories were developed in-house based on a “fit-for-purpose” approach, i.e. the developed assay was considered applicable for clinical purpose based on the assay linearity, imprecision, accuracy and robustness, among other validation parameters. While LC-MS methods are considered or proposed

as reference methods for several proteins including urine albumin and whole blood Hb_{A1c} (5,6), they are restricted to selected reference laboratories and not available in routine setting. LC-MS-based platform was considered more cost-effective compared to traditional analyzer due to the negligible reagent cost (7). However, the initial capital investment in LC-MS instrument, personnel, training, and the method development and validation process might be prohibitive. In spite of these challenges, the application of LC-MS-based protein assay in clinical laboratory, as well as in clinical trials, is becoming more popular (8).

In this review, the clinical utility and historical aspects of LC-MS-based Insulin-like Growth Factor I (IGF-I) assay in clinical laboratory are reviewed and discussed. The anticipated challenges of the platform in future application for other circulating proteins will be discussed.

BIOCHEMISTRY AND CLINICAL UTILITY OF INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factor (IGF) is a class of circulatory peptide hormones identified in 1957 and subsequently described as “non-suppressible insulin-like activity” in 1963 (9,10). Two members of IGF family, IGF-I and IGF-II, share a similar domain structure as well as sequence homology with insulin, and are able to bind with insulin receptor on cell surface (11–13).

Insulin-like growth factor 1 (IGF-I) is a 70-amino-acid single chain polypeptide which is synthesized and secreted by the liver in response to pituitary growth hormone (GH). While the precursor, pre-pro-IGF-I, is structurally variable and may be composed of two classes of C-terminal signal peptides and three classes of N-terminal E-domains, the mature IGF-I in circulation is a conserved polypeptide with defined A, B, C and D-domains (12). Because of these well-defined properties, it is possible to generate a pure preparation of mature IGF-I as a reference material. The National Institute of Biological Standards and Control (NIBSC) has thus established the first WHO International Standard for IGF-I for immunoassay in 2008 with defined measurement uncertainty (NIBSC code 02/254).

IGF-II, on the other hand, is a 67-amino-acid single chain polypeptide which share approximately 70% homology with IGF-I (14). The circulating IGF-II level is independent of gender- and age-specific variation, and is relatively stable compared to that of IGF-I (15). The determination of IGF-II is essential for calculating IGF-II/IGF-I ratio, which is a useful parameter for the investigation of non-islet cell tumor hypoglycemia (NICTH) (16).

Circulating levels of serum total IGF-I are widely used in the diagnosis of GH disorders, including acromegaly and GH deficiency. Pituitary GH is known to be secreted on a pulsatile manner, in diurnal rhythm, and is subjected to different physiological and environmental stimuli including fasting, exercise and feeding. The short half-life of GH (20 minutes) further increases the variability of the circulating level. On the other hand, IGF-I is synthesized in a more stable manner, does not exhibit diurnal rhythm, has a longer half-time, and therefore is a more reliable biomarker of GH disorders (17). To highlight the importance of IGF-I in the management of GH disorders, it was recommended by the Endocrine Society that serum level of IGF-I should be used as a first line screening test for acromegaly, followed by GH measurement with an oral glucose loading as a confirmation test. The management goal of acromegaly was also established biochemically by normalization of serum IGF-I level. Similarly, the Endocrine Society also recommended using IGF-I level to diagnose, to document, and for treatment monitoring of persistent GH deficiency (18)(19).

IGF-I QUANTITATION BY IMMUNOASSAY

Traditionally, IGF-I was measured by using commercial immunoassay. However, due to the reported lot-to-lot reagent variation in IGF-I assay and the global supply disruption of immunoassay kit, the reliability of immunoassay for GH disorders was questioned (20). Patients with GH disorders required regular measurement of serum IGF-I for management and treatment monitoring. It is important to have a consistent and reliable assay for long term patient management.

At least 2 types of IGF-I sequence variants were reported. One of these variants was confirmed to be pathogenic (21). Apart from the issue of assay stability, immunoassay is not able to differentiate between wild-type IGF-I and genetic variants of IGF-I. Failure to differentiate between sequence variants may cause a falsely high measured value of wild-type, bioactive IGF-I. Patient management may be affected if pathogenic IGF-I variants were not promptly identified. Therefore, an alternative non-immunological platform for serum IGF-I quantitation would be valuable.

LC-MS-BASED PLATFORM FOR IGF-I MEASUREMENT

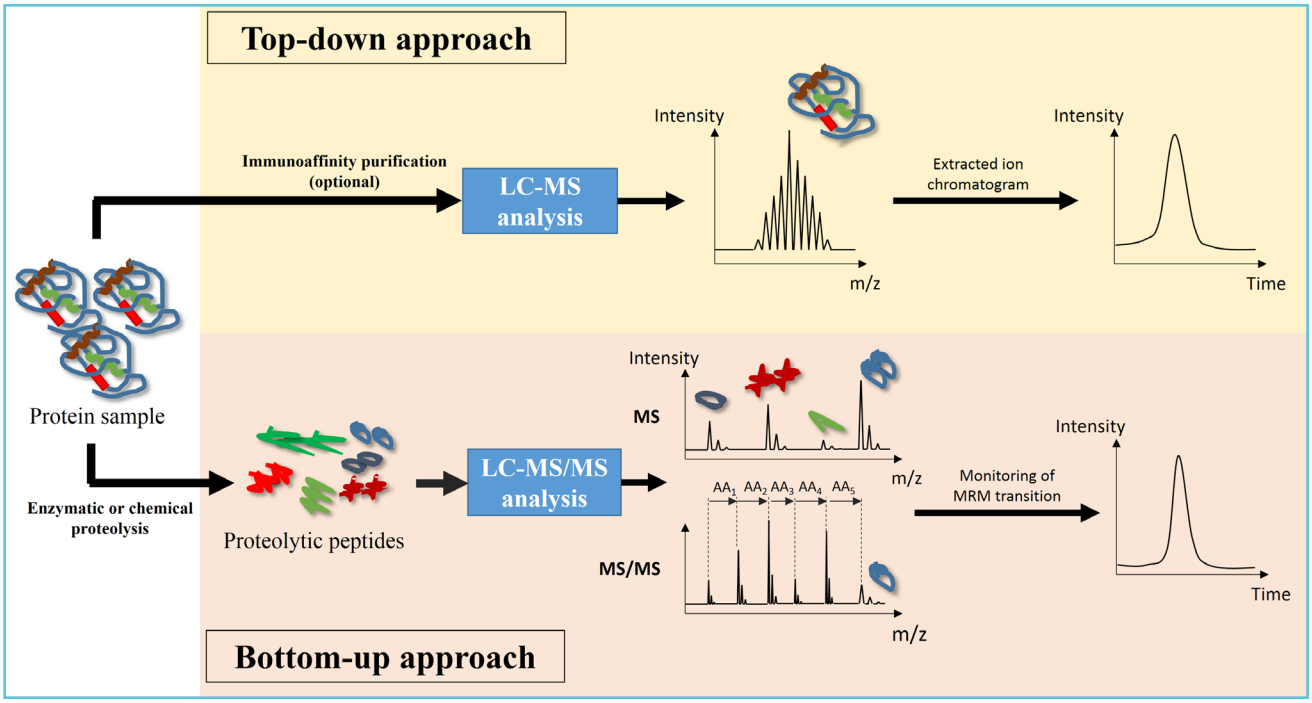
An alternative platform considered for serum IGF-I quantitation is LC-MS. While the use of LC-MS in IGF-I quantitation in the clinical setting is relatively new, the method was widely investigated in the past decade for sport science and doping control. The assays were first reported in 2001 by de Kock *et al.* and by Bobin *et al.* Two approaches exist for LC-MS analysis of proteins. In "top-down" approach, the ions entering the MS instrument carry the complete amino acid sequence information of the respective intact protein. In most cases the ions being analyzed are intact protein without proteolysis by enzymatic or chemical method. On the other hand, for "bottom-up" approach, the ions entering the MS strument only carry partial amino acid sequence of the intact protein. The ions are usually peptides generated by protease digestion, each representing a fragment of the intact proteins (Figure 1). Both determination of proteolytic peptides after enzymatic digestion ("bottom-up" approach) and analysis of intact protein ("top-down" approach) were described for the characterization and quantitation of IGF-I respectively (22,23).

BOTTOM-UP APPROACH

The bottom-up approach is based on the assumption that the generation of proteolytic peptides are stoichiometrically related to the parent proteins. By quantitating the proteolytic peptides, the concentration of parent proteins can be derived. de Kock *et al.* reported the use of endoproteinase Glu-C and Asp-N for the generation of peptide mass fingerprint and subsequent MS/MS analysis of peptide fragments for the characterization of IGF-I. The bottom-up approach was also reported by Kirsch *et al.* in 2007, and Kay *et al.* in 2009 (24,25). In the report by Kirsch *et al.,* tryptic peptides were generated from human plasma, with the addition of a stable isotope-labeled peptide as the internal standard, followed by analysis in a single reaction monitoring (SRM) experiment. Kay *et al.* adopted a similar approach, but introduced an acetonitrile precipitation procedure in order to enrich IGF-I prior to digestion. This approach was further elaborated by the same group in 2013 (24). Instead of acetonitrile precipitation, an offline SPE device was used for IGF-I enrichment, followed by trypsin digestion and SRM experiment.

The generation and measurement of proteolytic peptides offered a unique advantage over top-down method in terms of analytical simplicity. Proteins with diverse physical properties and

Figure 1 Schematic diagram demonstrating the workflow of top-down and bottom-up approach in LC-MS-based protein analysis



In top-down approach, intact protein with complete amino acid sequence information is analyzed in MS experiment; in bottom-up approach, proteolytic peptides, each carrying partial amino acid sequence of the intact protein, is analyzed by MS and MS/MS experiment.

molecular weights were converted into a pool of peptide mixture, which was relatively similar in terms of solubility, chromatographic behavior, molecular weight and ionization efficiency. A general analytical approach was capable of simultaneously determining multiple proteolytic peptides, and thus the parent proteins. This was reported by Such-Sanmartín *et al.* in 2015 in which five proteins (IGF-I, IGF-II, two IGF binding proteins and leucine-rich alpha-2-glycoprotein), with diverse molecular weights and glycosylation states, were simultaneously quantitated for anti-doping analysis and for a cancer study (26).

