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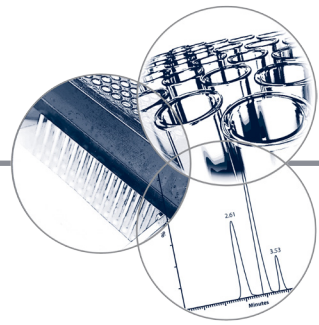

Immunogenicity and PK/PD evaluation in biotherapeutic drug development: scientific considerations for bioanalytical methods and data analysis

With the advent of novel technologies, considerable advances have been made in the evaluation of the relationship between PK and PD. Ligand-binding assays have been the primary assay format supporting PK and immunogenicity assessments. Critical and in-depth characterizations of the ligand-binding assay of interest can provide valuable understanding of the limitations, for interpreting PK/PD and immunogenicity results. This review illustrates key challenges with regard to understanding the relationship between anti-drug antibody and PK/PD, including confounding factors associated with the development and validation of ligand-binding assays, mechanisms by which anti-drug antibody impacts PK/PD, factors to consider during data analyses and interpretation, and a perspective on integrating immunogenicity data into the well-established quantitative modeling approach. Through recognizing these challenges, we propose some opportunities for improvements in the development and validation of fit-for-purpose bioanalytical methods.

Even though substantial advances have been made in the understanding of PK, PD and **immunogenicity** of **biotherapeutics** in the past decade, there are still several gaps that need to be addressed, especially in the context of new generation of novel constructs like bispecific modalities, antibody–drug conjugated (ADC) to small molecules or toxins and recombinant fusion proteins. The increasing complexity and novelty in the structure of biotherapeutics calls for a modification or redesign of the existing **ligand-binding assay** (LBA) formats. The evolution of biotherapeutics from partially human, chimeric, humanized and finally to fully human was intended to engineer molecules with closer resemblance to self-proteins that are more tolerant to immune responses. However, new aspects of impurities from cell lines or cell systems, such as *Escherichia coli* and yeast were also introduced with the innovative engineering. The desire for less-frequent dosing and high potency also led to the generation of high-concentration formulations, where the biotherapeutics could potentially conglomerate and form dimers, trimers or aggregates during long-term storage or during reconstitution from lyophilized forms. All of these aspects exacerbate the immunogenicity risk and, potentially, leading to reduced efficacy/exposure of biotherapeutics.

The immunogenicity to biotherapeutics measured as **anti-drug antibody** (ADA) response can impact the exposure (PK), response (PD) and drug safety (toxicity findings and adverse events). Furthermore, immunogenicity has been shown to be associated with a reduction or loss in efficacy; therefore it is a factor to consider in the clinical setting. LBA formats are usually employed to determine the level of biotherapeutics immunogenicity quantitatively or qualitatively. However, the reliable assessment of PK/PD and ADA can be influenced by the sensitivity, specificity, accuracy and precision of the bioanalytical assays, including the potential interferences by drug and/or immune reactive material present in the study samples. An increased understanding of the impact of ADA on PK and PD will enable an adequate dose selection for long-term toxicology studies, as well as first-in-human dosing. The prospective awareness of the doses where immune response can pose an obstacle, can be useful in selection of low or high doses that can reduce or overcome the unwanted response, respectively.

This review illustrates several key challenges with regard to understanding the ADA and PK/PD relationships, including the factors to consider during PK/PD data interpretation, the possible mechanisms by which ADA impact the

Yow-Ming C Wang[1], Vibha Jawa[2] & Mark Ma*[3]

[1]Office of Clinical Pharmacology, Office of Translational Sciences, Center for Drug Evaluation & Research, US FDA, MD 20993, USA
[2]Department of Clinical Immunology, Amgen, Inc., CA 91320, USA
[3]Department of Pharmacokinetics & Drug metabolism, Amgen, Inc., One Amgen Centre Drive, Thousand Oaks, CA 91320, USA
*Author for correspondence:
E-mail: mark.ma@amgen.com

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Key Terms

Immunogenicity: Ability of an antigen, can be the biotherapeutic, to elicit immune responses, which can be humoral and/or cell-mediated immune responses.

Biotherapeutics: Therapeutic material produced using biological means, including recombinant DNA technology. Most biotherapeutics are proteins that are engineered in the laboratory for pharmaceutical use.

Ligand-binding assay: Bioanalytical method for support of macromolecules in preclinical and clinical development, which relies on specific protein binding to the analyte of interest.

Anti-drug antibody: Antibody that specifically targets biotherapeutics

PK/PD, confounding factors associated with the development and validation of LBAs and considerations for quantitative model-based analysis of immunogenicity impact. The review also highlights the opportunity for improvements in order to develop and validate fit-for-purpose bioanalytical methods, while recognizing these challenges.

PK/PD evaluations & data interpretation

PK characteristics of biotherapeutics are often complex, involving linear and nonlinear processes. The nonlinear PK commonly involves target-mediated dispositions [1,2]. PD characteristics of biotherapeutics are also often complex involving multiple functional attributes of the molecular structure, such as Fab region of monoclonal antibody (mAb) for target engagement, FcγR binding site for antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), receptor internalization, and so on. While PK data combined with relevant PD data allow for the elucidation of exposure–response (E–R) relationships of desired effects; PK data combined with safety/toxicity data provides information on E–R relationships of undesired effects. In order to obtain E–R relationships that are useful and impactful for decision-making in development programs, two elements are essential. The first is the selection of clinically meaningful PD markers, because the resulting E–R relationships will have enhanced probability of being correlated with clinical outcomes [3,4]. The second is the selection of the most relevant bioanalytical assay that can produce concentration data reflective of the moiety(ies) associated with PD responses [5].

E–R relationships are often the foundation for decision-making at milestones of development programs. For instance, preclinical E–R facilitates the decision of advancing into clinical development and of the first-in-human dose selection; E–R data from Phase II dose-ranging studies facilitate the selection of dose(s) for confirmatory Phase III studies; the demonstration of E–R in pivotal Phase III studies can serve as the evidence of effectiveness [6]. Hence, characterizations of PK and PD properties are usually conducted over a wide range of doses in order to derive the E–R relationship.

Mechanisms behind immunogenicity of biotherapeutics

The immune responses to biotherapeutics can affect the overall exposure and/or toxicity findings in preclinical studies. Immunogenicity is monitored in most preclinical and clinical studies via the measurement of ADA [7]. It is well-recognized that immunogenicity of biotherapeutics can vary across products and across study populations, depending on many influencing factors, such as the product origin (e.g., foreign or endogenous), the route of administration [8], concomitant medications [9], patient-related factors such as genetic makeup, diseased state of the individual and immune-suppressed versus immune-reactive state of the individuals. The outcome of immunogenicity can also vary, ranging from little to no impact to serious health implications [10,11]. In instances where the impact of immunogenicity was not clearly delineated during clinical development, postmarketing studies may be conducted to provide further immunogenicity information following chronic dosing.

The humoral immune response to proteins is characterized by the generation of antibodies that could be T-cell-dependent or -independent. The T-cell-independent antibody responses may be generated when B-cells recognize a repeated pattern (motif) in the therapeutics and respond by transiently producing low-affinity, predominately IgM antibodies [12]. On the other hand, high-affinity antibodies generated in conjunction with T-cell help are referred to as T-cell-dependent or thymus-dependent antibodies.

The T-cell-dependent antibody response is an outcome of interplay of antigen presenting cells, T-cells, secreted cytokines and B-cells. The mature immune response to the exogenously administered biotherapeutics is also influenced by the genetic factors associated with each individual, such as HLA haplotype expression and T-cell/B-cell repertoire. The T-cell-independent low affinity responses are primarily observed within a couple weeks after the first dose of the biotherapeutics. Subsequently, chronic multiple dosing with the biotherapeutics can be associated with the T-cell-dependent antibody responses that can lead to a persistent memory cell response. Once the humoral response is triggered, the onset and magnitude of such a response can also differ, depending on the nature of biotherapeutics, the dose route and concentration as well as immune competency of the diseased individual. While very low and high doses might not be impactful, moderate doses might provide a consistent antigenic stimulus to mount a long-lasting response that matures

into long-term T-cell-driven B-cell response. The impactful antibody response can also be observed in biotherapeutics that have higher nonhuman antigenic epitopes. Such molecules can be associated with a fast onset and persistent responses that can impact exposure, compared with biotherapeutics that lack such nontolerant epitopes. Similarly, the immune state of an individual can influence the immune responses; especially true in immune compromised and/or suppressed individuals due to chemotherapy, concomitant medications such as steroids and other immunosuppressants that can modulate the immunogenicity of the biotherapeutics.

The magnitude of ADA as well as onset (when ADA is first observed) can impact the *in vivo* exposure and the efficacy of the biotherapeutics. The longevity of the response can also impact the overall exposure, especially if the ADA response matures from a binding response to a neutralizing response, due to affinity maturation or potential epitope spreading.

Bioanalytical approaches in PK & ADA assessment

The LBA format has been the gold standard for quantification of biotherapeutics and characterization of immune response to biotherapeutics [13–16]. With the advent of innovative technologies and novel reagents, LBA methods have evolved to overcome resource constraints and avoid extensive development for providing robust, sensitive and specific data [17–20]. Importantly, an increased rigor and depth of assay characterization allows for a more explicit understanding of the limitation of LBA and the associated data interpretation in support of biotherapeutic development.

Multiple assay platforms and formats are available for LBA development. However, selection of the most appropriate assay platform and format can still be challenging. Critical assay reagents, assay conditions and a combination of other contributing factors can manifest in bioanalytical error, misleading PK and ADA evaluation [15]. Hence, a thorough understanding of these challenges and designing proper experiments to assess the impact of the data is imperative. Such approaches would enable the choice of the most appropriate LBA for the pertinent question being posed.

■ Assays for immunogenicity assessment

The measurement of clinical and preclinical immunogenicity involves detection and characterization of pre-existing and treatment-induced ADA. Various analytical methods have been used to detect or monitor the presence of ADAs, including ELISA, radioimmunoassay or radioimmunoprecipitation assay, surface plasmon resonance, and electrochemiluminescence-based platforms [21–23]. The sensitivity and specificity of most of these technologies depend on the specific binding of the ADA to its target drug via the antigen binding site, that is, the idiotype or complementary-determining region. Due to this interaction, only the nondrug complexed ADA can be detected. The inability to detect ADA due to circulating therapeutic is one of the major concerns during ADA assessments in multiple dose clinical studies and in preclinical studies in general. Several approaches have been considered to reduce the effect of excess therapeutic on the ADA and PK LBAs, including the pretreatment of samples with mild acid to dissociate therapeutic-ADA immune complexes or with protein G/L depletion of serum to remove excess-free mAb-based therapeutic, or the use of platforms such as Gyrolab™ and ImmunoCAP®. The bridging-based Meso Scale Discovery® and Gyrolab platforms are designed to tolerate excess biotherapeutic interference caused by the incorporation of an acid dissociation pretreatment of the sample [24].

■ Assays for PK evaluation

To support PK, TK and PD analyses, the quantification of uncomplexed concentration of biotherapeutics is desirable. The term uncomplexed/free refers to the active form of the biotherapeutic where the functional region of molecule is not masked by interfering ligands such as ADAs, soluble forms of targets or receptors. An estimate of the uncomplexed form of the biotherpaeutic provides a true measure of the PK. The LBA can theoretically measure uncomplexed, complexed or total (i.e., the sum of analyte in uncomplexed and complexed forms) concentration of therapeutic mAbs depending on reagents, format and experimental conditions. Antigen-capture assay, bridging assay, anti-idiotipic antibody capture assay, generic assay using anti-Fc antibodies as a capture reagent [5] and competitive assay are the five most commonly used LBA formats [5,19,25–27].

For biotherapeutics, the immune-complex of the drug and ADA can exist *in vivo* through a noncovalent binding; therefore, a biomatrix sample may contain uncomplexed drug, drug–ADA complexes (e.g., mono- and/or

bivalent complexes for mAbs) or uncomplexed ADA. The drug–target complexes can also exist in the sample if the target is a soluble ligand or shed receptor. Although typically, the complex is very low due to the low endogenous levels of target/receptor. Unlike ADA–drug complex, the drug–target complex often only takes a small portion of the total therapeutics measurement, unless the target level is significantly increased in diseased or post-treatment subjects. In principle, LBA formats can be designed to measure all forms of drug using proper assay reagents. For data interpretation, ADA is differentiated into two categories: binding ADA and neutralizing ADA. For PK/PD evaluations, the primary interest is the non-neutralizing ADA-bound drug because it is the bioactive form with the assumption of drug–ADA complexes being inactive moiety. Therefore, the PK assay can be designed with employment of anti-idiotipic antibody or therapeutic target as a capture reagent (Figure 1).