To reliably quantitate an analyte by LC-MS, several information was necessary including retention time on chromatographic column, accurate mass of parent ion, and accurate mass of fragment ions after fragmentation. The bottom-up approach utilized triple quadrupoles MS in MRM mode for the detection of proteolytic peptides. Similar to small molecule analysis, this approach has been well characterized and the instrument can accommodate the m/z range of peptides and their fragment ions. In terms of instrumentation, clinical laboratories equipped with triple quadrupoles MS can adopt the bottom-up approach readily.

However, while the bottom-up approach provides a relatively general analytical platform for diverse range of protein, it requires a more stringent quality control protocol to monitor analytical variation arising from enzymatic digestion to peptide purification. This was highlighted by the observation that all three groups generated the calibration curve from pooled plasma via standard addition approach in order to ensure the reproducibility of enzymatic digestion in

the endogenous matrix. Since the endogenous concentration of IGF-I cannot be certified externally, the spike-in method may also undermine the accuracy of the bottom-up assay.

TOP-DOWN APPROACH

Bobin *et al.* reported the use of ESI-ion-trap with deconvolution to measure the intact IGF-I neutral mass for quantitation, as well as the use of matrix assisted laser desportion ionization (MALDI) - time of flight (TOF) for the identification of an oxidized IGF internal standard by the "top-down" approach (14). In plasma sample analysis, the group utilized immunoaffinity column for sample purification, and successfully quantitated IGF-I level in equine plasma samples with +/- 10 ng/ml deviation from nominal value obtained from immunoassay. The work demonstrated a comparable quantitative performance between immunoassay and MS assays, and was also the first application of mass spectrometric immunoassay (MSIA) for IGF-I analysis in biological samples.

Other top-down quantitative approaches were subsequently reported by Nelson *et al.* in 2004 (27), and Bredehoft *et al.* and Popot *et al.* in 2008 (28,29). The work by Nelson *et al.* and Bredehoft *et al.* further expanded the workflow of MSIA by modifying it into a pipette tip format and antibody-coated magnetic beads, respectively. Popot *et al.* derived the deconvoluted peak area in the mass chromatogram using an in-house protein transposing model for the quantitation of IGF-I in horse plasma, with better precision and accuracy compared to the use of deconvoluted peak height alone. An overview of LC-MS-based IGF-I assays revealed that immunoaffinity extraction was necessary for the top-down approach to achieve sufficient sensitivity and precision for quantitation, potentially due to the limitation of instrumentation at that period. However, the use of antibodies may affect the recovery of the analyte for LC-MS detection, as well as introducing a variable to the analysis.

In 2011, a dilute-and-shoot top-down approach, without the use of immunoaffinity extraction, was reported by Bystrom *et al.,* using a simple acid-alcohol extraction process and online solid-phase-extraction (SPE) as the sample preparation method (30). By using a high resolution TOF-MS, a mono-isotopic peak of IGF-I within the $[M+7H]^{7+}$ cluster was monitored by narrow mass extraction, which generated a specific mass chromatogram for quantitation. A limit of quantitation of 15.6 ng/ml was achieved without using immunoaffinity purification, and interference was detected by using the isotope ratio of IGF-I. Comparatively, the limit of quantitation was reported as 50 ng/ml by Bredehoft *et al.* in 2008. These were the first few reports on the use of IGF-I LC-MS assay for clinical purpose. LC-MS-based age- and gender-specific reference interval, as well as the assay comparison with immunoassay, was subsequently published by the same group in 2012, thereby providing the fundamentals of LC-MS-based IGF-I assay in clinical laboratory (15). This reported method was also adopted by Hines *et al.* in 2015 using Orbitrap, a high resolution mass analyzer, for clinical application (31).

The use of top-down approach in routine clinical laboratory setting is preferred since it utilizes a simpler sample preparation procedure and therefore introduces less variation, compatible with liquid handling robot for automation, and ultimately can accommodate a higher sample volume. However, the accurate mass information on fragment ions is usually absent. The instrument's relatively slower MS/MS duty cycle may not be able to provide sufficient data points for quantitation, and the m/z range of intact protein and their fragment ions may exceed that of the instrument. Multiplex analysis was also limited to proteins with similar physical properties,

for example IGF-I and IGF-II. Therefore, the top-down approach requires a high-resolution mass analyzer to provide high mass accuracy of parent ions, as well as to resolve single isotopic peaks of multiple charged proteins to provide specificity of quantitation. With the advance of high resolution MS in recent years including TOF and Orbitrap instrument, it is expected that the top-down approach will be more commonly used and affordable for the clinical laboratory.

An overview on the imprecision and linear range of IGF-I assays reported in the literature is provided in Table 1.

DETECTION OF SEQUENCE VARIANTS AND OXIDIZED PROTEINS

While mature IGF-I is a non-glycosylated protein and is relatively conserved in its amino acid sequence, two types of variants have been reported independently using the top-down approach. Hines *et al.* in 2015 reported an A70T IGF-I variant in a cohort of 1720 samples, with an estimated prevalence of 0.6% in the studied population (31). The variant was identified from the outliers in a correlation study with immunoassay platform, in which subjects with either a heterozygous or homozygous variant showed 50% and 100% reduction in wild-type IGF-I compared to immunoassay respectively. The other variant was suggested by Oran *et al.* in 2014 to be the A67T IGF-I variant, which was found to be present in 9 out of 1054 (0.85%) samples (32). Using MSIA in pipette tip format in combination with an automatic liquid handler, the group used MALDI-TOF MS for the top-down quantitation of IGF-I, and the A67T variant was identified as a twin peak with a shift of 30 m/z unit; however, this A67T variant was not confirmed by genetic sequencing. It should be noted that the antibodies reported was unable to differentiate between wild-type IGF-I and these two variants, as the immunoassay utilized in Hines *et al.* study quantified wild-type and A70T variants as a single entity, while the antibody used in MSIA in Oran *et al.* study captured A67T for MS analysis as well. Interestingly, by using the same principle, Oran *et al.* also speculated a glycosylated IGF-I in the circulation in the same study with a mass shift of approximately 700 Da, although it was not confirmed by tandem MS/MS fragmentation or other structural analysis.

Other pathogenic changes in the IGF-I sequence have been identified previously. A partial deletion of IGF-I in exons 4 and 5 was described in 1996, causing growth failure and mental retardation (33). The deletion rendered the mutated IGF-I undetectable by radioimmunoassay. IGF-I with a single nucleotide polymorphism of T-to-A transversion in the untranslated region of exon 6 was described in 2003, which causes dysregulation of IGF-I mRNA processing and IGF-I deficiency (34). A V44M variant was identified in 2005 which reduced the affinity of IGF-I to IGFBP-3 by 90 folds, leading to severe growth and mental retardation (21). Immunoassay was not able to differentiate between the V44M variant and wild-type IGF-I.

Protein and peptide were known to be susceptible to chemical modification, including oxidization of methionine, and carbamylation of lysine and N-terminal in the present of urea (35,36). Cory *et al.* in 2012 reported the present of significant amount of oxidized IGF-I in the commercial QC pools, which exhibited a shift of 2.285 m/z value and was not observed in human serum samples (15). Our group also observed the presence of oxidized IGF-I in The Royal College of Pathologist of Australasia (RCPA) External Quality Assurance (EQA) materials, which was absent in human serum samples (data not showed).

The presence of protein variants and chemically altered proteins represented an important group of analytes of unknown significance. The

Table 1 Overview on the imprecision and linear range of LC-MS-based IGF-I assay reported in literature

Year	MSIA	Quantitative approach	Calibration curve	Max. imprecision of quantitation	Linear range (ng/ml)	Reference
2001	N	Top-down	Pure standard	Not available	30 - 500	(23)
2001	N	Top-down	Pure standard and standard addition in plasma	Not available	310 - 1480	(22)
2004	Y	Top-down	Pure standard	Not available	7.8 - 1000	(27)
2007	N	Bottom-up	Standard addition in serum or plasma	30.00%	2000 - 8000	(24)
2008	Y	Top-down	Standard addition in serum or plasma	12.00%	50 - 1000	(28)
2008	Y	Top-down	Known plasma samples	8.05%	318 - 898	(29)
2009	N	Bottom-up	Standard addition in serum or plasma	17.00%	15.6 - 2000	(25)
2011	N	Top-down	Standard addition in artificial matrix	5.20%	15.6 - 2000	(30)
2012	N	Top-down	Standard addition in artificial matrix	6.50%	15 - 2000	(15)
2012	Y	Top-down	Standard addition in urine matrix	18.50%	Not available	(43)
2013	N	Bottom-up	Standard addition in serum or plasma	Not available	254 - 4230	(44)
2013	Y	Bottom-up	Pure standard	7.36%	1 - 1500	(45)
2013	N	Top-down	Standard addition in human or chicken whole blood	11.00%	50 - 600	(46)
2014	N	Bottom-up	Standard addition in rat serum	5.60%*	100 - 1000	(42)
2014	N	Top-down	Standard addition in rat serum	4.80%	50 - 1000	(47)
2014	Y	Top-down	Standard addition in artificial matrix	9.75%	5 - 1500	(32)
2015	N	Bottom-up	Standard addition in serum or plasma	< 15%	50 - 3200	(26)
2015	N	Top-down	Not available	Not available	Not available	(31)

*MSIA: mass spectrometric immunoassay; *Average value calculated from 4 laboratories.*

reported IGF-I mRNA with mutations in exon 4, 5 or 6 encoded mature wild-type IGF-I, as only the untranslated regions were affected. The A70T and V44M IGF-I variants (and the genetically unconfirmed A67T variants) in circulation were chemically different to wild-type IGF-I in terms of amino acid sequences, but were recognized by immunological method as wild-type IGF-I in the original reports. Immunoassay relies on the recognition of epitope by antigen binding sites on the antibody for quantitation. Unless the variant region or the chemically modified residue were recognized by the antibody or interfere with the antibody binding, they will be detected as wild-type proteins, and may contribute to clinically discordant results.

LC-MS-based platforms provide an ideal methodology for the simultaneous detection and quantitation of protein variants and chemically altered proteins, since these protein species are detected unambiguously as distinct peaks with specific m/z values on the mass spectrum. However, it should be noted that LC-MS-based platforms are also limited by their inability to differentiate isobaric compounds. As in the case of A67T and A70T IGF-I variants, protein variants with different amino acid sequences but with the same empirical formula will have the same observed m/z value on the mass spectrum, and their detection relies solely on their difference in chromatographic behaviours.

The detection of protein variants and chemically altered proteins is also limited by the choice of proteolytic peptides in the bottom-up approach, especially for targeted triple quadrupole instrument, as the quantitative peptides may not cover the variable amino acid sequence. In such cases the protein variants will be detected as wild-type proteins, similar to the immunoassay. Depending on the measurand of interest, the non-specific detection of protein variants or chemically altered proteins may be advantageous when it is the total protein species, instead of the specific form, that is of clinical interest. On the other hand, if the quantitative peptides cover the variant region, the measured native peptides will reflect the level of wild-type proteins in the circulation, but the information carried by the variant or chemically modified peptides may be lost in SRM mode unless its presence was anticipated and monitored for simultaneously with the native peptide. Care must be taken when developing LC-MS-based protein assays for clinical applications, as well as when interpreting the results for clinical decision.

INSIGHT FOR OTHER PROTEIN QUANTITATION WITH LC-MS PLATFORM

Comparison of the two approaches and MSIA for different proteins

Other complex circulating proteins are expected to be technically challenging for LC-MS-based quantitation, for example proteins with diverse post-translational modification (PTM) such as transferrin and haptoglobin; proteins composed of different subunits such as human chorionic gonadotrophin (hCG) and insulin; and proteins with a combination of the above such as immunoglobulins and thyroglobulin. These proteins may exist as multiple glycoforms or isoforms with different molecular weights, similar to that described above for the IGF-I variants. Multimeric proteins are prone to dissociation during sample handling and LC separation, and the analytical approach should consider all subunits of interest. As demonstrated by the IGF-I assays, different information can be obtained by the selective use of different analytical approaches and strategies.

It is obvious that proteins with diverse PTM cannot be easily quantitated by the top-down approach due to the variable molecular weights. In this regard the use of bottom-up approach may be advantageous since a consensus peptide

can be used, similar to the case of protein variants. An excellent example is the reference method for glycated hemoglobin HbA1c, which utilized the endoproteinase to generate a terminal hexapeptide for LC-MS/MS quantitation (6). Another recent application was the quantitation of thyroglobulin, which is a 660 kDa dimeric glycoprotein with multiple glycoforms. LC-MS/MS quantitation of total thyroglobulin has been achieved by using a combination of the bottom-up approach, MSIA, and enrichment of peptides without N-glycosylation sites (37).

Immunoaffinity capture involves the use of antibody binding to concentrate and purify the protein of interest before either top-down or bottom-up analysis. While it improved the sensitivity and specificity of the LC-MS assay, it also introduces variation in the analytical process. The immunoaffinity process is subjected to temperature, pH and ionic strength of the reaction buffer, stability of the antibody, and conjugation of the antibody to the solid phase support. It is well-known that circulating proteins in serum covers a large dynamic range, and immunoaffinity purification becomes inevitable when the protein of interest is present at picomolar to femtomolar concentration levels, as in the case of thyroglobulin or growth factors (38). At this concentration, the use of the top-down or bottom-up approach alone is not feasible due to the matrix suppression effect from highly abundant proteins. There has been rigorous discussion on MSIA, or hybrid ligand-binding assay (LBA)/LC-MS technologies, for biopharmaceutical compounds for regulatory purpose (39,40). Commercial immunoaffinity proteomics assays were also available for clinical application (41). It will be expected that MSIA will play a crucial role in the analysis of low abundant proteins with clinical significance.

Standardization and harmonization

Similar to other analytical platforms, the quality of LC-MS-based protein assays cannot be guaranteed if they are not traceable to higher reference materials or methods, or not harmonized among existing platforms. The bottom-up approach reported by Kay *et al.* in 2013 exhibited positive bias compared with immunoassays (Passing-Bablok regression slope = 1.37), while negative bias was observed in the top-down approach by Bystrom *et al.* (Deming linear regression slope = 0.81) and Hines *et al.* (Least square linear fit slope = 0.84). The apparent discrepancy may be attributed to the different regression models used for comparison, different reference materials used for calibration in two assays, or the use of different patient cohort.

A systematic study was carried out by Cox *et al.* in 2014 which compared the inter-laboratory agreement of IGF-I measurement using the bottom-up LC-MS platforms and immunoassays (42). A standardized sample and calibrator preparation procedure was used across five laboratories, and two designated tryptic peptides were measured by the different LC-MS instruments in each laboratory. Instead of using human serum, the standardized method used rat serum as the matrix for the preparation of the human IGF-I calibration curve, which was not subjected to the variation of endogenous rat IGF-I concentrations. Results showed that considerable discrepancy was observed between laboratories, especially for high concentration samples. The study concluded that the use of in-house prepared calibrators may contribute a significant degree of imprecision across different laboratories, even though the calibrators were traceable to the same reference material.

These studies not only highlighted the lack of harmonization in IGF-I measurement between

immunoassays and between different LC-MS platforms, the observation is also applicable to other LC-MS-based protein assays as well. It is expected that the between-assay discrepancy is higher in the bottom-up approach than in the top-down approach, since it is subjected to more analytical variables and especially the choice of quantitative peptides. In an effort to provide a more standardized MS-based proteomic assay, the National Cancer Institute Clinical Proteomic Tumor Analysis Consortium has established an Assay Portal which allows open access to standard operating procedures, reagents, experiment parameters and validation data on different bottom-up assays, developed by the research community (https://assays.cancer.gov/).

FUTURE PERSPECTIVE AND CONCLUDING REMARKS

In the past decade, there has been a growing trend for the implementation of LC-MS-based technology in clinical setting, including steroid analysis, immunosuppressant therapeutic drug monitoring, and toxicology screening. Due to instrumentation advancement including increased sensitivity and resolution, quantitation of macromolecules like proteins or peptides is also possible using LC-MS.

The analysis of IGF-I provides an excellent example of macromolecule determination by LC-MS platform in the clinical setting, which is proven to be a flexible and valuable analytical tool for protein quantitation, and is able to provide additional information on proteins that was not accessible by traditional assays. Like other analytical platforms, the adaptation of LC-MS technology for protein determination requires a continuing effort in validation and standardization for clinical application.

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In this issue: Recent Developments in the Clinical Application of Mass Spectrometry

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Progress in metabolomics standardisation and its significance in future clinical laboratory medicine

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ABSTRACT

Today, the technology of 'targeted' based metabolomics is pivotal in the clinical analysis workflow as it provides information of metabolic phenotyping (metabotypes) by enhancing our understanding of metabolism of complex diseases, biomarker discovery for disease development, progression, treatment, and drug function and assessment. This review is focused on surveying and providing a gap analysis on metabolic phenotyping with a focus on targeted based metabolomics from an instrumental, technical *point-of-view* discussing the *state-of-the-art* instrumentation, pre- to post- analytical aspects as well as an overall future necessity for biomarker discovery and future (pre-) clinical routine application.

1. INTRODUCTION

The well-established field of metabolomics aims to comprehensively identify qualitatively and/or quantitatively detectable, endogenous metabolites in biological systems. It is the study of the complete biochemical phenotype of a cell, tissue, or whole organism mainly using analytical platforms such as: nuclear magnetic resonance spectroscopy (NMR), liquid chromatography–mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). Metabolomics interrogates biological systems since it is an unbiased, data-driven approach that may ultimately lead to hypotheses providing new biological knowledge. The term 'metabolomics' typically describing the state of an organisms' metabolism, was first coined by one its pioneers, Jeremy Nicholson, Chair in Biological Chemistry at Imperial College, London. United Kingdom [1]. The term 'metabolomics' was concerned with measuring the metabolites in one sample and may be derived from only one cell type. The term 'metabonomics' (common term used almost a decade ago) was defined as the global study of the systems that regulate metabolism, including variations over time, nevertheless, metabolomics is generally the accepted term used to date [1]. This can be confirmed in the field by new developments of metabolomics core units and research centers in academia with an increased interest in the pharmaceutical and biotechnology industries. These developments and the progressive increase in the number of publications in the field of metabolomics, justifies the growing interest of metabolomics in biomarker discovery and its applications in complex (long-term developing) diseases with open diagnostic, predictive, patient stratification, treatment response, relapse questions, in metabolic disorders (e.g. metabolic syndrome, hypertension, diabetes [2]), neurological disorders (Alzheimer´s disease [3-5]), cardiovascular diseases (heart failure [6, 7], inflammatory diseases (rheumatoid arthritis [8]), oncology [9-13] as well as in toxicology and drug assessment [14]. It is evident that metabolomics is a promising tool to aid in providing insight to answer a biological question, especially in biomarker discovery, however one must assume that it is unlikely there is a single *'golden'* endogenous biomarker that predicts or diagnoses disease. The consensus in the research community is that most studies in the scientific literature document that *'metabolic signatures'* will be the answer. By definition, a metabolic signature contains a panel and/or combination of affected endogenous metabolites and not just an individual metabolite, which is plausible due to the relevance of affected metabolic pathways. It also appears that metabolic signatures can provide an improvement in statistical outcomes and robustness of candidates in biomarker discovery. As a consequence, the instrumental technologies need to possess cross-platform capabilities established by MS and/or NMR and need to be reliable and robust for high-throughput routine analyses. This is the case for *state-of-the-art* LC-MS/MS instrumentation (as detailed below), which are routinely used in the clinical environment for toxicology screening, therapeutic drug monitoring, vitamin and hormone quantitative analysis [15, 16].