Traditionally, the interference from ADA is evaluated as part of the specificity component of the PK assay validation. The experiment often involves spiking various levels of polyclonal ADAs into QC samples containing a range of known amounts of the biotherapeutic. Resulting biotherapeutic concentrations in spiked QC samples are subsequently compared with those of unspiked QC samples at the same concentrations to calculate the percentage reduction, which represents the impact of excess ADA on the PK assay.

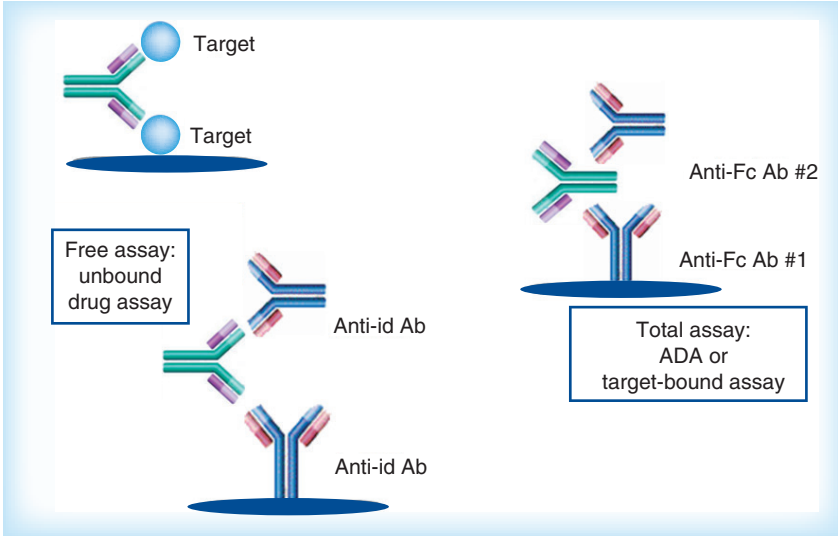

Figure 1. PK assay formats for quantification of uncomplexed and total biotherapeutics.
Ab: Antibody; ADA: Anti-drug antibody; id: Idiotipic.

Recently, novel biotherapeutics including bispecific mAbs, multispecific fusion proteins and mAbs conjugated with small-molecule drugs have entered the biopharmaceutical industry [28–30]. These novel constructs pose unique bioanalytical challenges. For instance, the nonself designs in these novel constructs and their complex mechanisms of action that involve bispecific molecular features, or involve both the small- and large-molecule components, pose increased immunogenicity risk. For instance, a potential exists for these product to have an increased immunogenicity risk attributed to their novel nonself design and complex mechanisms of action involve bispecific molecular features or involve both the small- and large-molecule components in the case of ADCs. A paradigm change in bioanalytical approaches is needed in terms of methodologies to deploy and address the complexity of mechanisms of action. A successful bioanalytical strategy will need to consider multiple assays to address distinct components of these novel constructs for epitope mapping of potential new nontolerant regions in the sequence-like sites where the linkers connect with conjugates such as toxins, peptides and so on. Extensive effort should be dedicated to thorough evaluations of potential interfering factors, such as ADA against or circulating targets for each structural component of the novel constructs. Designing fit-for-purpose method validation approach is imperative for efficient deployment of high performing assays supporting clinical and preclinical studies. [31,32].

Currently, there is no industry consensus or regulatory guidance regarding how to evaluate the impact of ADA on PK assays during assay validation. However, companies developing large-molecule bioanalytical methods should investigate ADA impact on PK bioanalytical assays during method validation. In principle, the fact that an ADA interferes with the PK assay would suggest that the PK assay reagents may not interact with the drug molecule in complex with the ADA, or interact with lower sensitivity. A quantitative understanding of this interaction has potential impact on PK data interpretations. The ADA impact should be assessed using the most appropriate surrogate ADA and performing relevant reagent-binding characterization. To enable the selection of the most relevant and appropriate positive controls, a wide range of positive controls that reflect both anti-idiotype and antiframework reactivity, both low- and high-affinity antibodies and with polyclonal

versus monoclonal specificity, should be considered during PK assay validation. A limitation of this approach, however, is that the interference will depend on the characteristics of the polyclonal ADA response, including the immunodominant epitope, which might be different in human subjects compared with the polyclonal standard. The *in vitro* evaluation may therefore not replicate ADA effects on drug disposition and clearance. For the bivalent or multivalent novel constructs, relevant controls against each functional domain are also needed during PK assay development for testing the relevant interference to each component.

However, given the complexity of the dynamic binding equilibrium occurring in the body, and multiple sources of perturbation of the equilibrium during bioanalysis, it is clear that *ex vivo* measured concentration of the particular forms of interest (uncomplexed, complexed or total drug) may differ from the actual *in vivo* concentration. To fully understand the differences between *ex vivo* and *in vivo* concentration, further experimental investigations may be necessary.

Additional challenges in evaluating the influence of ADA on PK assay remain, besides the potential for the lack of a relevant surrogate ADA to serve as a positive control. Such as: the capture reagent may compete with the ADA in an immune complex leading to the dissociation of the ADA–drug complex and an overestimate of uncomplexed drug concentration; and the assay incubation time and sample dilution procedures may also impact the complex stability. Therefore, early assessments using proper tools would help to understand the reported concentration data in terms of the uncomplexed drug, and facilitate appropriate use of the data by PK scientists in subsequent data analyses.

Immunogenicity: an important factor in clinical study & beyond

Immunogenicity data are collected from study subjects at multiple time points over the course of a study. Each of the study samples is evaluated for the presence of ADA. Typically, the sample collection schedule is designed with the consideration of the natural time course of an immune response, in addition to the logistics of study visits for other safety assessments. Because the IgM peaks at approximately 2 weeks and IgG at approximately 4 weeks after dose administration, 2- and 4-week postdose are two early time points commonly included. While the sampling frequency beyond a month is more variable across programs, quarterly scheduled assessment of ADA within the first year of treatment would provide useful insight into the immunogenicity profile of the product of interest. Although data interpretation can be aided by measuring both ADA and PK from the same time point, as mentioned previously, it would be important to understand the impact excess levels that either one has on their accurate measurement within their respective LBAs.

Because of the heterogeneity of ADA induced by the treatment of biotherapeutics, clinical impacts of ADA may vary widely and may require multiple assays to characterize. Figure 2 illustrated the heterogeneity with two classes of biotherapeutics, namely: mAbs and some enzyme-replacement therapeutic agents that rely on receptor-mediated cellular uptake to reach the target action site

In practice, such complex pictures of immunogenicity are often presented in a simplified format, such as a qualitative determination of positive or negative ADA, which is frequently accompanied by an inconclusive impact assessment. Therefore, further characterization of the magnitude of ADA, its isotype depicting its maturity, dissociation and association rates defining its affinity, and time of its onset can all be extremely helpful, not only for the interpretation of clinical outcome data, but also for the immune monitoring and management of patients who developed immunogenicity upon treatment. For instance, in the case of products with high immunogenic potential,

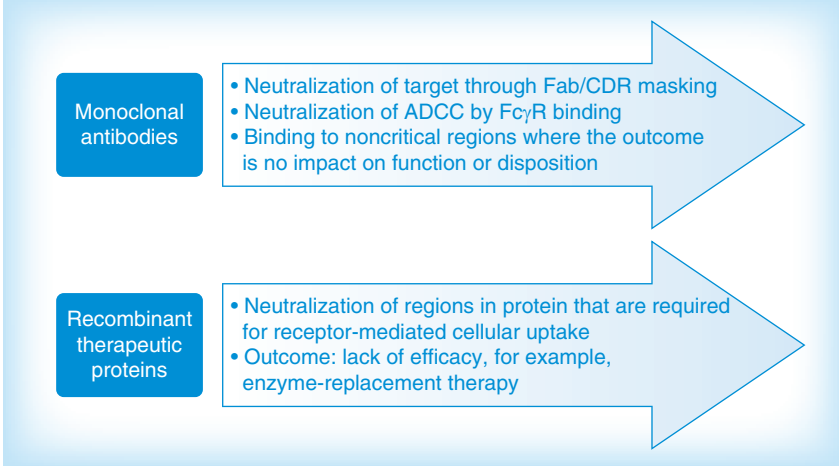

Figure 2. Heterogeneity of anti-drug antibody response and its impact on different modalities of biotherapeutics.
ADCC: Antibody-dependent cell cytotoxicity; CDR: Complementarity-determining region.

such as enzyme replacement therapeutic agents, immune-tolerizing regimens [33] are being explored or employed to maximize the treatment benefits in patients. In the case of anti-TNF mAbs, the development of ADA has been associated with the loss of efficacy, which led to either discontinuation of treatment or the switch to a different biotherapeutic agent [34–36].

Approaches used to evaluate impacts of ADA on PK, PD & efficacy

To assess the clinical impact of ADA, the timing of clinical assessment (e.g., PK, PD, efficacy and safety) in relation to the overall ADA sampling schedule is an important factor to consider at the stage of protocol design. In the premarketing clinical trials, the evidences of effectiveness are primarily based on the efficacy data collected at one particular time point defined for evaluating the primary endpoint, or in some cases at additional time points defined for evaluating the co-primary endpoint or the secondary endpoint(s). As such, the most common approach to assess the impact of ADA formation on efficacy is comparing the efficacy results in the subgroup with positive ADA (ADA+) to the subgroup with negative ADA (ADA-) [9]. For subgrouping the ADA status, it is appropriate to consider the overall ADA status throughout the study, including the time of the primary endpoint evaluation, because ADA+ observed at earlier time points prior to the time of the efficacy assessment can have impact on the overall efficacy. Therefore, a common practice is to derive one single ADA status from results of multiple ADA samples where a subject is deemed ADA positive when at least one of the ADA samples is found to be positive for ADA [9]. Similarly, the comparison between subgroups of ADA+ and ADA- subjects is the most common approach used to evaluate the impact of ADA on PK, PD and safety. There are caveats to this approach, as a transient ADA response might not be strong enough to impact efficacy, the magnitude of the response might be low; even if qualitative ADA result was positive, S/N or titer might be too insignificant [37,38]. Additionally, impact of ADA titers on PK, PD, safety and efficacy can also be considered. Evaluation of these endpoints in ADA+ subjects with titers above and below the median is one such approach. Given immunogenicity assays typically quantify the ADA titer in each sample to draw a conclusion on its categorical ADA status, the ADA titer data would logically be more informative than ADA status. However, ADA titer data are less commonly used for correlating with PK, PD or efficacy and further investigations would be useful.

However, alternate approaches can be utilized for evaluating the effects of ADA on PK, as PK measurements are often made at more than one time point during a trial. For some rare diseases where study population is limited, full PK profiles are collected after the first dose administration and again at steady state after multiple dose treatment in the pivotal trial. In such cases, a second approach could be conducting intra-subject comparison of PK data before and after the formation of ADA, in addition to the comparison of PK in ADA+ and ADA- subgroups. The current product labeling of idursulfase contains an example of such an approach, as it indicates the exposure was reduced or not measurable after repeated dosing. A few variations may be derived from this approach. For instance, the intra-subject comparison of PK data can be done based on the ADA titers, a continuous variable, instead of the dichotomous variable of ADA+ vs. ADA-. A more sophisticated variation of this approach, which demands higher intensity in data collection, is to use semi-mechanistic models that describe the temporal data on PK and ADA titers, where systemic exposure to a biotherapeutic is considered a driving force for the production of ADA and the ADA plays a role in the elimination of the drug (Figure 3). Such models are similar to PD-mediated or target-mediated disposition models, where drug exposure results in an increase in the production of targets, which then play a role in drug elimination [39,40].

A third approach may be considered for products that are indicated for the treatment of chronic diseases and involve a large population in the pivotal trials, where PK samples are collected in sparse manner, for example, multiple trough samples collected over time. In such cases, PK properties of the product are commonly described using population PK (PopPK) modeling approach, which allows exploration/evaluation of the impact of intrinsic factors (e.g., body weight, age and sex) and extrinsic factors (e.g., concomitant medications) on the PK properties. These intrinsic and extrinsic factors are typically incorporated into the PopPK model as time-invariant covariate. Inclusion of the ADA status (ADA+ vs ADA-) in the PopPK analysis as a covariate is a logical extension of the well-established technology, since immunogenicity resembles an intrinsic factor reflecting the potential for an individual to

mount an immune response to the exogenously administered biotherapeutics.