Metabolites may have a concentration range in the pico-millimolar range, a mass range of the order of ~1000 amu and polarity of molecules ranging from highly hydrophilic to hydrophobic. There is no single methodology able to separate, detect, and quantify the range of a chemically diverse range of metabolites [17], therefore multiple analytical techniques and sample preparation strategies are necessary to capture most of the metabolome [18]. A typical metabolomics workflow is comprised of sample harvesting and metabolic quenching; metabolite extraction, data acquisition, interrogation and bioinformatic analysis. For sample harvesting

and metabolic quenching, many methods have been published in the literature for various biological systems [19-22]. Individual differences of varied sample types (e.g. WT (Wild-type)/ Control vs KO (Knock-out)/salinity stress), will determine the techniques used at each of these steps. The aim of a typical metabolomics experiment is to analyze as many metabolites as possible. To date, there are several established analytical platforms which details the semi-quantitative detection (relative intensities) of metabolites, however the field of metabolomics is now spearheading towards the absolute quantitation of metabolites in biological systems. As a significant number of metabolites are present in an organism, the data acquired is substantially large requiring interrogation and needs to be processed and treated (eg. normalized and statistically transformed) to obtain a meaningful biological interpretation. Multivariate data analyses such as Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA), Hierarchical Clustering Analysis (HCA), Heatmaps, Volcano plots and pairwise *t*-test, are routinely employed to extract information from large metabolomics data sets [23]. Once the statistically significant variables (metabolites) are identified, correlations between metabolites and responses, groupings and all the experimental data can be used, permitting to constructing a hypothesis or explain observations [23]. The differences between the samples and the identified metabolites connected with them will provide a holistic vision about the interrogated biological system.

Since its establishment in the late 90s [24, 25], metabolomics has proven to be a valuable tool in the analysis of biological systems where it has been used in an ever increasing number of diverse applications such as identifying key genes for important traits, to clarify events of physiological mechanisms and to reveal unknown metabolic pathways in crops [26]; response of salinity in chickpea cultivars [27] and salinity research [28]. It has been applied to assess meat quality traits in pig, cattle and chicken [29]; produce, preserve, and distribute high-quality foods for health promotion [30]; beer [31]; and natural products [32, 33]. By performing global metabolite profiling, also known as *"untargeted"* metabolomics, new discoveries linking cellular pathways to biological mechanisms are being revealed and are shaping our understanding of cell biology, physiology and medicine. These pathways can potentially be targeted to diagnose and treat patients with immune-mediated diseases [34]; understanding the physiological changes occurring in *"normal"* aging and the molecular multi-mechanistic processes involved during senescence [35]; human related studies, that is, genomics, epigenomics, microbiomics, transcriptomics, proteomics and metabolomics (systems biology) [36]; forensic science [37]; response to high intensity exercise [38] and to aid in precision medicine for patients with multiple sclerosis [39].

In this review, the choice and characteristics of all major metabolomics technologies will be addressed together with a discussion on current trends and requirements of biomarker discovery in a clinical environment as well as future (pre-) clinical routine capabilities. Finally, the current state of knowledge with respect to metabolomics standardization and a gap analysis, which needs to be addressed to bring metabolic signatures to clinical routine applications, will be presented.

2. GC-MS and LC-MS

2.1 Gas Chromatography –Mass Spectrometry (GC-MS)

It is generally assumed that GC-MS is only amenable for the analysis of volatile compounds or those classes of lipophilic compounds extracted from apolar, organic solvents. This is not

the case for the well-established 'polar GC-MS metabolomics', hydrophilic compounds can be made volatile due to chemical derivatisation which selectively alters known functional groups making them amenable for GC-MS analysis. Nevertheless, GC-MS, though limited to the analysis of compounds smaller < 1,000 Da, can unambiguously, comprehensively resolve >400 compounds including sugars, sugar alcohols, sugar phosphates, amino and organic acids, amines, sterols and fatty acids in one acquisition and is typically suited to the analysis of primary metabolites - those involved in fundamental biological processes (e.g. glycolysis, TCA cycle and amino acid synthesis) of the growth and development of a cell. To increase the number of identified metabolites, authentic standards are required to match mass spectra and retention time with metabolites in the sample [40]. In addition to the highly reproducible electron impact (electron impact ionization, EI) mass spectra, some commercially available libraries provide retention times or retention time indices under standardized conditions for each metabolite [41] increasing confidence in metabolite identification. One of the greatest advantages of GC-MS is that the ionization mode used in this technique is highly standardized and reproducible across GC-MS systems from different vendors worldwide (based on 70 eV ionization) which allows for the establishment of comprehensive GC-MS mass spectral libraries such as the NIST (http://www.nist.gov/srd/nist1a.htm), Agilent's FienLab (http://fiehnlab.ucdavis.edu/db) [42], or publically available [Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de) [41], which contain TMS (tri-methylsilylated)-derivatized metabolites. Undoubtedly, for more than a decade and a half, GC-MS has been accepted as the *"work-horse"* platform due to its notable separation, reproducibility, robustness, ease of establishment and operation and its relatively low costs and standardization worldwide.

Recently, there has been a focus within the metabolomics community to obtain quantitative data for biological studies since they describe accurately the actual concentration of the metabolites of interest. In the current literature, >90% of published metabolomics studies are semi-quantitative with <10% of published metabolomics studies using absolute quantification. In one well-known example, a quantitative database was curated by Psychogios *et al.,* who systematically characterized the human metabolome through the Human Metabolome Project [43]. In another study, Schwarz and colleagues identified and quantified 476 metabolites in cerebrospinal fluid as part of an integrative metabolome-proteome CSF database towards biomedical research [44]. Boutara *et. al.,* utilized a number of analytical multi-platform (NMR, GC-MS, DFI/LC-MS/MS, ICP-MS and HPLC) analyses which led to the identification of 445 and quantification of 378 unique urine metabolites or metabolite species. An online database containing 2651 confirmed human urine metabolite species, 3079 in total, concentrations, related literature references and links to their known disease associations are freely available at http://www.urinemetabolome.ca [45].

2.2 Liquid Chromatography –Mass Spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is a complimentary analytical platform used to identify metabolites that generally do not require chemical derivatisation, typically suited to the analysis of higher molecular weights metabolites in their 'intact' form. There are also derivatization reagents available which provide greater selectivity and sensitivity for the analysis of 'targeted' metabolites classes [46-48]. Depending on the metabolite(s) or metabolite classes of interest requiring LC-MS analysis, the choice of extraction solvent is crucial in separating polar and apolar metabolites, achieved

through either a monophasic or bi-phasic solvent extraction (e.g. chloroform, methanol and water) covering the polarity range.

Once a sample has been extracted prior to carrying out a LC-MS analysis the chromatographic separation of eluting metabolites requires subsequent optimization. A number of stationary phases [(e.g. ion exchange, reversed phase (C18), hydrophilic interaction (HILIC) chromatography and aqueous normal phase separation)] with varying solvent systems in either isocratic or gradient elution are used to separate and attempt to capture the metabolome. The most commonly used ionization modes include, electrospray ionization (ESI) and atmospheric chemical pressure ionization (APCI). Depending on the metabolite classes to be targeted, a number of LC-MS based platforms and modes can be used. For example, the lipophilic extract can be profiled in an untargeted manner using Liquid Chromatography–Quadrupole Time of Flight–Mass Spectrometry (LC–*Q*TOF–MS) which allows for the identification of intact lipid species with its corresponding high resolution mass spectra. Quantification of each lipid species can be achieved using Multiple Reaction Monitoring (MRM) using authentic standards on a Liquid Chromatography–Triple Quadrupole–Mass Spectrometry (LC–*QQQ*–MS), a rapidly important field referred to as lipidomics. The LIPID MAPS Structure Database (LMSD, www.lipidmaps.org/data/structure) encompasses structures and annotations of biologically relevant lipids.

Subsequently, the polar extract which may contain secondary metabolites or higher molecular weight metabolites and those that are not GC-MS amenable can be separated by reversed phase (C18) chromatography. The lipophilic or non-polar extract can be separated using hydrophilic interaction (HILIC). Further to these separation methodologies, positive and negative, soft ionization modalities need to be applied to cover both positively $[M+H]^+$ and negatively $[M-H]^-$ charged metabolites present. Each mode can result in 500 - 3000 mass features detectable represented with their accurate mass, isotopic pattern, and retention time. Accurate mass (as obtained with a *Q*TOF or FT-type instrument, and isotopic pattern as well as additional secondary and tertiary MS^n can assist with the structural elucidation of the metabolite of interest. Nevertheless, the unambiguous identification is not always possible as a number of metabolites can have exactly the same molecular formula and mass ultimately requiring the isolation and complete elucidation by NMR by first principles.

For LC-MS based untargeted metabolomics a number of databases currently exist. As mentioned, the HMDB Version 3.6 (www.hmdb.ca/) is a comprehensive, web-accessible electronic database containing information on metabolites found in the human body. For food compounds, FoodDB (http://foodb.ca/), however its mass spectral information is also duplicated in the HMDB. METLIN is a metabolite repository is a web and freely accessible electronic database (http://metlin.scripps.edu/) to facilitate metabolite annotation through MS analysis. MassBank is an open-community mass spectra repository designed for public sharing of reference mass spectra from authentic chemical standards for metabolite annotation (www.massbank.jp). mzCloud is an open community of academic and industrial partners who provides MS/MS and MS^n spectral trees that can be freely searched (https://www.mzcloud.org/). For further details please *see* [49].