However, immunogenicity data differs from traditional intrinsic factors, such as body weight, age and sex, because normal subjects were ADA- at the start of the study before dose administration and developed ADA after treatment. The formation of ADA takes time, which can be variable from subject-to-subject and dependent on intrinsic biological factors. Therefore, treating ADA status as a time-invariant covariate in the PopPK models may not be appropriate and can lead to underestimation of the magnitude of ADA impact on PK. For example, infliximab PopPK analysis indicated that ADA+ subjects had a 24% higher clearance than ADA- subjects [41], whereas the infliximab product label in the US states that ADA+ subjects did not have measurable serum concentrations. As demonstrated in recent literature, it is technically feasible to implement ADA status as a time-varying covariate in PopPK modeling [38,42]. However, challenges exist in terms of assigning which PK samples are associated with positive ADA. This is because blood sampling for immunogenicity determinations is more sparse than blood sampling for PK evaluations in typical study designs; as such PK samples at some time points may not have accompanying ADA data to confirm the assignment of ADA status. Adopting a strategy to collect simultaneous samples for both PK and ADA assay may enable the use of ADA status as a time-varying covariate in PopPK analysis. However, a potential hindrance to this approach is the presence of high biotherapeutic concentration that can interfere with ADA detection/determination due to technical limitations of LBA [43].

Another more challenging issue with using PopPK approach is illustrated in the example of ADA formation rendering infliximab-treated subjects with no measurable infliximab serum concentration. When concentrations are not measurable or below the LLOQ of the LBA, they are traditionally set to zero value. Because PK data are customarily log-transformed for the PopPK modeling analysis, PK data points with zero values are customarily omitted in the analysis datasets with the exception of some cases where the first sample falling below the LLOQ was set to half of LLOQ. Therefore, besides addressing the issue of capturing the timing of ADA formation with simultaneous measurement of PK and ADA, technical improvements in PopPK modeling are probably required to face the scenario where immunogenicity reduces drug concentrations to not detectable/quantifiable.

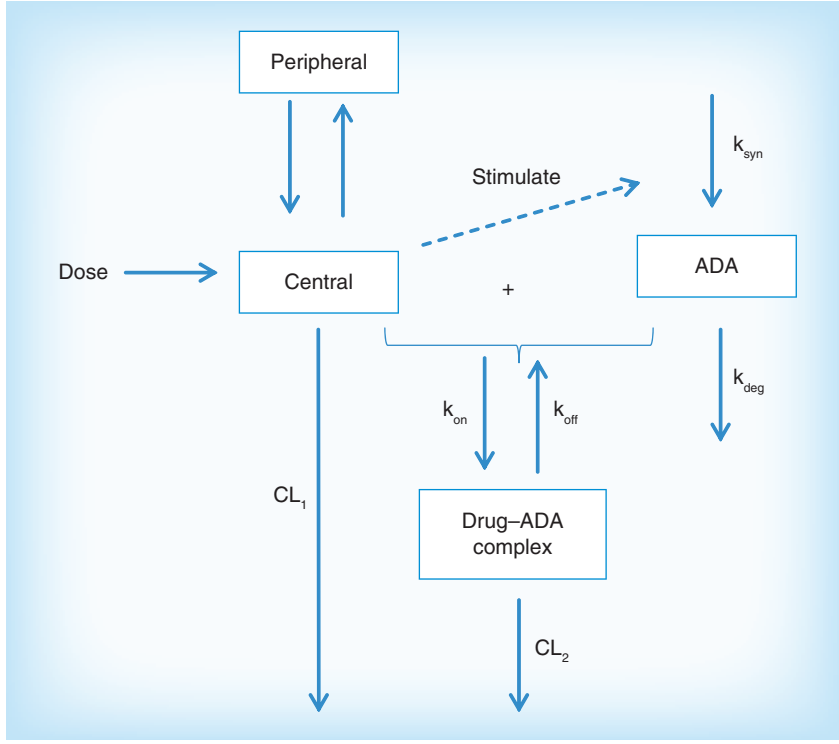

Figure 3. PK-immunogenicity model.
ADA: Anti-drug antibody; CL: Clearance; k_{deg}: Degradation rate constant; k_{off}: Dissociation rate constant; K_{on}: Association rate constant; k_{syn}: Synthesis rate constant.

In summary, a wide range of methods are available and/or have been applied to evaluate the impact of immunogenicity in preclinical and clinical studies. The choice of methods may depend on the study design with respect to immunogenicity sampling, PK sampling and clinical assessments. Depending on the data availability and the understanding of the pharmacology, model-based analysis may be feasible; however, results should be interpreted with an understanding of the potential methodological limitations. To conclude, the field of biotherapeutic development needs revisiting, related to the development and validation of PK assays, especially in the face of newer impediments such as innovative constructs. Such challenges have increased the risk of factors that can confound the PK estimations. Immunogenicity to biotherapeutics is one of the most obvious factors that needs to be integrated into the biotherapeutics development strategy. Specifically, the characterization of immune response for titers/magnitude, understanding epitope specificity, and evaluating the impact on drug exposures at early and late stage of immune

maturity can aid in PK/PD estimate assessments and for future study designs. With the increase in innovative constructs, the need for improved analytical assays and better interpretative models will also increase. This review provides an overview of the gaps and our insights on potential means to address them for successful development of biotherapeutics.

Future perspective

The understanding of impact of immunogenicity on drug levels, exposure and efficacy can be considerably enhanced with the advent of innovative LBA that use well-characterized antibody clones, platforms with improved drug tolerance and statistical models with an ability to predict immunogenicity and its outcome much before the onset of such a response. A prospective evaluation of the LBA assays for their ability to capture the bioactive drug would enable a more accurate estimation of drug exposure and efficacy. The limitations associated with interfering factors in LBA might be overcome by employing assay platforms such as high-throughput microfluidics, predictive integrated PK, ADA and PD models, eliminating the need for bioanalytical assessments.

Disclosure

This manuscript contains the scientific opinions of Y-MC Wang who is an employee of the US FDA; however, these opinions are her own and do not necessarily reflect the official views of the FDA.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

- Assessment of PK and PD properties requires good understanding of the mechanisms of action and PK dispositions as well as thoughtful design of bioanalytical methods.
- Immunogenicity is best determined through controlled clinical trials. Immunogenicity assays should be robust, sensitive, specific and validated. Furthermore, assays should be able to tolerate the drug concentrations expected during treatment.
- The quality of bioanalytical data generated by anti-drug antibody and PK assays relies on a careful consideration of the fit-for-purpose assay design and thoughtful method validation.
- Evaluation of PK and immunogenicity of novel modalities with multiple epitope specificities will require multiple assays for assessment of individual components.
- The use of anti-drug antibody titer data, instead of categorical anti-drug antibody status, and the use of model-based analysis (e.g., population PK analysis) are alternative approaches that are less mature.

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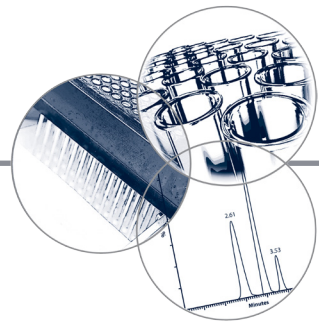

An ultrasensitive method for the quantitation of active and inactive GLP-1 in human plasma via immunoaffinity LC–MS/MS

Background: Measuring endogenous levels of incretin hormones, like GLP-1, is critical in the development of antidiabetic compounds. However, the assays used to measure these molecules often have analytical issues. **Results:** We have developed an ultrasensitive, highly-selective immunoaffinity LC–MS/MS (IA LC–MS/MS) assay capable of quantitating endogenous levels of active (7–36 amide) and inactive (9–36 amide) GLP-1 in human plasma. We performed fit-for-purpose validation of the assay by assessing the following assay performance characteristics: inter-assay precision, sensitivity, spike recovery, dilution linearity, absolute recovery, matrix effect, immunoprecipitation efficiency, and food effect. **Conclusion:** We have developed a robust analytical method for the quantitation of endogenous active and inactive GLP-1 in human plasma. In addition, we employed this method to measure the typical changes in GLP-1 levels after food intake. The sensitivity of this assay is better than another LC–MS/MS GLP-1 assay previously reported and many commercially available immunoassays. This important analytical tool could be used to qualify and/or harmonize the different immunoassays used for the quantitation of GLP-1.

GLP-1 is part of a family of incretin hormone peptides. It is secreted from endocrine cells located in the intestinal mucosa in response to the presence of nutrients in the gut lumen [1]. It is a potent regulator of glucose homeostasis by enhancing glucose-dependent insulin secretion [2]. GLP-1 is derived from the proglucagon gene, which is post-translationally processed in a tissue-specific manner into several different peptide products including: glucagon, GRPP, major proglucagon fragment, glicentin, oxyntomodulin, GLP-1, GLP-2, IP-1 and IP-2 [3,4].Once in circulation, two NH2-terminal amino acids of **active GLP-1** are rapidly cleaved by dipeptidyl peptidase IV (DPP-IV) into inactive GLP-1, resulting in a half-life of approximately 1–2 min for active GLP-1 [5]. Because of their ability to extend the biological effects of incretin hormones such as GLP-1, orally-administered DPP-IV inhibitors have been developed and utilized in the clinic as a novel class of antihyperglycemic agents for the treatment of Type 2 diabetes [3].

Measuring endogenous levels of incretin hormones like GLP-1 is critical for the development of antidiabetic compounds. However, the assays employed in the measurement of these molecules often have analytical issues [3,6]. For instance, GLP-1 is present at very low concentrations (<10 pM) in human plasma and, is a hydrophobic peptide with a very short half-life in circulation, both of which contribute to sensitivity and stability challenges [7]. There are currently a few immuno-based assays for the measurement of **incretins**. However, because of the complexity of proglucagon biology as referenced above, the selectivity of these methods has been questioned. To that end, here we demonstrate the development of an ultrasensitive, highly selective **immunoaffinity LC–MS/MS** (IA LC–MS/MS-based platform to enrich and measure endogenous levels of active (7–36 amide) and inactive (9–36 amide) GLP-1 in human clinical plasma samples.

Materials & methods

Reference materials

Human active GLP-1 (7–36) amide and human inactive GLP-1 (9–36) amide standards were purchased from Anaspec (Fremont, CA, USA). The IS, stable isotope-labeled GLP-1 (7–36) amide and inactive GLP-1 (9–36) amide, were obtained from Sigma-Aldrich (St. Louis, MO, USA), with each IS containing one phenylalanine and one leucine, labeled with ^{13}C and ^{15}N, resulting in a mass shift of 16 amu, which provides adequate separation (+4 amu at a +4 charge state) from the unlabeled molecules.

Reagents

The following critical reagents were obtained for the development of the assay: Water and

Derek L Chappell[1‡], Anita YH Lee[2‡], Jose Castro-Perez[2], Haihong Zhou[2], Thomas P Roddy[2], Michael E Lassman[1], Sudha S Shankar[3], Nathan A Yates[2,4], Weixun Wang[2] & Omar F Laterza*[1]

[1]Clinical Development Laboratory, Merck Research Laboratories, Rahway, NJ, USA
[2]Molecular Biomarker Laboratory, Merck Research Laboratories, Kenilworth, NJ, USA
[3]Experimental Medicine, Merck Research Laboratories, Rahway, NJ, USA
[4]Current affiliation: Biomedical Mass Spectrometry Center for the Health Sciences, University of Pittsburgh, Pittsburgh, PA, USA
*Author for correspondence: E-mail: omar_laterza@merck.com

[‡]These authors contributed equally

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Key Terms

Active GLP-1: Predominant circulating biologically active form of GLP-1 [GLP-1(7–36) amide] associated with insulin secretion and glucagon inhibition.

Incretins: Gastrointestinal hormones that regulate insulin release as a result of food uptake.

Immunoaffinity LC–MS/MS: Using an antibody to enrich a target analyte from a complex sample matrix prior to MS analysis.

Fit-for-purpose validation: Efficient validation approach in which an assay is validated based on how the data will be generated and the intended application.

acetonitrile were purchased from Honeywell Burdick & Jackson (Morris Township, NJ, USA), Formic acid from Thermo Pierce Scientific (Rockford, IL, USA), RIPA buffer from EMD Millipore (Billerica, MA, USA), Tosylactivated M-280 Dynabeads from Life Sciences (Grand Island, NY, USA), anti-GLP1 monoclonal antibodies (HYB147–06 and HYB147–13) purchased from Thermo Pierce Scientific, a rabbit polyclonal antibody against GLP-1 obtained from Bachem (King of Prussia, PA, USA) and an anti-active-GLP1 monoclonal antibody (ABS033–04) from Enzo (Farmingdale, NY, USA).

■ Assay procedure

Anti-GLP1 monoclonal antibodies were conjugated to Tosylactivated M-280 Dynabeads according to the manufacturers' instructions and stored at 4°C until ready for use. All plasma samples were collected in P700 or P800 vacutainer tubes (BD Biosciences, CA, USA), immediately processed for plasma and stored at -80°C. Samples were thawed on ice prior to analysis. Samples were transferred to a 2 ml Protein LoBind Tube (Eppendorf®) and the volume was adjusted to 1 ml with 1×RIPA buffer. IS was added to a final concentration of 40 pM in sample. Anti-GLP1 antibodies (HYB147–06 and HYB147–13) conjugated beads were mixed in equal volumes, and 50 µl of mixed beads were added to each plasma sample. Samples were incubated for 1 h at room temperature with end-over-end rotation. After the incubation and with the aid of a magnetic device, the beads were washed twice with 2 ml of 1×RIPA buffer and twice with 2 ml of water. GLP-1 peptides bound to the beads were eluted with 30 µl of elution buffer (400 µg/ml BSA in 20% acetic acid, 10% methanol) for 15 min at room temperature with shaking. Eluates were spun over an Ultrafree®-MC 0.22 µm PVDF filter (Millipore, MA, USA) and analyzed by LC–MS/MS (Figure 1).

■ Chromatography

Chromatographic separation was performed using a nanoACQUITY UPLC® system (Waters, Hertfordshire, UK). The analytical column was a TRIZAIC nanoTile™ C18 85µm × 100 mm column with a 1.8 µm particle size and onboard trap, and maintained at 45°C. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B consisted of acetonitrile with 0.1% formic acid. A sample volume of 2 µl was injected and loaded for 2 min onto the trapping column at a flow rate of 8 µl/min 15% B. Following the trapping cycle the elution gradient occurs as follows: 15% B at t_0, ramp to 45% B from t_0 to $t_{3.0}$, ramp to 60% B from $t_{3.0}$ to $t_{5.0}$, ramp to 95% B from $t_{5.0}$ to $t_{7.0}$ and hold for 2 min before returning to 15% B at a constant flow rate of 1 µl/min. The retention time for active GLP-1 and IS was approximately 4.47 min, and the retention time for inactive GLP-1 and IS was approximately 4.64 min (Figure 2). The cycle time of the method and injection was approximately 15 min per sample.

■ MS

The mass detector was a Waters Xevo™ TQ-S triple quadrupole mass spectrometer fitted with a TRIZAIC nanoTile source (Waters). The mass spectrometer was operated in MRM mode. Samples were ionized via ESI in positive mode. The source was set at a temperature of 120°C. Ionization source parameters were as follows: capillary voltage 3.0 kV, cone 40.00 V, source offset 60.0 V. Gas settings were as follows: nanoflow gas 0.70 bar, collision gas flow 0.15 ml/min, nebuliser gas flow 7.00 bar. Dwell time per transition was set to auto (42 ms). Resolution settings were low mass 2.8 and high mass 15.0 for both Q1 and Q3. The following MRM transitions were monitored with collision energies given in parenthesis: active GLP-1 *m/z* 825.4→946.3 (30 V); active GLP-1 IS *m/z* 829.5→946.3 (30 V); inactive GLP-1 *m/z* 773.3→852.5 (21 V); inactive GLP-1 IS *m/z* 777.3→857.6 (21 V). Data was processed using TargetLynx™ software (Waters). A calibration curve was generated for both active and inactive GLP-1, each run by plotting response (defined in TargetLynx software as the peak area ratio of analyte to IS multiplied by the designated calibration concentration for each point) versus the concentration of analyte (pM). Concentrations of unknown samples were plotted against the known standard curve to determine nominal concentrations.

■ Method optimization

Four antibodies were evaluated for use in the immunoprecipitation (IP) step of the assay. The two mouse monoclonals (HYB147–06 and HYB147–13) along with the rabbit polyclonal, bind the two peptides of interest, active GLP-1 (7–36) and inactive GLP-1 (9–36), while the fourth antibody tested (ABS033–04) only binds the free *N*-terminus of GLP-1 (7–36). Antibody selection was made based on the Octet® RED96 System platform (Pall Corporation, NY, USA). The Octet Red system uses bio-layer

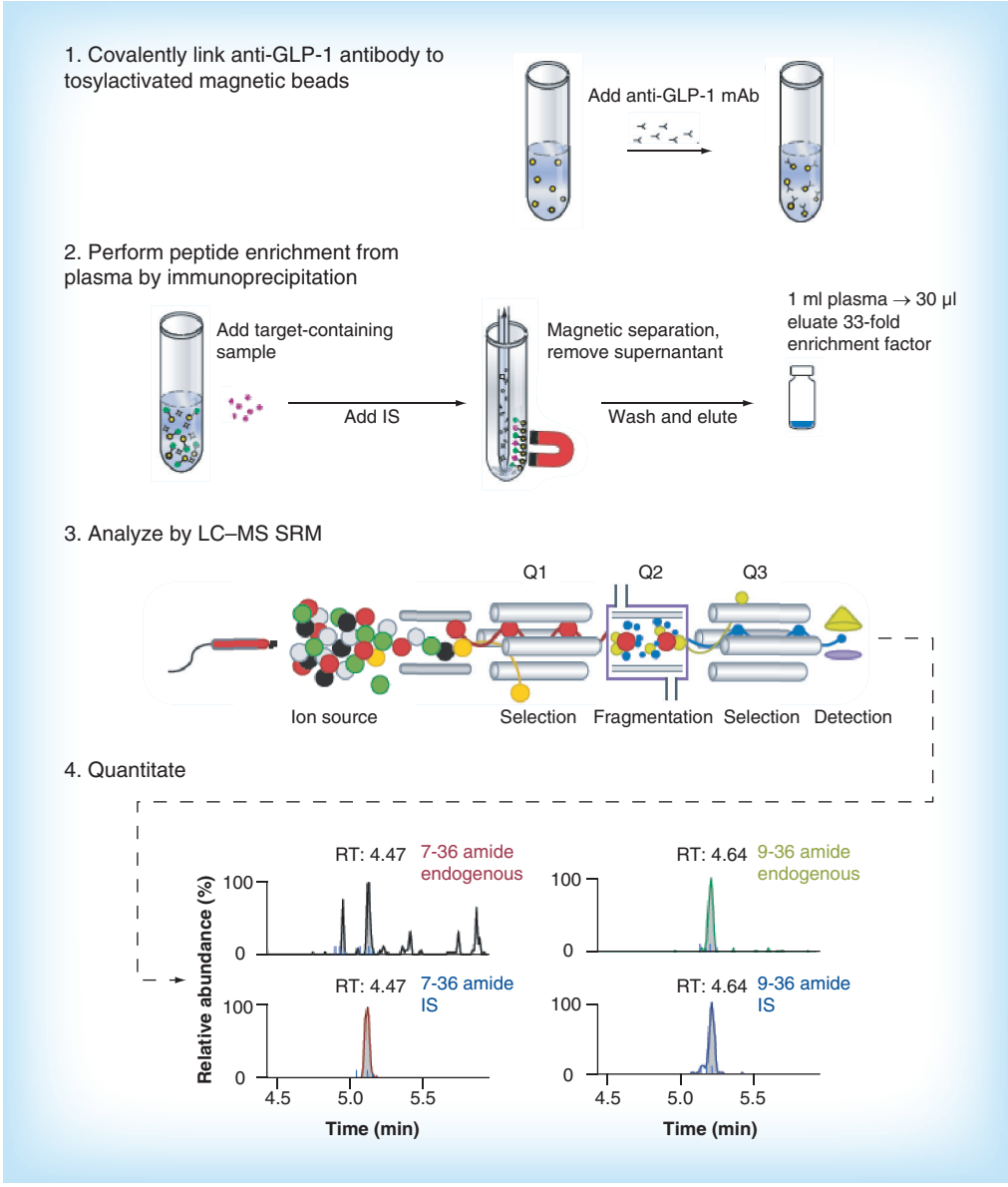

Figure 1. Immunoaffinity-LC–MS/MS-based assay platform to measure endogenous levels of active (7–36 amide) and inactive (9–36 amide) GLP-1.
mAb: Monoclonal antibody; RT: Retention time.

interferometry, a label-free biosensor technology that gives real-time information regarding protein interactions in a fluidics-free instrument environment. Briefly, streptavidin-coated tips that house the biosensor required for this measurement, are dipped into wells containing the biotinylated analyte of interest (biotin-GLP-1 7–36, and biotin-GLP-1 9–36) during the 'load' step. After a baseline equilibration step, the tips are dipped into wells containing the desired antibody to measure the association rate. Finally, the dissociation rate can be measured by dipping the tips into a buffer of choice. For the final step we chose our IP wash buffers (1×RIPA and H_2O) to confirm a lack of dissociation during these wash steps, of which there was none.

■ Assay validation

The following assay performance characteristics were assessed as part of the **fit-for-purpose validation** of active and inactive GLP-1 assay: inter-assay precision, sensitivity, spike recovery, dilution linearity, absolute recovery, matrix effect, IP efficiency and food effect. Three QC samples with varying active and inactive GLP-1 levels were prepared for assay validation

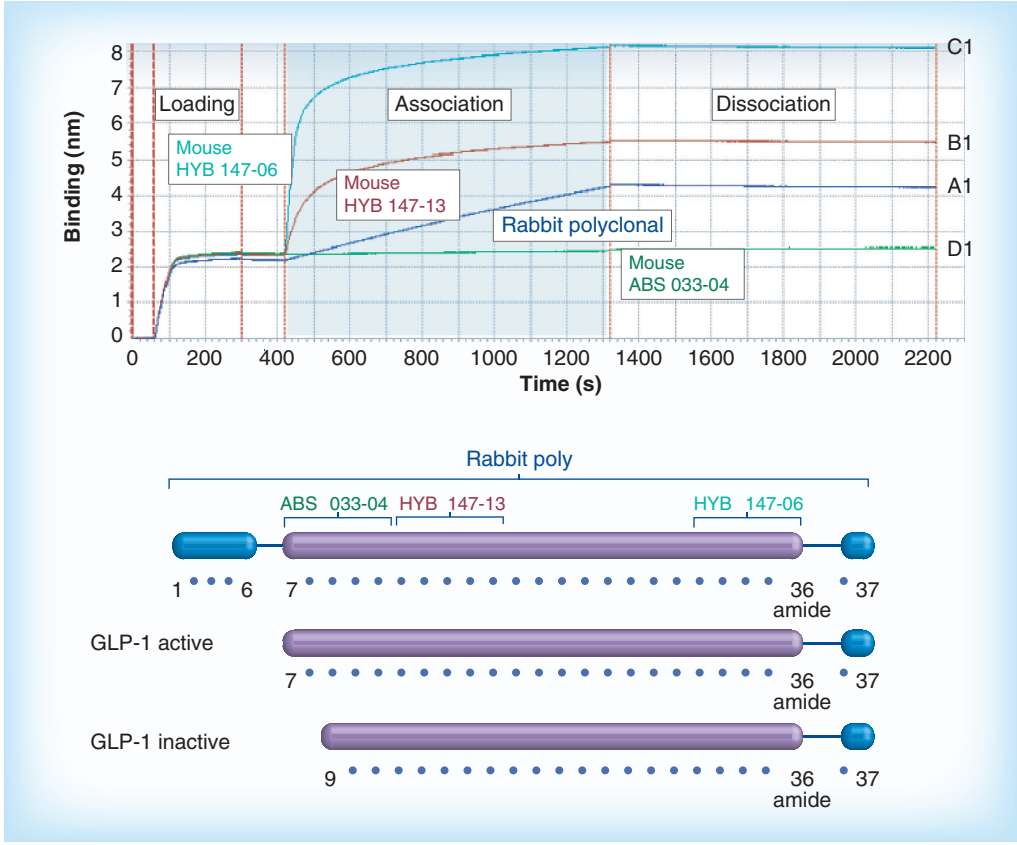

Figure 2. Antibody selection process using the Octet® instrument. The biotinylated protein of interest is first loaded onto strepavidin-coated tips ('loading'). The tips are then dipped into each antibody during the 'association' step, followed by buffer during the 'dissociation' step. The bottom part of the figure shows the approximate binding sites of the different antibodies tested.

purposes: low, medium and high. The QC samples were prepared using pooled plasma collected in p700 tubes from healthy volunteers. GLP-1 was spiked into the medium and high QC samples to achieve the desired levels. Inter-assay precision was determined by measuring the QC samples over n = 4 runs. Sensitivity, as defined by LOQ, was calculated based on precision and bias of the lowest standard concentration. Spike recovery was determined by spiking two levels of active and inactive GLP-1 (50 pM and 200 pM) into plasma samples and comparing the GLP-1 concentrations in spiked samples versus the unspiked samples. Total assay recovery was determined by comparing the IS peak areas in samples spiked with IS at the beginning of the assay procedure versus the peak areas of IS in buffer. Matrix effect was determined by spiking IS into the elution buffer of processed plasma sample that contained no IS initially. The IS peak areas of this sample were compared with the IS peak areas found in the elution solution containing IS. IP efficiency was determined by comparing the IS peak areas in samples spiked with IS prior to the IP step versus samples spiked with IS after the IP step. Dilution linearity was measured by serially diluting the Medium QC sample with 1×RIPA buffer from 1:2 to 1:32.