2.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

For decades NMR has proven to be the summit in the structure elucidation of organic compounds but the last decade and a half has seen its integration in metabolomics given that it is *non*-destructive, providing highly accurate quantification of

metabolites and unambiguous structure elucidation of compounds. Furthermore, NMR is regarded the *'gold standard method'* of choice for the structural elucidation of unknown compounds if accurate mass measurements and mass fragmentation pattern analysis has not resulted in sufficient information. Though, ^{1}H NMR is highly reproducible and signal intensity is directly related to the molar concentration, a drawback is that it has low sensitivity and resolution compared to MS-based methods [50]. For literature describing NMR metabolomics *see* [51-54].

3.0 NEED AND GAP ANALYSIS IN STANDARDIZATION/HARMONIZATION IN TARGETED METABOLOMICS

Today there is a motivation in the research community that the standardization and harmonization in metabolomics are mandatory to deliver comparable, reliable, high-quality and precise quantitative data [15, 55-59]. These requirements need to establish a set of standards to guarantee longitudinal, robust *lab-to-lab* and *inter-instrument* comparability and bring emerging applications of metabolomics into future routine analyses. Biomarker signatures identified using untargeted metabolomics profiling needs to be translated to targeted-based metabolomics later in the biomarker validation workflow to allow for the absolute quantitation and improved comparability of studies. The following gap analysis summarized in Table 1 below, presents the requirements for targeted metabolomics studies, related metabolic signature validations as well as a first outlook for the requirements in routine clinical applications.

3.1 Metabolomics study design

Published biomarker (signatures) discovery case/control studies today are often quite limited in sample cohort sizes and lacking in appropriate validation studies for confirmation. It is critical to invest in validation studies using a greater cohort of samples including the development of valuable biomarker/metabolite associations, which are powerful for future clinical applications. Research focused on population based cohorts is able to fulfil this requirement. Standardized targeted metabolomics analytics (e.g. kits) acquired in the same data formats is certainly advantageous, due to ease in comparing data and translating results, increasing statistical power. Secondly, studies are often not well designed to address biomarker specificity based on appropriate control cohort selection. Frequently, only healthy controls are included. Consideration of all relevant disease control cohorts as delimitation controls are mandatory to test and approve specificity of biomarker candidates resulting in increased value for future clinical applications (e.g. consideration of alternate cancers in women, benign breast diseases, endometriosis, polycystic ovary syndrome (PCOS) for breast cancer studies). In addition case/control studies needs to be translated to longitudinal studies, both retrospective as well as prospective, to investigate the biomarker performance for predictive value and to improve knowledge about the follow-up of individuals. Additional factors which can contribute to a cohesive study design are the inclusions of typically used clinical (less invasive) biofluids as potential target matrices to allow for parallel data translation/interpretation from bodily compartments to systemic biofluids (e.g. plasma, serum, dried blood spots), which are appealing in (pre)clinical applications.

3.2 Pre-analytics

The consideration of pre-analytical aspects in biomarker discovery and the validation is mandatory for the robust and reliable performance of detecting biomarkers. Avoiding "artificial" interferences and to investigate how biomarker candidates are affected and sensitive to sample generation (e.g. venepuncture), transport and storage (short-term

Table 1 Requirements for improved metabolomics biomarker studies and for future clinical applications

Study design	a) Number of samples in a cohort are often limited b) Validation studies are missing c) Gap of disease specificity in biomarker studies: case/control studies require inclusion of more disease and gender related delimitated controls not only healthy controls to prove and deliver specificity d) Translate case/control studies to longitudinal studies (population based, retrospective followed by prospective) e) Inclusion of typically used clinical (less invasive) biofluids as matrices into study protocols to ensure data translation/interpretation from body compartment to systemic biofluids
Pre-analytics	a) Pre-analytical quality markers based on endogenous metabolites (stability markers for sample generation and storage), appropriate database is required also to prove biomarker candidates robustness, standardized pre-analytics
Analytics	a) Quantitative metabolomic data b) Standardization (e.g. kits) (from sample to results including sample preparation, analysis, technical validated analytical results to deliver *lab-to-lab* comparability, *inter-instrument* comparability, long-term comparability c) Gap of reference materials and reference laboratories, round-robin/ring trial tests d) Gap of standard materials (external, internal standards) e) Established QMS system in the analytical laboratory (ISO 9001, ISO 17025, GLP etc.)
Post-analytics	a) Gap in standardized data pre-processing for statistical data analysis: • identification of pre-analytical affected samples in the study • normalization, batch correction, data cleaning (e.g. LOD imputation), confounder adjustment and multivariate outlier detection b) Standardized data formats
Needs for future clinical applications (bench to bed side)	a) High performing biomarker signatures in defined/standardized biological matrices for clinical question b) Translate disease/metabolite association to causality c) Reference methods/kits (medical device regulatory, FDA, CE/IVD), reference laboratories (e.g. CLIA) d) Traceability and commutability of standards and reagents (e.g. calibrators) e) Standardized sample/sampling device f) External quality assurance programs (proficiency tests, ring trials) g) Certified reference materials (for metabolic signatures /metabolite panels)

mainly at room temperature, mid/long-term (biobanking) in accordance to sample collection time and storage) are important. The value of targeted based metabolomics has the potential to monitor pre-analytical sample treatment based on the analysis of endogenous metabolites concentrations and signatures (e.g. sums, ratios) demonstrate the feasibility for targeted metabolomics [60-64]. The best approach is to test a panel of markers in combination and in parallel, to identify affected samples with high confidence when all quality markers (combinations) are identified. In our opinion, an appropriate database is required to collate (non)pre-analytical affected endogenous metabolites, allowing to evaluate the value and quality of biomarker candidates from a pre-analytical perspective.

3.3 Analytics

The automation, standardization and harmonization of providing quantitative metabolite data in metabolomics is required to bring together *lab-to-lab*, inter-instrument and long-term robust analytical analyses into biomarker discovery and development, critical in further developing biomarker signatures into the clinical environment. Commercially available kits are available for targeted metabolomics (e.g. Biocrates Life Sciences), which can *fill-the-gap*, by delivering standardized data formats. Biomarker discovery need to apply the rules which are standard practice in routine clinical analyses today to bring new biomarker signatures into the (pre-) clinical routine [15, 56, 59]. A second aspect to consider is the current gap of broad target analyte coverage of available standards (external and internal standards) and (certified) reference materials (covering metabolite classes) as well as appropriate reference laboratories. This gap is obligatory, not only for quantitative data but also to enable quantitation with approved accuracy and traceability. One of the initial activities of the National Institute of Standards and Technology (NIST) in collaboration with NIH (National Institute of Health) confirms the awareness for the need of standard reference materials (e.g. SRM 1950) [15, 65, 66]. External and internal standards are essential to define quantitative linear range, sensitivity, selectivity, and correction of matrix effects of the metabolites in the assay resulting in improved analytical accuracy and precision. Finally, an established quality system including consideration, for example, sample entrance and storage control, instrument and quality performance tests within the laboratory organization will and should assist in common laboratory quality rules.

As previously discussed, it is evident that GC-MS based metabolomics is very-well standardized with commercial and publically MS-databases and methods available. However, with new, sensitive instrumentation, enhancements in LODs, mass accuracy and resolution, dynamic ranges and the push for 'quantitative' data, existing libraries may need to be revisited because of the differences in mass accuracy and the relative intensities of fragment ions that can affect mass spectra similarity scores. As previously mentioned, there is numerous metabolomics applications and technologies and there has been discussions and consortiums formed to attempt to standardize aspects in metabolomics.

The bottleneck lies predominantly with LC-MS based profiling typically due to the complexity of variables including: extraction, chromatographic method or solvent system to comprehensively profile the whole metabolome in a single analytical run. Establishing unbiased analytical methods are not trivial due to a number of factors such as: issues in combining data from different MS analyses which hinder correlation of data obtained in different instruments/laboratories which include large scale studies acquired overt time, instrument drift and maintenance; analyte quantification is a problem compared to conventional targeted

methods; data acquired in untargeted LC-MS profiling from same/different laboratories acquired on different instruments cannot be easily compared. Standardized protocols from the phase of study design, sample collection and handling, up to the phase of chemical and statistical analysis remains an issue to be resolved [67]. The standardization of metabolomics data formats (more easier in targeted metabolomics compared to profiling) is absolutely necessary to simplify data comparability. A logical consequence is also the need and consideration of standardization in data pre-processing including data filtering (e.g. agreement of common rules for limit of detection (LOD) and lower limit of quantitation (LLOQ) imputation), sample batch correction, and apply pre-analytical quality marker signatures over the sample data set) before any downstream analysis can begin.

There have been a number of worldwide initiatives which have attempted to standardize LC-MS methodologies and protocols including column stationary phases, elution solvents and gradient, ionization modalities (positive/negative), MS scan setup including accuracy of mass detection with efforts focussing on creating LC-MS bases mass spectral information including accurate mass, MS^n fragmentation pattern and more importantly, retention time under specific LC conditions [68]. More recently, work carried out by Wolfer *et. al.,* described an approach that enabled the generation of reliable quantitative structure retention relationship models tailored to specific chromatographic protocols. The methodology, applied to 442 experimentally characterized standards, employed a combination of random forest and support vector regression models with molecular interaction descriptors [68]. This retention time prediction framework could be replicated by different laboratories to suit their profiling platforms and enhance the value of standard library by providing a new tool for compound identification [68].

3.4 Post-analytics

The *push* for metabolomics standardization began over 10 years ago with the standard metabolic reporting structure initiative (SMRS) and the Architecture for Metabolomics consortium (Armet) which focused particularly on NMR based metabolomics [69]. In 2005, the metabolomics standards initiative (MSI) focused on a community-agreed minimum reporting standards providing initial efforts on the descriptions of the experimental metadata describing a metabolomics study [70]. Founded by the community, standards and infrastructure for metabolomics still require storage, exchange, comparison and re-utilization of metabolomics data. From this, 5 working groups (WG) were created focussing on: metabolomic pipeline; biological context metadata WG, chemical analysis WG, data processing WG, ontology WG and exchange format WG, with the task of collecting relevant metabolomics standards and a forum for discussion [71, 72]. In order to implement agreed and acceptable guidelines on reporting identified metabolites, an application platform such as a metabolomics repository in addition to a journal publication was required. In 2012, MetaboLights (http://www.ebi.ac.uk/metabolights) was the first general purpose database in metabolomics, developed and maintained by the European Bioinformatics Institute (EMBL-EBI) which combines small molecule 'reference' layer with information about individual metabolites, chemistries, spectrometry and biological roles with a study archive, where primary data and metadata from metabolomics studies are ontologically tagged and stored [73]. Such depositions receive a stable identifier for each study, which can be quoted in related publications and can be used to access the data on a long term. Making metabolomics data publicly accessible allows it to justify researchers' findings in a peer-reviewed publication, increases the possibility of wider collaborations within the metabolomics community and ultimately gives a study higher visibility

and increased citation [73]. More recently, the Framework Programme 7 EU Initiative 'coordination of standards in metabolomics' (COSMOS) is developing a robust data infrastructure and exchange standards for metabolomics data and metadata [72]. The data deposition and exchange workflow in the COSMOS consortium will be formally defined, agreed, and documented in relation with MetaboLights and all partnering databases in Europe and worldwide that would like to participate. The COSMOS consortium ultimately develops the standards and infrastructure for and with the metabolomics and fluxomics community. These efforts will directly enable the implementation of COSMOS important deliverable—that of a robust data infrastructure and mechanisms for standards metabolomics data representation and data/meta-data exchange that will enrich metabolomics science [72].

The general community agreement is that the challenge of metabolomics is the accurate identification of large numbers of metabolites in various untargeted profiling techniques. The metabolomics community has been discussing the challenges of metabolite identification and minimum reporting criteria for some time, and the Chemical Analysis Working Group of the MSI proposed some basic guidelines in 2007 [74]. While spectral standardization within a particular database such as METLIN is helpful, the diversity in acquisition is also beneficial for metabolite annotation (isomers), as it can highlight similar/dissimilar fragmentation processes across analytical conditions. Public databases are more often enough curated *in-house* in different laboratories (academia and commercial), applying a multitude of different analytical methods, reflecting the analytically diverse nature of the metabolomics community. Standardization of spectra acquisition using one particular ionization source, mass analyzer, and/or fragmentation technique would only be essential for a small percentage of groups.

3.5. Needs for future clinical applications

Targeted based metabolomics with respect to the detection of biomarker signatures for newborn screening, a *proof-of-concept* adopted more than 10 years ago will continue to be important in future (pre)clinical applications. Current studies provides new insights into numerous chronic and long-term developing diseases such as (cardiovascular disease, hypertension, cancer, metabolic disorders e.g. diabetes, inflammatory bowel disease, autoimmune disease, neurological diseases). Targeted metabolomics will continue to provide new knowledge about the commonalities, for example: insulin resistance, mitochondrial function, and inflammation and differences of these diseases and demonstrate strong metabolic causes to individual disease pathophysiology. The importance of the microbiome and the acquired dysbiosis of the gut microbiota [75-81] and immune system/acquired immune competence [82] are important drivers for the paradigm shift to the understanding of disease, wherein targeted metabolomics can be considered as the *gold standard* tool to measure and quantify related alterations in the metabolic phenotype. However, several factors need be considered for the successful translation of new biomarker signatures in routine analyses. (Pre-)clinical applications require a defined and standardized biological matrix and sample introduction. Furthermore, disease/metabolite associations has to be translated to understand causality and affected biological pathways providing understanding, which might assist in future therapy approaches. Nevertheless, there needs to be a *push* towards enhancing regulatory requirements with traceable and commutable reference materials [16], standards, methods, kits (medical device regulatory, FDA, CE/IVD), and/or reference laboratories (CLIA), external quality assurance programs to improve the robustness and validity of clinical data in future.

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eJIFCC

National External Quality Assurance Program Pakistan (NEQAPP) –a milestone in proficiency testing in Pakistan

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Key words:
National External Quality Assurance Program Pakistan, NEQAPP, proficiency testing, medical laboratories

ABSTRACT

Objective

The objective of this study was to highlight current status and importance of National External Quality Assurance Program Pakistan (NEQAPP).

Study Design: Cross sectional study

Place and duration of study

Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology (AFIP) from August to October 2015.

Methods

The study data was extracted from electronic NEQAPP database. Results from 2014-2015 were evaluated for clinical chemistry, hematology, microbiology, and immunoassay programs. Frequencies of unsatisfactory results of individual analytes as well as of all the participating laboratories were calculated.

Results

Failure rate of newly enrolled laboratories were more as compared to those which were participating for the last two years. The percentages of unsatisfactory results of all laboratories were 19% and 15% in 2014

and 2015, respectively. Fifteen analytes were selected according to their increasing percentage of participation. Failure rate was highest for alkaline phosphatase (35%) followed by creatinine (22%) and urea (20%) in two years analysis. Performance of laboratories in each quarter was evaluated depending upon number of analytes in which they fail to pass. The major failures were due to clerical and technical errors as determined during data compilation of results.

Conclusion

There is an increase in trend of participating in NEQAPP by health care laboratories which is a step towards laboratory quality management system in Pakistan. Nonetheless, there is a need for improving quality of laboratory results.

❖❖❖❖❖

INTRODUCTION

National external quality assurance programme Pakistan (NEQAPP) is a system designed to objectively assess the quality results obtained by medical laboratories in Pakistan. The primary aim of this proficiency testing (PT) program is to strengthen standards of clinical laboratories in Pakistan by providing medical professionals with a comprehensive quality and cost effective external quality assessment (EQA) scheme at a national level and to reduce the risk of errors in laboratory results. This will help provide better patient care and quality results of clinical laboratories in Pakistan along with fulfilling regulatory and accreditation requirements[1]. Erroneous lab results have great impact in delaying appropriate patient care along with increasing cost of diagnosis and management[2].

NEQAPP program runs in a twelve-month cycle. Samples are sent to registered laboratories on a quarterly basis. Results of a laboratory are judged against a comparator mean of instrument and method, or a pre-determined result as in the case of culture sensitivity for the microbiology program.

The aim of this study is to highlight efforts taken to improve quality of laboratories by inculcating proficiency testing (PT) philosophy through NEQAPP, importance of laboratory PT in medical science and appraise current situation of quality reporting in our laboratories. As such, our study will help all those concerned with medical laboratories in Pakistan and encourage a quality reporting culture according to international standards for better patient management.

METHODS

We conducted this cross section study at the Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. The study data was extracted from the electronic NEQAPP database. Results from January 2014 – December 2015 were evaluated for clinical chemistry, hematology, microbiology and immunoassay programs. Frequencies of unsatisfactory results of individual as well as all of the analytes were calculated for different laboratories.

RESULTS

There were 88 laboratories enrolled with NEQAPP in 2011, which increased to 140 laboratories in 2015. Ninety-six percent of the enrolled laboratories participated in the clinical chemistry programme followed by hematology (30%), microbiology (28%) and immunoassay (14%).

Failure rates of newly enrolled laboratories were higher than those which were participating for the last two years. Laboratories were grouped as defence, public and private laboratories. The percentage of unsatisfactory results of participating laboratories was 19%, 15% and 10% in the years 2014 and 2015. Fifteen analytes were

selected according to their increasing percentage of participation. This included ten parameters from clinical chemistry (glucose, alkaline aminotransferase, bilirubin, creatinine, cholesterol, albumin, triglyceride, urea, alkaline phosphatase and amylase), three from hematology (hemoglobin, red blood cell count and white blood cell count) and two from immunoassay (thyroid stimulating hormone and human chorionic gonadotropin). Failure rates were highest for alkaline phosphatase (35%) followed by creatinine (22%) and urea (20%) during the two years of the study period. In clinical chemistry, failure rates of the ten analytes for defence laboratories was 19%, 19.5% for public laboratories and 13.5% for private laboratories in the year 2014. Whereas failure rates declined to 8.5% in defence laboratories, 11% in public laboratories and 10.5% in private laboratories in the year 2015.

Reasons for unsatisfactory results were evaluated and classified into five main categories: methodological (21%), clerical (wrong entry of results or unit) (42%), technical (20%), PT material stability (9%) and random errors (8%). It has been observed that failure rates were low in laboratories which are supervised by technically qualified professionals in laboratory management and quality control, use of automation, standard methodologies and in those laboratories which are frequently participating in national and international proficiency testing programs for quite some time.

DISCUSSION

Proficiency testing program helps in improving and maintaining analytical inter laboratory agreement[3]. Good analytical agreement between laboratories is required as patients/clinicians move from one area/hospital to another. Irrespective of the setting, i.e., large reference laboratory with the latest equipment and professional staff or a small laboratory, a laboratory must report proficiency results of adequate quality to meet the stated guidelines. This can only be ensured by participating in external quality assessment (EQA) and taking appropriate actions when results do not meet acceptable performance. Although once considered a theoretic entity, PT is now a regulatory requirement for laboratory licensing by health authorities[4]. Moreover it is a prerequisite for getting the laboratory accredited as per ISO 15189[5].

Material used for proficiency testing are provided by external agencies, either mandated legislative bodies or voluntary organizations[6]. These materials are used to check the quality performance of a laboratory relative to its peers in terms of standard of performance that is usually expressed as a total variation from a target value for each sample. These samples are intended to reflect the laboratory's performance with patient samples. Erroneous proficiency results indicate that the laboratory is incapable of meeting the accepted standard of performance and can ultimately lead to the loss of the laboratory's license to perform that specific test or entire class of tests.