■ Clinical assay application

To demonstrate the utility of this method in the clinical setting, we chose to measure samples from a food effect study. GLP-1 is associated with appetite and satiety signaling, gastric secretion inhibition and delayed gastric evacuation. An increase in GLP-1 release can be stimulated by overfeeding [8]. Food effect was assessed in six individuals by measuring samples collected before and after a meal tolerance test. Specifically, subjects had their plasma drawn (t_{-10min}) and were administered the test meal, which consisted of one can of Ensure® and one Power bar. They were instructed to complete

consumption of the entire meal within 15 min, while consuming the last portion of the meal within the last 2 min. Plasma was then collected after the 15 min had expired (t_{0min}) and at the following intervals for the next 3 h: t_{10min}, t_{15min}, t_{30min}, t_{60min}, t_{90min}, t_{120min} and t_{180min}. All protocols underwent Ethics Committee review and approval, and all subjects provided informed consent prior to undergoing study procedures and activities.

Results

Two monoclonal antibodies (HYB147–06 and HYB147–13) with high affinity and low dissociation properties were selected for the immunoenrichment step (FIGURE 2). The LOQ of the LC–MS/MS assay was determined to be 0.5 pM based on a %CV <20% and a %bias <5% of the lowest standard. The S/N ratio for the low QC was approximately 70 and 837 for active and inactive GLP-1, respectively. A standard curve

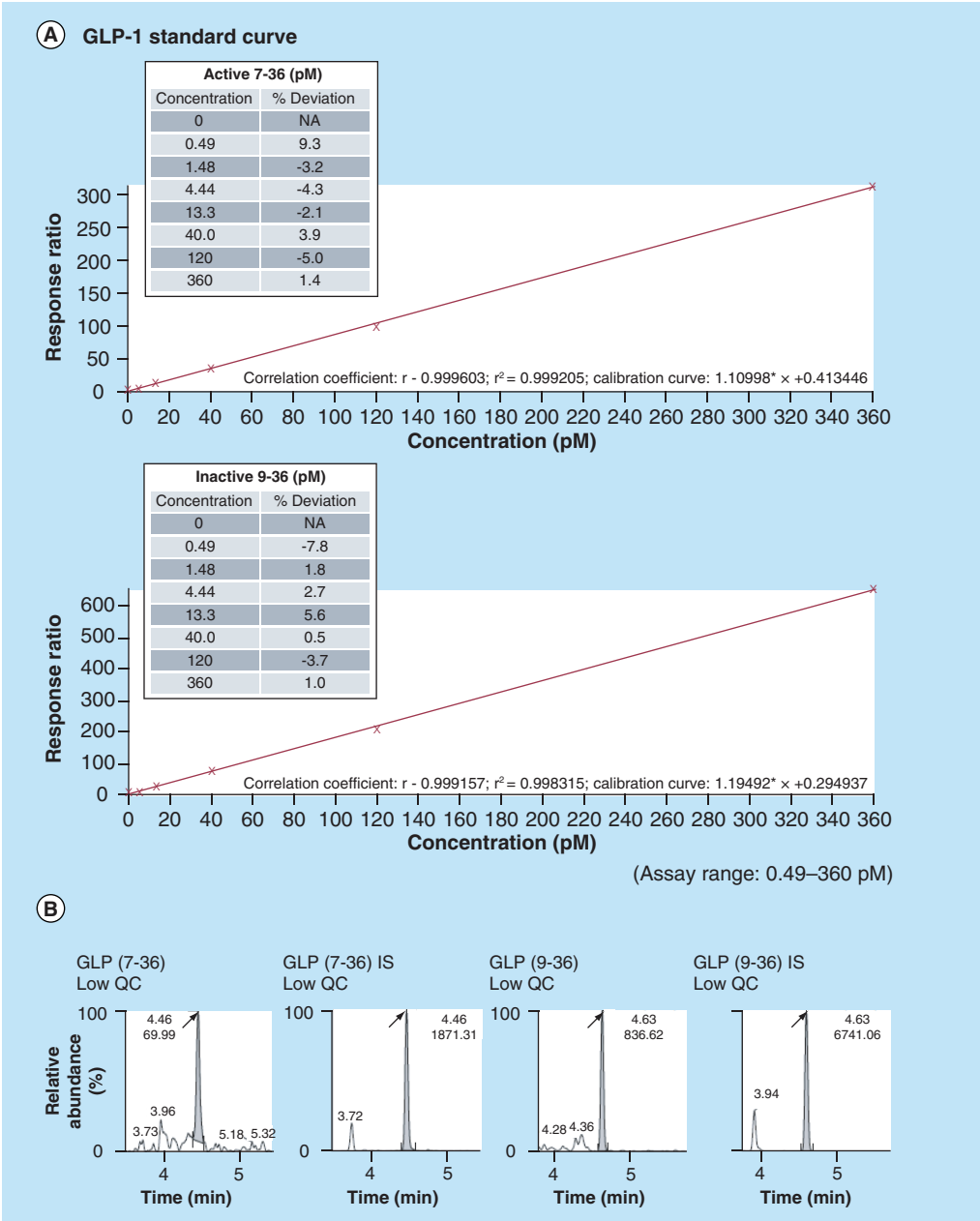

Figure 3. Standard curve and representative chromatograms. (A) Representative standard curves for active (7–36 amide) and inactive (9–36 amide) GLP-1 ranging from 0.49–360 pM. **(B)** Chromatograms from the low QC sample are shown for both active (7–36 amide) and inactive (9–36 amide) GLP-1, along with their respective IS. Each chromatogram lists the analyte along with retention time and S/N ratio.

Table 1. Inter-assay precision in human plasma.

	Active GLP-1 (7–36)			Inactive GLP-1 (9–36)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
Mean (pM)	1.5	55.5	110.8	3.1	55.6	110.7
%CV	9.2	6.7	8.8	9.9	11.6	11.1
N	4	4	4	4	4	4

representing a range of 0.49–360 pM for both active and inactive GLP-1 is shown in Figure 3A, along with representative chromatograms from the low QC sample (Figure 3B). Inter-assay precision ranged from 6.7 to 9.2% and 9.9 to 11.6% for active and inactive GLP-1, respectively (Table 1). Spike recoveries for the low and high spike levels were calculated to be 111 and 113% for active GLP-1, and 110 and 113% for inactive GLP-1 (Table 2).

Total assay recovery of active and inactive GLP-1 was determined to be 48 and 37%, respectively. The matrix effect for active and inactive GLP-1 was determined to be -36 and -52%, respectively. IP efficiency of active and inactive GLP-1 was determined to be 75 and 76%, respectively. The assay was observed to be linear up to at least a 1:32 dilution (Table 3). Endogenous GLP-1 concentrations from 6 individuals ranged from <0.5(LOQ) to 1.5 pM for active, and between 0.5 and 3.9 pM for inactive. The results of the food effect experiment are shown in Figure 4. An increase is observed in both active and inactive GLP-1, with a peak effect occurring 10 min after completion of the meal.

Discussion

Lack of concordance and/or standardization in GLP-1 measurements has been previously investigated [9]. Specifically, the need for an internationally recognized GLP-1 standard, as well as the importance of characterizing the specificity of commercially available immunoassays was highlighted [9]. Some of the issues raised were that commercially available GLP-1 assays do not necessarily give results equal to an extensively documented method as developed by Orskov *et al.* [10].An extensive comparison of commercially available methods for detection of GLP-1 by Bak [11] highlights many of the challenges related to GLP-1 measurements. Assay sensitivity and the way it was determined varied depending on the vendor. Sensitivity as described in a commercial kit insert may be based on the signal of the background, signal of analyte in buffer or signal of analyte in sample matrix and, most likely, will not factor precision into the estimation. All nine kits tested had at least one of the following analytical issues: insufficient sensitivity in plasma, plasma interference/matrix effect or poor spike recovery [11]. None of the kits tested was able to measure spiked concentrations of active and inactive GLP-1 in plasma below 10 pM while demonstrating acceptable spike recovery (80–120%), a clear example for the need of improved assay platforms to measure GLP-1 in human plasm [11].

To our knowledge, this is the first LC–MS/MS assay that has been developed and validated with the required sensitivity to measure endogenous levels of active and inactive GLP-1 in human plasma and it is our intention that this method could aid in the harmonization or characterization of GLP-1 assays. There are many components of this assay which, when combined, result in a highly selective and sensitive tool for the analytical characterization of GLP-1. The three key elements that result in better sensitivity and selectivity are: the enrichment of the analytes from a complex matrix through the use of monoclonal antibodies specific to GLP-1; the specificity afforded by

Table 2. Spike recovery for low (50 pM) and high (100 pM) concentrations of active and inactive GLP-1 in control human plasma.

	Active GLP-1				Inactive GLP-1			
	Endogenous concentration (pM)	Spiked concentration (pM)	Spike amount (pM)	Recovery (%)	Endogenous concentration (pM)	Spiked concentration (pM)	Spike amount (pM)	Recovery (%)
Low spike	1.4	57.0	50	111	3.1	57.9	50	110
High spike	1.4	114.8	100	113	3.1	116.1	100	113

Table 3. Dilution linearity in human plasma.

Dilution factor	Active GLP-1		Inactive GLP-1	
	Corrected concentration (pM)	Change from undiluted (%)	Corrected concentration (pM)	Change from undiluted (%)
Undiluted	59.9	NA	64.3	NA
2	60.3	0.7	61.0	-5.2
4	60.5	1.0	65.4	1.6
8	58.1	-3.1	66.8	3.9
16	44.2	-26.3	64.6	0.5
32	49.0	-18.3	65.6	2.0

the mass spectrometer to reliably quantitate both active and inactive forms of GLP-1 in the same sample; and the sensitivity of advanced MS instruments to reproducibly measure the samples in the sub-pM range.

The use of antibodies to enrich an analyte of interest prior to MS analysis has been extensively documented for a variety of applications [12–14]. Similar to immunoassays, IA LC–MS/MS benefits from utilizing specific antibodies with high association rates and low dissociation rates. The experiments performed on the Octet system confirmed the selection of two monoclonal antibodies based on favorable binding kinetics. Incubation times for the immunocapture step vary and are performed overnight in many cases. Based on the antibody affinity data and tight inter-assay precision we observed, the decision was made to perform the immunocapture step for 1 h, which results in an assay with quick turnaround time. The assay, including sample addition, immunocapture and elution can be performed in approximately 2 h. This is a significant improvement considering existing total GLP-1 immunoassays can last up to 4 days. Beyond the relatively short assay time, one of our future goals is to automate the assay in a 96-well plate format to increase throughput and make the assay easily transferable to partner laboratories. After

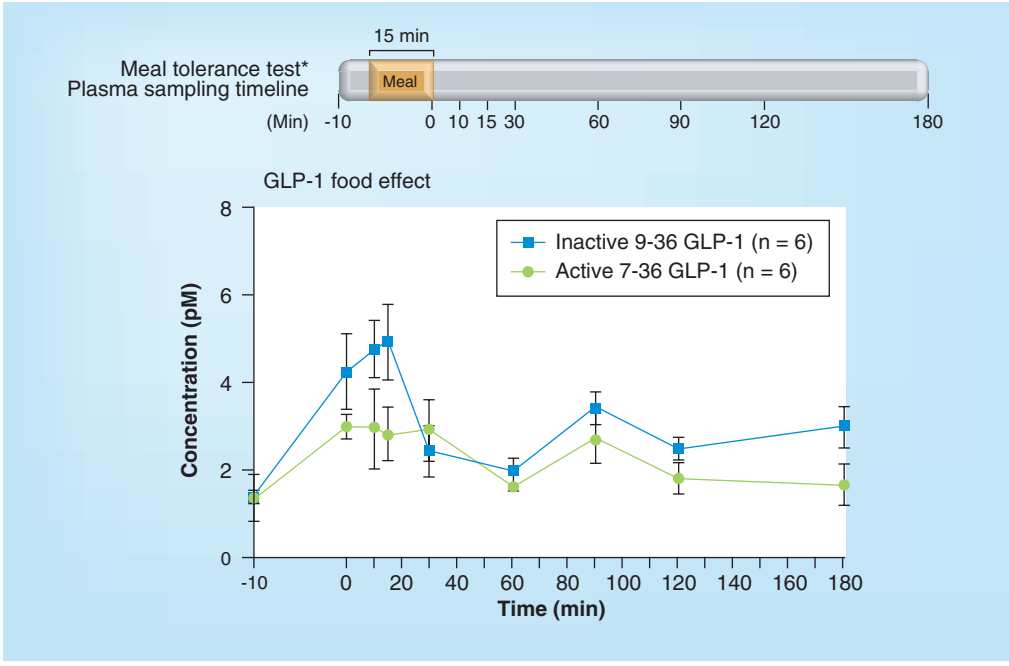

Figure 4. GLP-1 food effect measured by immunoaffinity-LC–MS/MS. Following an overnight fast, subjects (n = 6) were administered a test meal (one can of Ensure® and one power bar) and instructed to complete consumption of the entire meal within 15 min. Plasma was sampled at t = -10 min (prior to meal challenge), 0, 10, 15, 30, 60, 90, 120 and 180 min following the completion of the meal.

the antibody selection and conditions for the immunoaffinity step were optimized, the authors focused on adapting the method to fit the most sensitive robust detection platform available to them. In this case, we opted for the TRIZAIC nanoACQUITY UPLC® system with nanoTile™ coupled to the Xevo™ TQ-S. Nanoflow LC is often selected as the front end to provide chromatographic separation in the quantification of extremely low abundance analytes, because of the optimal ionization efficiency and low background. While producing relatively clean spectra with low solvent background, nanoflow techniques are usually hampered by long gradient times and can be difficult to achieve consistent peak shapes and reduced dead-volumes, due to the very delicate columns and fittings. Others have demonstrated the TRIZAIC microfluidic system to be more robust and stable than traditional micro- and nano-flow separation devices [15]. In addition, for selected analytes, we have demonstrated a >60-fold increase in sensitivity with the TRIZAIC system compared with traditional 2.1 mm columns on traditional flow sources [16]. The TRIZAIC nanotile system proved to be relatively easy to use because of its plug-and-play interface. By using the trizaic tile with onboard trapping, we were able to keep our gradient times relatively short (9 min), while maintaining low background and great resolution.