External quality control program is usually selected by the laboratory and purchased from an external company. It is an external check of the analytical methods performance in an acceptable manner to produce clinically acceptable patient results within the stated criteria[7]. QC samples provide us with data that represent the accuracy and precision of each method at the level of analyte present in each control. A laboratory must interpret that data in order to make daily decisions about the acceptability of each batch of patient samples, and ongoing decisions about the overall acceptability of method performance.

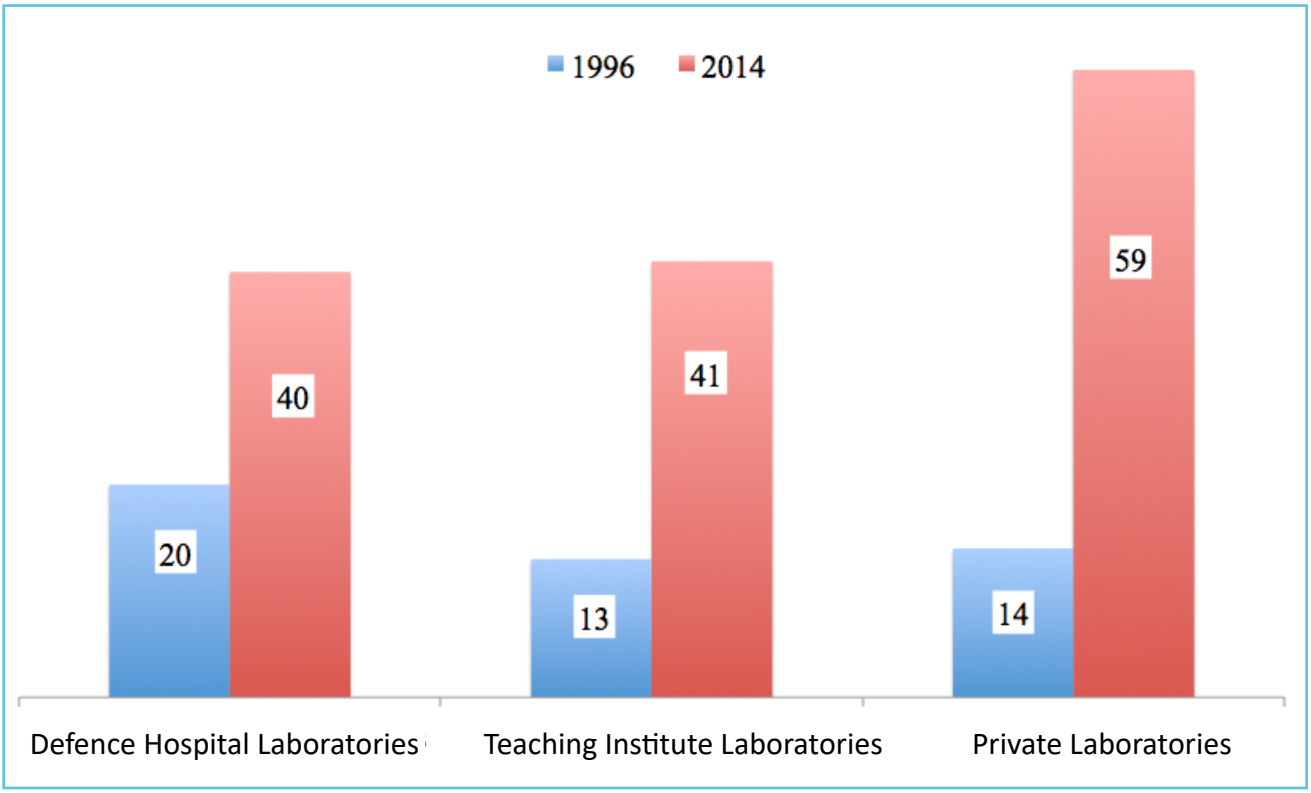
In 1996, at AFIP, Rawalpindi, participation in the clinical chemistry survey of NEQAPP was commenced with the aim to expand it to other

disciplines of Pathology. The Pakistan National Accreditation Council has launched the Medical Laboratory Accreditation Scheme in Pakistan in compliance with international organization of standardization (ISO) 15189 (International Standard for Medical Laboratories). PT is considered mandatory for clinical laboratory accreditation. It was at that time that NEQAPP program was expanded to hematology, microbiology and immunoassay to support the scientific and medical communities in Pakistan. Since then it has gained immense popularity and at present more than 140 clinical laboratories of Pakistan including 40 defence hospital laboratories, 41 teaching institution laboratories (medical colleges and postgraduate medical institutes), 59 public and private sector laboratories are voluntarily participating in this program (Fig. 1).

NEQAPP is a user friendly, cost effective program with immediate availability of customer service and technical support. Laboratories have an option of submitting their test results electronically (NEQAPP Online) or on paper (Results Forms) via post. Laboratory performance is displayed in each laboratory specific Sample Report. Acceptable performance in this national program is identified as falling within 2 standard deviation index (SDI) from ones comparator mean. Results outside ± 2 SDI are considered as unacceptable and highlighted as 'Fail' (Fig. 2).

In the last three years there is an increasing trend of ISO certification. This program should be adopted as mandatory requirement by regulatory authority for running and scrutinizing quality assurance of medical laboratories in Pakistan. Quality reporting can be ensured by selecting appropriate instrument with test method validation at instrument installment, improving transport and storage of reagents/calibrator/controls, scheduled equipment maintenance and ensuring quality checks[8].

Figure 1 Increasing trend of participation in NEQAPP



There is improvement in quality of PT results due to improvement in pre-analytic steps, laboratory automation, maintaining internal quality control and following standardize protocols for laboratory reporting.

There are certain problems, as follows, which need to be highlighted for running the proficiency program effectively. Laboratories not submitting results on due date, incomplete methodology details, failing to participate in all four quarters of a cycle, submitting results in units other than prescribed by NEQAPP, less use of electronic result submission and failing to update laboratory corresponding details especially in case of public laboratories.

CONCLUSION

NEQAPP program plays a pivotal role in improving the quality of laboratory services in Pakistan. In the last three years there has been a significant improvement in pass percentage of participating laboratories. No health care facility can be totally self-reliant in terms of maintaining quality and this gap can be filled by participating in an external quality program, giving the true picture of level of quality reporting.

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Figure 2 NEQAPP

NEQAPP

National External Quality Assurance Program Pakistan (NEQAPP)

Clinical Chemistry

Report

AFIP Rawalpindi

Your Laboratory No:	A-31	
Report for Cycle:	Cycle 4	January 2013 - April 2014
Sample No:	sample 4	
Sample Date:	01 / 10/ 2013	

AFIP Rawalpindi

Pathologist, AFIP Rawalpindi Cantt Rawalpindi

Coordinator NEQAPP. Dept of Chemical Pathology & Endocrinology AFIP Rawalpindi
For Details of Individual Analyte Report, Please Visit www.neqapp.net

National External Quality Assurance Program Pakistan (NEQAPP)

Clinical Chemistry

LAB	Cycle Name	Sample	Sample Date
A-31	Cycle 4	sample 4	01 / 10/ 2013

Sr.No	Analyte	Your Result	Group Mean	SDI	
18	Albumin	48 g/l	45.54	0.9	Pass
19	Alkaline Phosphatase	235 U/L	375.29	-1.6	Pass
20	ALT	131 U/L	127.56	0.4	Pass
21	Amylase	483 U/L	554.38	-1.2	Pass
22	AST	155 U/L	161.64	-0.6	Pass
23	Bilirubin (Direct)	26.03 umol/l	26.1	-0	Pass
24	Billirubin (Total)	69 umol/L	73.67	-0.9	Pass
25	Calcium (Total)	3.19 mmol/l	3.01	0.9	Pass
26	Chloride	113.7 mmol/l	115.67	-0.6	Pass
27	Cholesterol	6.13 mmol/L	5.93	0.7	Pass
28	Creatine Kinase	382 U/L	407.04	-0.6	Pass
29	Creatinine	532 umol/L	476.95	1.2	Pass
30	Gama Glutamyl Transferase	106 U/L	108.67	-0.2	Pass
31	Glucose	16.1 mmol/L	15.11	1.4	Pass
32	Iron	197 ug/dl	186.2	0.3	Pass
33	Lactate Dehydrogenase	578 U/L	563.65	0.2	Pass
34	Magnesium	1.47 mmol/l	1.32	1	Pass
35	Osmolality	310 mosm/Kg	312.5	-0.7	Pass
36	Phosphate (Inorganic)	2.34 mmol/l	1.95	1.2	Pass
37	Potassium	6.46 mmol/l	6.17	1	Pass
38	Protein (Total)	71 g/l	72.16	-0.2	Pass

Pass; SDI ± 2.0

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Letter to the editor: Successful ISO 15189 accreditation in the Bethzatha Advanced Medical Laboratory in Ethiopia

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INFO

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LETTER TO THE EDITOR

Many laboratories are taking a more focused approach to quality management system, i.e. the ISO 15189:2012 guideline (1). The Ethiopian standard agency (ESA) adopted ES ISO 15189:2013, in an effort to improve patient care through quality laboratory practices (2). In Ethiopia, a laboratory that meets this guideline can be accredited by Ethiopian national accreditation office (ENAO), indicating the highest level of confidence in the quality of services provided by the laboratory (3). Bethzatha Advanced Medical Laboratory was established as a private company in 1996, with a mission to provide quality medical laboratory services. It is well equipped with high-tech laboratory equipments (Fig.1). It has highly qualified professionals such as pathologist, clinical laboratory specialist, medical laboratory technologists and technicians. The laboratory is also structured into different departments.