In contrast with previous immunoassays cited in the literature, we have demonstrated a robust quantitative measurement in plasma at the low-pM level, along with acceptable spike recovery (110–113%) with two different levels of analyte. The suboptimal analytical performance historically associated with the majority of GLP-1 assays can be attributed to problems with immunoassays in general such as: lack of concordance in measurement of the same analyte via different methodologies (kits, standards and platforms), production of autoantibodies that may block the epitope on the analyte of interest, antireagent antibodies and the hook effect [17]. Based on these potential pitfalls, we targeted an IA LC–MS/MS method to obtain the specificity required for accurate measurement of GLP-1. Many properties of IA LC–MS/MS are the same as immunoassays, with the added benefit that a mass spectrometer provides an extra level of selectivity due to the specific precursor-product ion fragmentation patterns for any given molecule and the ability to concentrate the sample for increased sensitivity. IA LC–MS/MS is also a preferred option when only one antibody reagent is available or multiple antibodies compete for the same epitope. Wolf *et al.* previously developed an IA LC–MS/MS method to measure glucose-dependent insulinotropic peptide (GIP1–42, GIP3–42), along with active and inactive GLP-1 [18]. This was a novel assay for the time and a great early application for IA LC–MS/MS, however, the LOQ of the assay (11 pM) was not low enough to reliably measure endogenous levels of active GLP-1 in human plasma. In comparison, the present assay is approximately 20-fold more sensitive (0.5 pM vs 11 pM). In addition to increased sensitivity, our assay has the advantages of a shorter gradient time, 9 min compared with 35 min, increased specificity resulting from the decision to use monoclonal antibodies and MRM over polyclonal antibodies and a single quadrupole mass spectrometer, and finally improved chromatographic separation and resolution [6]. The precision, linearity, and spike recovery data presented here for active and inactive GLP-1, validate this method as a sensitive and specific platform.

In addition to specificity and sensitivity, another advantage of the assay is the ability to multiplex the measurement of active and inactive GLP-1 from the same sample. This is significant if one is making decisions based on ratio of active to inactive or active to total GLP-1. This ratio has been thoroughly characterized in the development of DPP-IV inhibitors. As described earlier, active GLP-1 is quickly cleaved by DPP-IV, resulting in the inactive form 9–36. Therefore, inhibition of DPP-IV should result in an increase of active GLP-1, and account for a greater percentage of total GLP-1 measured. This has been previously demonstrated by Herman *et al.* for the development of Januvia®, a DPP-IV inhibitor for the treatment of diabetes [19]. Active and total GLP-1 measurements, as performed by ELISA and radioimmunoassay, respectively, demonstrated a significant increase for all doses of sitigliptin (Januvia) over placebo for the active:total ratio, with no impact on total GLP-1 levels [19]. It is also worth noting that lot-to-lot variability was observed for the measurement of active GLP-1, an issue that tends to be more problematic in immunoassays than in LC–MS platforms. We now propose to use one assay to measure both GLP-1 forms to alleviate concerns due to differences in

sample handling, since the same procedure and reagents will be used for both measurements. We acknowledge that the 1 ml plasma requirement is a relatively large volume compared with other biomarker assays regardless of platform. We have demonstrated that less volume may be used if levels are not expected in the lower range of the assay. If 1 ml of sample is not attainable, IA LC–MS/MS can also be used to validate or invalidate existing immunoassays that require less volume. This comparison model is especially advantageous when there are questions regarding the specificity of a given immunoassay. For any given analyte, immunoassays may be preferred due to higher throughput, smaller sample volume requirements or better sensitivity, in which case the IA LC–MS/MS assay can aid to build confidence and reinforce the quality of data provided by immunoassays. Cross-validating assays on multiple platforms is a growing trend that has been presented more and more in the literature [20,21]. In the end, the preference of one platform over the other will be determined by the question of how the data will be used.

In summary, we have presented a specific and sensitive method for the quantitation of active and inactive GLP-1 in human plasma using IA LC–MS/MS. The sensitivity of this assay is better than any other LC–MS/MS GLP-1 measurement and many commercially available immunoassays. This important analytical tool could be used to qualify and/or harmonize the different immunoassays used for the quantitation of GLP-1.

Future perspective

The measurement of proteins and peptides by MS-based methods has become an important tool in the analytical laboratory and will continue to grow over the next few years. Immunoassays often recognize a mixture of the protein or peptide of interest that may include fragments and/or post-translational modifications. This may lead to less selective methods for the 'same' analytes, yielding different results when different antibody pairs are used. This, in turn, may lead to paramount standardization and harmonization efforts that could be very involved, as it was the case with the cardiac troponin assays. MS methods for the quantitation for proteins/peptides are more selective and may not suffer from this lack of agreement between methods. This has been the thought for many years. However, the technology did not have the required sensitivity to measure many analytes of interest. Improved MS technologies, such as the one presented here will enable scientists to measure low abundant analytes with exquisite sensitivity and specificity. These methods may be first used as reference methods and will eventually become more mainstream.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The measurement of incretin hormones, such as GLP-1, is critical in the development of antidiabetic compounds.
- There are multiple immuno-based assays for the measurement of GLP-1. However, because of the complexity of proglucagon biology, the selectivity of these methods is often questioned.
- We have developed an ultrasensitive, highly selective immunoaffinity-LC–MS/MS assay capable of measuring endogenous levels of active (7–36 amide) and inactive (9–36 amide) GLP-1 in human plasma samples.

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Review

Bioanalysis

Analytical protocols based on LC–MS, GC–MS and CE–MS for nontargeted metabolomics of biological tissues

Invasive, site-specific metabolite information could be better obtained from tissues. Hence, highly sensitive mass spectrometry-based metabolomics coupled with separation techniques are increasingly in demand in clinical research for tissue metabolomics application. Applying these techniques to nontargeted tissue metabolomics provides identification of distinct metabolites. These findings could help us to understand alterations at the molecular level, which can also be applied in clinical practice as screening markers for early disease diagnosis. However, tissues as solid and heterogeneous samples pose an additional analytical challenge that should be considered in obtaining broad, reproducible and representative analytical profiles. This manuscript summarizes the state of the art in tissue (human and animal) treatment (quenching, homogenization and extraction) for nontargeted metabolomics with mass spectrometry.

Shama Naz[1], Délia Chaves Moreira dos Santos[1,2], Antonia García[1] & Coral Barbas*[,1]

[1]CEMBIO (Center for Metabolomics & Bioanalysis), Facultad de Farmacia, Universidad San Pablo CEU, Campus Monteprincipe, Boadilla del Monte, 28668 Madrid, Spain

[2]Laboratório de Tecnologia Farmacêutica, Faculdade de Farmacia Universidad Federal de Minas Gerais, Brazil

*Author for correspondence:
Tel.: +34 91 3724 711
cbarbas@ceu.es

Background

The field of metabolomics is an emerging and promising omic science in systems biology, which aims to depict the metabolic profile in complex systems through the combination of data-rich high-throughput analytical techniques and multivariate data analysis. Metabolomics investigates single component effects on a biological system and offers a holistic approach in the exploration of the molecular details of multiple factors on an entire biological organism. Metabolomics techniques allow for a high-throughput analysis of small molecules in biofluids and tissues, giving metabolic profiles of the end products. Comparison of metabolic profiles from different phenotypes can be supportive in the identification of metabolic changes and as well as helping to understand the molecular mechanism, integrated biochemical pathways and disease progression [1–3]. There are many metabolites in biological systems that change much faster than nucleic acids or proteins. Hence metabolites seem to depict more satisfactory changes in biochemical effects in any organism, representing a closer approach to determine biological end points than genomics, transcriptomics and proteomics. Two different approaches have arisen in this field: a targeted and nontargeted approach. The first approach can be defined as the targeted measurement of a selection of metabolites known to be involved in a given biochemical pathway that reflects the dynamic response to genetic as well as physiological modifications or the changes due to external stimuli in unicellular to multicellular biological systems [4,5]. On the other hand, the nontargeted approach is the global unbiased analysis of all small molecules that collectively constitute the entire metabolome and serves as a direct signature of biochemical activity in any sample of interest, giving more information than the targeted approach as it analyses all possible metabolites [6,7]. The nontargeted approach has been applied to different biofluids (e.g., urine, plasma/serum) and tissues [8–13]. Even though collection of tissue is invasive, tissue metabolomics has many advantages over biofluids. Metabolomics modifications and upstream regulations are first seen in tissue. Moreover, the pairwise comparison of tissue taken from diseased and nondiseased regions could reflect the

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Key terms

Multivariate data analysis: It involves the observation and analysis of more than one statistical outcome variable at a time, taking into account the effects of all variables on the responses of interest.

Nontargeted analysis: Nontargeted metabolomics is the analysis of all possible small molecules present in a biological system for a particular physiological state in response to external or internal stimuli.

interactions despite any individual differences. Global determination of metabolite concentrations in the tissues provides novel anatomical aspects of pathological conditions that cannot be obtained from target-specific fluid measurements. Providing more relevant information than systematic biofluids, tissue metabolomics has a greater importance in biomedical research. So far, many studies have already shown the applicability on a variety of animal tissues for metabolomics including liver, kidney, lung, brain and spleen from both rodents and other models [14–17]. Tissue metabolomics has been facilitated by advances in NMR and high-resolution MS. Although NMR is characterized by high-technical reproducibility, fast analysis and robust quantification of compounds, the intrinsic limitations of NMR are its poor sensitivity and signal overlap. The high-resolution magic angle spinning NMR spectroscopy is an ideal technique for the investigation of intact tissue specimens (10–50 mg) and permits spectra to be obtained with a resolution comparable to that observed in solution in a time that does not exceed 0.5 h for a routine analysis. However, poor sensitivity hampers the detection of low concentrations of metabolites, which could be important chemical biomarkers. By contrast, MS-based nontargeted metabolomics with separation techniques, such as LC, GC and CE, provides higher sensitivity and molecular specificity [15]. For nontargeted tissue metabolomics analysis, separation with LC coupled to MS enables the most comprehensive metabolite coverage achievable to date but there is no single analytical technique that covers the entire spectrum of the metabolome. Thus multiplatform approaches including different separation techniques or even direct infusion and MS are all being employed in order to extend metabolite coverage [10,16]. Recent advances by coupling GC (GC×GC) and MALDI-TOF-MS have also been applied in nontargeted analysis [18,19]. The use of GC×GC has been shown to produce comprehensive enhanced metabolic coverage compared with conventional GC–MS and NMR [20,21]. One of the critical steps in nontargeted metabolomics studies is the structural characterization of identified metabolites especially when the compound of interest is of low concentration. A recent article has been published

Figure 1. General workflow involved in nontargeted tissue metabolomics.

Table 1. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (A–B).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Adipose	CE–MS	Understanding the mechanism of visceral fat accumulation and metabolic syndrome between post- and pre-menopausal women	Addition of IS containing methanol in frozen adipose tissue, homogenization, addition of water and chloroform (500, 200, 500 μl), centrifugation, filtration by 5 kDa millipore filter, lyophilization, resuspend in water before analysis	[51]
Adipose	LC–MS	To identify pathways activated by feed restriction and to understand the contribution of insulin in chicken adipose physiology	Tissue homogenizing in liquid N_2 cooled mortar and pestle, metabolite extraction with chilled methanol containing IS, centrifugation, injection of supernatant	[52]
Bladder	LC–MS	Identification of bladder cancer associated metabolic signature and disclosing the precarcinogenic metabolic process	Tissue homogenization in ice cold 80% methanol containing IS, metabolite extraction with ice cold methanol:chloroform:water, deproteinization, drying the extract, resuspending in solvent before injection	[53]
Bone	GC–MS	Identification of metabolic pathways involved in the growth of bone metastases in order to improve cancer prognosis	Tissue metabolite extraction with water:methanol:chloroform containing IS using bead mill, extract drying and derivatization prior to injection	[54]
Brain	GC–MS	Comprehensively evaluating therapeutic effects of antidepressants in a depression rat model	Tissue homogenization in water, addition of solvents, IS and derivatizing reagent in the supernatant, ultrasonication, addition of chloroform in the extract, adjusting the pH, repeating the derivatization process, drying chloroform layer with anhydrous sodium sulfate for subsequent GC–MS analysis	[55]
Brain	UHPLC–MS	To assess the relative analytical power and potential usefulness of UHPLC–MS for studying the global polar metabolite changes in subjects with Alzheimer's disease	Frozen tissue samples were lyophilized and milled to a fine powder, addition of 50% methanol, mixing, sonication, deproteinization by centrifugation, collection of supernatant for injection	[56]
Brain	LC–MS	Investigating the link between histology and neural metabolites in rodent models of HIV infection	Tissue homogenization in methanol, addition of IS after serial dilution with water, deproteinization, centrifugation, supernatant collection, drying, reconstituting in 50% acetonitrile, diluting 10,000-times before injection	[57]
Brain	LC–MS	Metabolomics study to observe the biochemical mechanism of venlafaxine in brain tissues of rat models of depression	Tissue homogenization in ice cold methanol, centrifugation, supernatant collection, drying reconstitution in water, addition of chloroform, centrifugation, injecting upper aqueous layer	[58]
Brain	LC–MS	Global metabolomics analysis of GDE1 (-/-) mice	Tissue homogenization with cold 80% methanol, sonication and centrifugation. Re-extracting the pellet, drying the combined extracts and reconstitution in water before analysis	[59]

IS: Internal standard; UHPLC: Ultrahigh-pressure liquid chromatography.

suggesting a workflow to overcome this limitation and increase the number of identified metabolites using LC–MS nontargeted metabolomics applied to brain tissue, liver and astrocytes, as well as nerve tissue [22]. The global metabolomics study involves differential comparison of a healthy subject or treatment group with-

out a prior knowledge of any metabolites. Therefore, possible applications of the nontargeted tissue metabolomics approach to a variety of tissues was applied in order to discover clinically relevant biomarkers using different tissue types (lung, liver, brain, pancreatic, adipose, heart and kidney) to understanding disease and other biological processes, the effect of nutrition on health, the understanding the mechanisms of drug action, metabolism or toxicity among others [10,23–29]. Researchers are also focusing on whole organ or animal profiling using a nontargeted approach [30].

The aim of this review is to discuss the different challenges in the MS-based nontargeted tissue metabolomics approach focusing on sample preparation protocols, discussing different separation techniques and their application to different tissue types to date.

Major challenges in tissue sample preparation

Tissue metabolites provide valuable insights into the biochemistry of disease, toxicity and response to drug administration and normal physiological characteristics because they contain an extraordinary amount of biological information, written in the language of cells, genes, proteins and metabolites [4,31,32]. However, sample preparation remains a crucial variable in obtaining the most accurate information. The choice of sample pretreatment methods, which is an essential step, affects not only the molecular features but also the biological interpretation of the obtained chromatographic data. The workflow describing nontargeted tissue metabolomics is presented in Figure 1. The most common steps involved in all tissue fingerprinting comprise homogenization and metabolite extraction, in order to measure multiple small molecules to produce unique metabolic profiles.

Origin-based tissue homogeneity

Compared to biofluids, tissue collection generates intrinsic challenges in nontargeted metabolomics. Muscle or fat samples may be expected to be quite homogenous but most other tissue types are not; the liver has

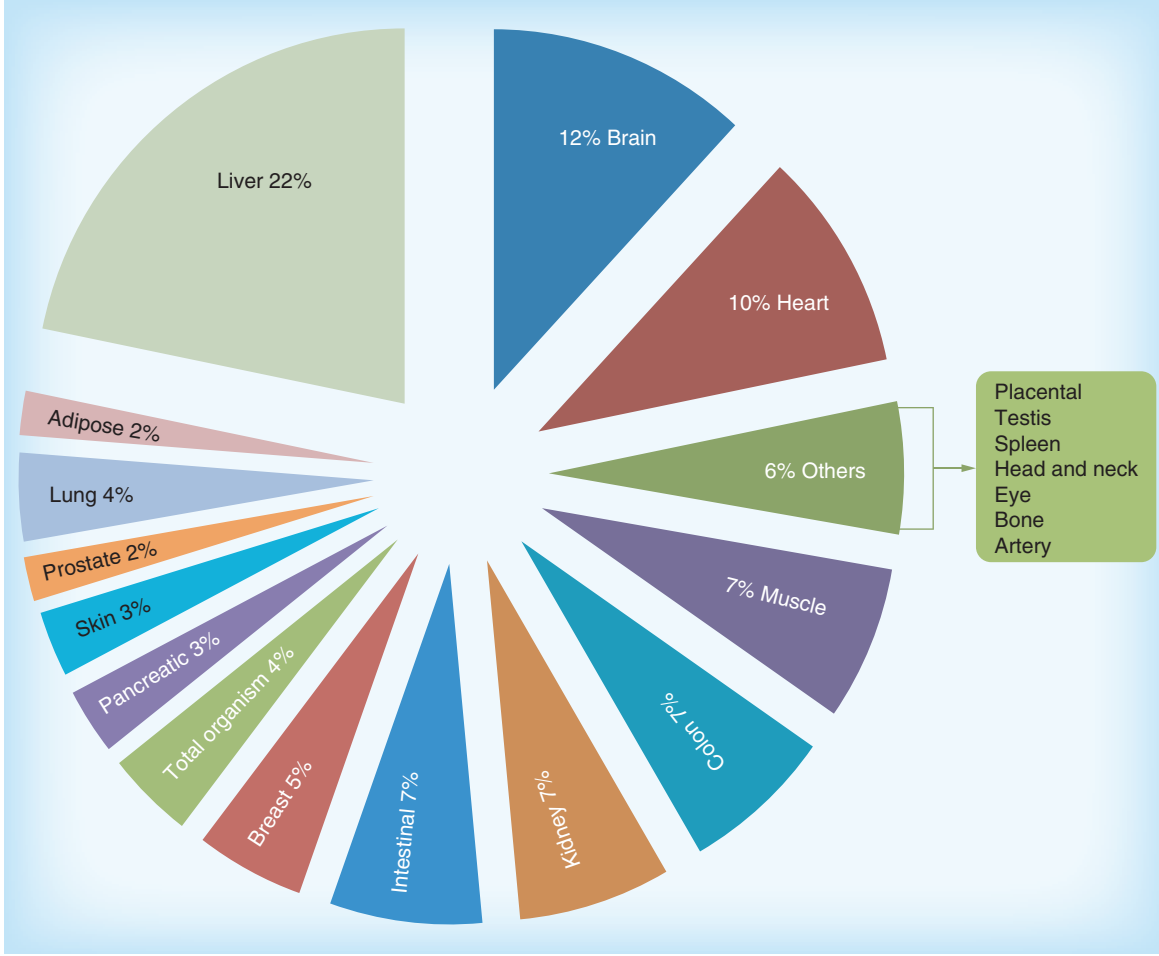

Figure 2. Percentage pie chart based on publications of different tissue types, searching keywords 'un/nontargeted metabolomics, fingerprinting, human and animal tissue' in NCBI PubMed.

five different topographic lobes and they have different levels of enzymatic systems, the kidney is another well-known heterogenic tissue type with medulla, cortex and multiple cell types with different structure and function throughout the nephron, and the brain is even more complicated [33]. Tissue from such organs could give rise to region-specific results thus the region should be well-defined before analysis is undertaken. Otherwise, the result may be erroneous and misleading. In cancer biology, tumors are being examined using a nontargeted approach in order to understand the different metabolites between tumorous and nontumorous region [34]. Although tumor tissue should be composed of the same cell type, regional differences are still present [35]. For example, in the tumor itself there may be regions that are well oxygenated, where as other regions that are not. Thus, during sample collection care should be taken to collect a sample from the same region for each sampling to avoid factors such as biological variability. Alternatively, whole tissue or cross-sectional tissue analysis can help in overcoming this inconsistency.

Tissue collection & quenching metabolism

Generally tissue samples are collected under anesthesia in a randomized manner following ethics guidelines. In order to avoid contamination from the anesthetic drug or blood, after collection the sample is usually thoroughly washed with deionized water or buffer [10,22,36]. Very interesting research has been published recently, where the effect of blood on liver analysis was evaluated by comparing perfused and nonperfused mice liver applying the nontargeted approach [37]. As blood circulates through different organs, it can carry other metabolites that are nonspecific to the liver and which could enhance the chances of overlapping or diluting the liver-specific metabolites. The magnitude of metabolite contaminations from blood were seen by elevated amounts of some amino acids, organic acids and sugars, with a clear overlap of blood and tissue metabolite profiles. Not only that, the study also confirmed the alterations in major blood related proteins through proteomics study.

One of the main limitations in tissue metabolomics analysis is the variation in metabolism within the tissue. The metabolism in tissue starts changing in seconds and metabolites are highly unstable at high temperatures and easily degrade during ultrasonication or homogenization. Thus quenching of metabolism to stop any metabolic reaction in the sample is mandatory. This quenching is usually obtained by using any one of the following, shock freezing with very low temperature (usually liquid nitrogen -195.8°C) or denaturing the enzymes with acid or solvents as fast as possible after tissue collection [38–41].

Key term

Quenching: Quenching is the inactivation of the metabolism. It should be rapid as compared with the metabolic reaction rates to have representative samples.

Homogenization

The first need of tissue extraction is the physical disruption of the tissue sample in order to enable proper access of the extracting solvent to the tissue as well as obtaining an homogenous solution. Conventionally, breakdown of the frozen tissue is achieved by grinding in liquid nitrogen with a cooled mortar and pestle, manual degradation of cold tissue with scissors [37] or by homogenizing the frozen tissue using an electric tissue homogenizer [41]. The mortar and pestle technique has been considered the gold standard but this method requires considerable care to transfer the now ground tissue and in addition it is very labor intensive and time consuming. In addition, weighting the frozen powder is hardly reproducible owing to water condensation, and following addition of the solvent for extraction a clot of the frozen powdered tissue can form. This clot is very nonhomogenously suspended and a further homogenization step is required. Moreover, there is a chance of sample carry over unless the mortar is thoroughly washed before the next sample. Compared with the mortar and pestle technique, homogenization with a probe tip avoids many of the previous problems; it is also susceptible to sample carry over unless the probe is washed thoroughly between extractions. To avoid these problems, mechanical disruption using the Qiagen (Hilden, Germany) tissue lyzer or Precellys (MI, USA) 24-bead-based homogenizer is also used. With these systems multiple tissue samples can be homogenized simultaneously in a high-throughput manner, probably representing the most convenient and repeatable methodology but often not suitable for hard tissues [42–44]. Irrespective of the homogenization method used, tissue preparation is very labor intensive and represents a considerable bottleneck for metabolite profiling. The proper disruption of tissue must be checked with the microscope. Moreover, these devices are effective for medium- to high-throughput extraction of metabolites. However, these devices focus on automating the homogenization process, whereas the addition of extraction solvents and sample filtration has not been fully streamlined. Apart from the techniques involved, the need of homogenization is closely linked with the objective of any research. The selection of solvent for homogenization depends on the category of metabolites to be analyzed and separation techniques to be applied. Usually homogenizations are carried out either with a separate solvent or along with the solvent used for extraction. Most of the methods published in the