Our medical laboratory was first recognized by SLMTA WHO-AFRO in 2012 as a 2-star laboratory. The second accreditation was an ISO 15189:2012 based accreditation. This accreditation process began in 2013 and was certified as ENAO-accredited in 5 analytes, i.e. glucose, creatinine, cholesterol, alanine amino transferase and aspartate amino transferase, in May, 2015.

Figure 1 Bethzatha Advanced Medical Laboratory in Addis Ababa

During the preparatory phase, first we offered awareness training for the top management about the benefit of accreditation. Secondly, we trained all the technical and non technical staffs about the12 quality essentials. We designed a Quality Management System (QMS) based on a quality manual. The system was fully operational after trainings were offered to all personnel. Since the implementation of this QMS, a lot of progress could be seen that brings about a well organized structure, smooth work relations, and efficient services.

Metrological traceability is the property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty (4). International Laboratory Accreditation Cooperation (ILAC) and its associate member, ENAO, have mandatory policy on metrological traceability, i.e. medical laboratories are required to have an established calibration program for critical equipments that directly or indirectly affect examination results (5,6). As a result, most of the subsidiary equipments were calibrated by National Metrology Institute of Ethiopia (NMIE). At last, Beckman Coulter master calibrator (where traceability was achieved through National Institute of Standards and Technology's (NIST's) Standard Reference Material (SRM)) was used to calibrate test methods.

Another critical criterion was Proficiency Testing participation (PT) (7) that made our journey to the accreditation process challenging. The German Society for International Cooperation (GIZ) provided us PT schemes on hematology and chemistry tests in support of the accreditation project since March 2013; however, it was terminated at the end of 2015. But, fortunately, our challenge was solved by the support of the Ethiopian Public Health Institute (EPHI).

Our laboratory monitors the progress of the QMS efficiently. The management reviews the pre-analytical, analytical and post-analytical processes. Using internal audit, as an assessment tool, the effectiveness of the action was evaluated.

To conclude, the management, and the staff in general, were dedicated and made an invaluable contribution towards the accreditation process becoming a success. Had we not obtained support of uninterrupted PT scheme from EPHI, things would not have been easy. Also, GIZ was a great help backing up our staff through trainings that facilitates the process of method verification and measurement uncertainty. Generally speaking, we have benefited enormously from the experiences we have been through in the

accreditation process. We hope our experience will inspire and shade a light to medical laboratories which might have not started the accreditation program yet.

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Book review: "Cardiovascular Disease and Laboratory Medicine"

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REVIEWED BOOK

"Cardiovascular Disease and Laboratory Medicine"
by Martin Crook, John Chambers, and Phil Chowienczyk

Editors: Natlie Walsham and William Marshall
Publisher: ACB Venture Publications
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EAN 9780902429567

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RECENSION

This slim text of 6 chapters is yet another title from the excellent series published by the Association for Clinical Biochemistry and Laboratory Medicine (ACB, UK).

The opening chapter rightly starts with a discussion of the risk factors of cardiovascular diseases (CVD) and their relative importance. There is a list of the emerging cardiovascular risk factor markers that are associated with inflammation, but most of these are not yet in clinical application. The authors do not make a recommendation of the most useful routine tests to assess the risk for CVD. It would have been helpful if C-reactive protein, a key risk factor, was discussed in a separate sub-section. The chapter also includes mention of non-laboratory techniques for assessing cardiovascular risk such as the ankle-brachia pulse index, B mode ultrasound, coronary artery calcification and magnetic resonance imaging.

A good part of this book is on the pathophysiology of CVD. While it is important for the understanding of the underlying changes in CVD upon which diagnostic tests are based, the reader would expect, from the title, an emphasis on the laboratory testing. The second chapter is mostly a description of the physiology,

metabolism and pathophysiology of the endothelium, haemostasis and thrombosis. It also deals with the clinical consequences and the diagnosis of thrombosis and its treatment. Other than D-dimer, there is little mention of the laboratory testing involved for these conditions.

Similarly, in Chapter 3, the reader is brought up to date on lipid metabolism, dyslipidaemias and their management and treatment. While this is welcome, the laboratory scientist cannot be blamed for feeling a little frustrated with the relatively inadequate treatment of the testing for dyslipidaemias. A short discussion on the analyses of various lipids and their inadequacies would have helped. Once again, the chapter does not have a separate heading for laboratory testing, which is instead lumped with metabolism and pathophysiology.

The discussion of blood pressure and hypertension in Chapter 4 follows the pattern of the two proceeding chapters. There is much material here which could have been safely deleted and replaced with more information on the laboratory testing. This chapter reflects more the clinical aspects of hypertension than the laboratory aspects. One is tempted to question the relevance of the inclusion of information on blood pressure measurements. Only about a page and a third is devoted to the laboratory assessment of hypertension but 4 full pages to drug treatment. This is a lop-sided emphasis of information that is less relevant to the laboratory scientist or doctor.

The authors would have done well in these chapters to follow the tone of Chapter 5, which deals with chest pain, including acute coronary syndromes (ACS). It describes the reasons for acute and chronic chest pains and the use of biomarkers in ACS. The interpretation of cardiac troponins levels for the diagnosis of different types myocardial infarction is discussed in excellent detail. The chapter also discusses the use of biomarkers such as fibrin degradations products in pulmonary embolism and aortic dissection. It elegantly describes the diagnosis of these conditions using biomarkers in conjunction with imaging and clinical parameters to arrive at a diagnosis.

The last chapter on cardiac failure deals with the pros and cons of the use of brain natriuretic peptide (BNP) in detail. As with the previous chapter, there is good interpretative information here. The authors speculate that multiple biomarkers will eventually be used to assess risk and guide management of heart failure.

Since some of the many abbreviations used in the book are not well known, having them listed with their full forms would have been helpful. It is odd that pages 30 and 64 are left blank. And, on page 91, there a probably typographical error where it states "six mRNAs" when the paragraph seems to imply "six MiRNAs" (micro RNAs), which is something quite different.

While the book does well to convey current knowledge, it does not live up to the expectation of the reader from the title. Throughout, greater emphasis should had been given to the laboratory tests, with separate sub-headings. Or perhaps, more accurately, the title should have read, "Cardiovascular Disease - Pathophysiology and Laboratory Medicine"!

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

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Book review: "Critical Care and Laboratory Medicine"

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REVIEWED BOOK

"Critical Care and Laboratory Medicine"
by Peter Gosling, Anne Sutcliffe, and Stephanie Dancer

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RECENSION

Care of the critically ill is a team effort where doctors and nurses heavily depend on laboratory measurements. The mortality rate of such patients is between 30 to 40%. The authors are, appropriately, an anaesthetist, a medical microbiologist and a clinical scientist, all of whom are key specialists that would make up the team. Their stated goal is to enable laboratory personnel to understand the contributions of the other team members.

The management of critical illness requires an appreciation of the physiological effects of the acute disease process, its ability to incite systemic inflammatory response, the needs of each affected organ, the impact and potential side-effects of interventions and the ability to adapt care to the individual patient's needs. The commonest cause of death in these patients is uncontrolled systemic inflammatory response syndrome (SIRS) leading to multiple organ dysfunction syndrome (MODS).

Most chapters provide an overview of the normal function of each organ, its pathogenesis and the essential role of the laboratory in understanding the process, the current strategies for the early identification of SIRS, the assessment of its severity, prevention,

diagnosis and treatment. The coverage of the book is comprehensive and includes the failure of the cardiovascular, respiratory, gastrointestinal (gut, liver and pancreas) and renal organs. The less common causes of admission into the intensive-care unit (ICU) such as failure of immunity, acute liver failure, acute pancreatitis, injury from burns and endocrine emergencies (such as diabetic ketoacidosis) also receive attention.

The importance of the microbiology laboratory is highlighted by way of a separate chapter that describes its support for the diagnosis, management and prevention of infection of patients in the ICU. As would be expected, routine tests that are associated with disease of the various organs or systems are described. For example, patients requiring intensive care are at risk of acute kidney injury (AKI). Its diagnosis and management are crucially dependent on serial measurements of the traditional analytes. In failure of the primary central nervous system, which is a common reason for admission to the ICU, the principle role of the laboratory in the investigation of the unconscious patient is to aid in the diagnosis of infective causes of primary brain injury. The metabolic, endocrine and toxicological causes of secondary brain injury and the relevant tests that need to be undertaken are also discussed. Stage III SIRS is associated with the failure of immunity, haemopoiesis and haemostasis. The clinical signs of these conditions when deranged are non-specific. To monitor these conditions and assess treatment, the routine laboratory investigations that are performed include haematological, coagulation, D-dimer and C-reactive protein measurements.

An attractive feature of the book is the attempt to keep the reader abreast with the newer tests and the current perspectives on regular tests. Several examples of this can be cited. Troponin I or T are not just specific and sensitive markers for myocardial damage but elevated levels in MODS are an independent risk factor for ICU mortality in patients without evidence of acute myocardial infarction. In AKI, new markers such as neutrophil gelatinase associated lipocalin (NGAL) have been proposed to enable early detection of the condition before functional abnormalities are manifest. While there are few circulating markers of brain injury or dysfunction, proteins such as the tau protein and neurone specific enolase have the potential to serve as circulating markers of brain injury. The markers of gut failure and the laboratory assessment of nutrition in critical illness are meant evaluate gut function and are important because of evidence that gut function and poor outcome are associated.

The book contains an index and a list of the abbreviations used, both of which are helpful. A succinct summary is provided towards the end of each chapter and this is followed by a short list of important references. There are elegant tables that illustrate and display key information. While two case studies are described, more would have enhanced the book. There is a moot point that may puzzle the attentive reader: both Stage 3 SIRS and stage III SIRS are mentioned. Are both forms of usage acceptable?

This book is devoted to an important group of patients where the support of laboratory medicine transcends its traditional boundaries. In most textbooks of laboratory medicine there is usually little mention support for the critically ill. As such, it should be an invaluable reference for anyone who is involved in the care of these patients.

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