Table 2. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (B–C).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Brain	LC–MS	To identify the molecular mechanism and potential biomarker for intrauterine growth restriction in a rabbit model	Addition of methanol:water mixture in tissue, ultrasonication, centrifugation, drying the supernatant, reconstituting in 60% methanol with 0.1% formic acid and supernatant injection	[62]
Breast	GC–MS	Investigating the metabolic alterations in breast cancer subtypes	Tissue homogenization and metabolite extraction with degassed isopropanol:acetonitrile:water, drying and resuspending the extract in 50% acetonitrile, centrifugation, drying and derivatizing supernatant for analysis	[72]
Breast	GC–MS	Identifying the metabolic changes in the central pathways in invasive carcinoma and metabolic markers for breast cancer	Tissue homogenization and metabolite extracting with isopropanol:acetonitrile:water, centrifugation, drying extracts, reconstituting in 50% acetonitrile, drying and derivatizing before analysis	[98]
Breast	LC–MS and GC–MS	Elucidating tumor and stromal genomic characteristics that influences tumor metabolism through genomic and metabolomic analyses	Sample preparation was carried out on a robot system using 96-well plates. Briefly, tissue homogenization in water, addition of ethanol acetate:ethanol (1:1) containing IS, centrifugation, repeated metabolite extraction from the supernatant with methanol:water (3:1) and dichloromethane:methanol (1:1), centrifugation, concentrating the supernatant before LC injection, and for GC drying and derivatizing	[68]
Colon	GC×GC–MS	Investigating the global metabonomic profiling of colorectal cancer	Addition of chloroform:methanol:water in tissue, ultrasonication, drying and derivatizing supernatant before analysis	[76]
Colon	GC–MS	To investigate the metabolic changes in colorectal cancer	Grinding frozen biopsy tissue, metabolite extraction with monophasic chloroform:methanol:water, centrifugation, drying and derivatizing supernatant before analysis	[74]
Colon	GC–MS	To classify tumor and normal mucosae metabolic profile	Tissue metabolite extraction with chloroform:methanol:water and IS, ultrasonication, centrifugation, drying the supernatant, addition of anhydrous toluene, drying and derivatizing before analysis	[78]
Colon	GC–MS	Metabolic profiling of human colon tissue in terms of its sample stability, reproducibility, selectivity, linear response and sensitivity	Addition of monophasic mixture of chloroform:methanol:water in tissue, ultrasonication, centrifugation, drying the supernatant, addition of toluene, drying and derivatizing before analysis	[20]
Colon	UHPLC–MS and GC–MS	To determine a distinct metabolic profile during experimental colorectal carcinogenesis	Tissue homogenization in water, metabolite extraction and deproteinization with methanol, centrifugation, direct injection of supernatant in UHPLC and for GC analysis drying and derivatizing	[65]
Colon	MALDI-TOF-MS and LC×LC–MS	Focusing on the molecular mechanism of colorectal cancer	Homogenization in water:methanol:chloroform, sonication, upper phase collection, repeating twice, mixing three upper phases, drying and reconstituting in 50% methanol before analysis	[70]

IS: Internal standard; UHPLC: Ultrahigh-pressure liquid chromatography.

literature have used methanol in different proportions (100%/80%/50%) as homogenization solvent. Some of them have used deionized water and the rest used the extraction solvent in order to perform homogenization and extraction together. In any case, the homogenization solvent should contain an important proportion of

Table 3. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (D–H).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Drosophila melanogaster	LC–MS	Establishing a baseline tissue map of *Drosophila melanogaster*, to show the interactions of different tissues within the whole organism	Tissue dissection in *Drosophila* Schneider's medium, monophasic solvent of methanol:chloroform:water, homogenization, centrifugation, collection of supernatant for injection	[41]
Eye	LC–MS	Characterizing the biochemical differences *in vitro* of different animal species	Addition of acetonitrile in the vitreous sample, deproteinizing by centrifugation, injection of supernatant in the system	[67]
Gastric mucosa	GC–MS	To test the hypothesis that distinct metabolic profiles are reflected in gastric cancer tissue and exploring potential biomarker for gastric cancer	Tissue lyophilization, metabolite extraction with mixture of methanol:chloroform, centrifugation, drying supernatant, addition of ethyl acetate, drying and derivatizing before analysis	[79]
Gastric mucosae	GC–MS	To identify the difference of metabolomic profile between normal and malignant gastric tissue	Metabolite extraction with monophasic mixture of chloroform:methanol:water and IS in tissue, ultrasonication, centrifugation, drying the supernatant, addition of anhydrous toluene, drying and derivatizing before analysis	[100]
Head and neck	GC–MS	Metabolomics analysis of squamous cell carcinoma of the head and neck	Tissue homogenization in methanol:water:chloroform, addition of IS and overnight shaking at room temperature, addition of water, centrifugation, drying the upper layer and derivatizing prior to analysis	[13]
Heart	CE–MS	To understand the chamber specific metabolism and pathophysiology in mouse heart	Addition of IS containing methanol in frozen heart tissues (atria, right ventricle and left ventricle tissue), homogenization, addition of water and chloroform, centrifugation, filtration, lyophilization, resuspend in water before analysis	[93]
Heart	GCxGC–MS	To optimize the metabolite extraction from mouse heart tissue for GCxGC-TOF MS analysis	Pulverized tissue in liquid N_2 with mortar and pestle, rehomogenization with chloroform:methanol on ice, centrifugation after adding 50% methanol, drying and derivatizing the aqueous layer for analysis	[19]
Heart	GC–MS	Investigating myocardial metabolic changes in depression rat model, to find the links between depression and cardiovascular disease	Tissue homogenization in methanol containing IS, centrifugation, drying and derivatizing supernatants before GC–MS analysis	[36]
Heart	LC–MS and CE–MS	To investigate the mechanistic basis of dilated cardiomyopathy in hamsters	Addition of IS containing methanol in tissue, homogenization, for CE injecting by drying the supernatant and resuspending in water from the homogenate; for LC: addition of water and chloroform (500, 200, 500 µl), centrifugation, filtration, lyophilization, resuspend in water before analysis	[64]

IS: Internal standard.

Table 4. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (H–L).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Hippocampus	ICR-FT-MS	Metabolomics analysis of hippocampus tissues to understand the mechanism of Alzheimer's disease in transgenic mice	Addition of 80% methanol in tissue, submerging in liquid N_2, thawing, sonication, centrifugation, supernatant collection, drying, reconstituting in 50% acetonitrile with 0.1% formic acid, diluting 100-times before injection	[25]
Hippocampus	LC–MS	Toxicological study of a neurotoxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) in a rat model	Tissue homogenization in methanol, centrifugation, drying and reconstituting the supernatant in 10% acetonitrile before injection	[101]
Intestine	UHPLC–MS	Identifying metabolite markers of intestinal tissue injury after ionizing radiation exposure in a murine model	Frozen tissue homogenization in 50% methanol containing IS, addition of acetonitrile, centrifugation, incubation on ice, drying and reconstituting the supernatant in water before analysis	[102]
Intestine (ileum)	LC–MS	Monitoring the metabolic events associated with the gradual development of Crohn's disease-like ileitis in a mouse model	Tissue homogenization in EDTA and BHT-buffer, addition of IS, acidification by citric acid, deproteinization with methanol:ethanol (1:1), centrifugation, drying organic phase, reconstituting in 20% acetonitrile before analysis	[63]
Kidney	LC–MS	To understand the metabolomic changes of ischemia/reperfusion-induced acute kidney injury and the protective effect of carnitine	Tissue homogenization in ice cold PBS, centrifugation, supernatant collection for injection	[66]
Kidney	LC–MS and GC–MS	To analyze the metabolic changes of three proximal tubule nephrotoxins	Tissue homogenization in water, deproteinization with methanol containing IS, centrifugation, supernatant injection in LC and for GC drying and derivatizing before injection	[29]
Kidney	UHPLC–MS and GC–MS	Analyzing the systematic alterations of renal cortex metabolites to explore the related mechanisms of diabetic kidney disease	Tissue homogenization in methanol containing IS, storing homogenates overnight, centrifugation, injection of supernatant in UHPLC and for GC derivatization before analysis	[40]
Liver	GC×GC–MS	Evaluating the effect of blood on liver fingerprinting analysis by comparing perfused and nonperfused mice liver	Tissue homogenization (24 different mixtures of five solvents), centrifugation, addition of IS and derivatization prior to analysis	[37]
Liver	GC×GC–MS	Assessing the feasibility of using commercially available software for nontarget processing of GC×GC–MS data	Addition of 80% methanol containing IS, ultrasonication, centrifugation drying and derivatizing the supernatant before injection	[77]
Liver	GC–MS	Evaluating metabolomics profile of hepatocellular carcinoma	Tissue homonization in chloroform: methanol containing IS, diluting with chloroform:methanol, addition of sodium chloride solution, centrifuging supernatant, drying and derivatizing both phases before analysis	[80]

BHT: Butylated hydroxytoluene; FT: Fourier transform; ICR: Ion cyclotron resonance; IS: Internal standard; PBS: Phosphate buffered saline; UHPLC: Ultrahigh-pressure liquid chromatography.

polar solvent to promote contact with the tissue whose composition is mainly water. A recent study on lung metabolomics applying the nontargeted approach has described the importance of the selection of homogenization solvent and how they affect metabolite coverage [10]. To avoid any metabolite loss during solvent extraction care should be taken during the homogenization and solvent selection.

Metabolite extraction

The main goal of metabolite extraction in tissue metabolomics analysis is to obtain reproducible results with the broadest possible range of metabolites. The primary step in metabolite extraction is the separation of unwanted compounds such as proteins. However the choice of metabolite extraction depends on the analytical tools and the metabolites of interest. Tissue extraction should be: as nonselective as possible, for wider metabolite coverage, reproducible, fast and easy, and should involve as few as steps as possible, enabling high-throughput analysis. Extraction of metabolites from tissues is often the most labor intensive and therefore the rate-limiting step, and extraction is usually achieved either by single phase or biphasic extraction. The biphasic separation has been found to be popular because polar and nonpolar small metabolites can be extracted simultaneously and each fraction can be analyzed separately. The biphasic method was first described by Bligh and Dyer using chloroform/methanol/water in a proportion to obtain two separate phases [45]. Further investigation on metabolite extraction strategies by Le Belle and coworkers also concluded that methanol/chloroform/water is the preferred method and concluded that this method may be mandatory for lipid-rich tissues [46]. Given the hazards associated with chloroform use, Matyash *et al.* proposed the usage of another organic solvent methyl-ter-butyl-ether, which has proven very useful in the extraction of polar and nonpolar metabolites [47]. In another study, dichloromethane was used as an alternative to chloroform and was proven to be superior [42]. Although multiphase extractions provide a higher number of extracted metabolites with higher concentrations, there are some metabolites that split in both phases creating problems during method validation. Moreover, metabolites that are very low in concentration can be lost owing to dilution between phases and two analytical runs are necessary. Considering these facts, a single extraction step would be ideal. Most published works regarding nontargeted tissue metabolomics have focused on single phase separation typically using methanol or a monophasic solvent mixture of methanol/chloroform/water as extraction solvent (Table 1). Twenty-four solvent mixtures from six different solvents (methanol, ethanol, isopropanol, acetone, chloroform and water) were tested for a liver study applying the nontargeted approach. The researchers proved that the extraction yield could not be improved either with acetone, isopropanol or water, but that a single phase solvent mixture of methanol/ethanol/water (8.5/1/0.5, v/v/v/) showed better extractions [37]. Our own study described that using one single phase and multiplatform analysis resulted in wider metabolite coverage in mouse lung tissue. Not only that, this method was validated selecting metabolites of different physicochemical properties, covering the entire chromatogram in three different platforms in terms of linearity, accuracy, precision and the method proved to be very reproducible [10]. Using either single or biphasic separation, tissue extraction involves multiple steps that include solvent additions, mixing and centrifugation. This is disadvantageous in terms of being time-consuming; furthermore, each step has the potential to introduce variation into the extraction protocol. The extraction yield and reproducibility does not only depend on the choice of solvent or phase separation. The addition of solvent during extraction also matters. The study of Wu *et al.* in developing a high-throughput methodology for the flatfish liver used three different solvent addition strategies [48]. The original slow stepwise addition, the addition of solvents in a more rapid two-step protocol, and an all-in one addition of all solvents simultaneously and the quality of these extraction methods were evaluated based on metabolite yield, extraction reproducibility and sample throughput. The results showed that the two-step method provided good quality data and more accurate snapshot of the liver metabolome [48]. The two-step protocol was later used by Masson *et. al.* in a nontargeted LC–MS-based study of liver metabolite profiling and was found to be optimal [42]. Even in our study a two-step solvent addition protocol has been followed [10]. In order to extract all possible metabolites (from polar to nonpolar), it is quite usual to extract a tissue sample using both aqueous and organic solvent. Adding them separately could enhance solvent specific metabolite extraction. Want *et al.* also provided detailed protocols for the preparation of animal and human tissue samples for obtaining nontargeted metabolic profiles based on the two-step method [49]. Another crucial point in nontargeted metabolomics is large-scale study sample preparation and analysis. Usually researchers attempt to reduce within-experiment analytical variation or any unavoidable sources of measurement error to be introduced, which is very likely for large-scale multi-batch experiments. In the quest to overcome this problem, the necessity for the development of robust workflows that minimize batch-to-batch variation have been well explained by Kirwan and co-workers [50].

Key term

Metabolic profiling: It is the targeted measurement of one/some known metabolites involved in a given biochemical pathway. Fingerprinting and profiling are frequently used indistinctively in the literature.

MS-based techniques involved in nontargeted tissue metabolomics

This is a reliable analytical method that helps to make a primary trustworthy assumption from the identified discriminant metabolites. Several separation techniques are available to use along with MS detection such as LC, GC, CE, but also direct infusion can be used with ion cyclotron resonance–Fourier transform (ICR-FT).

LC–MS

LC coupled to MS is a powerful tool for metabolomics because it allows for the separation and characterization of the majority of compounds. It can resolve different metabolite groups ranging from hydrophilic to hydrophobic. LC with MS detection has been used extensively in nontargeted metabolomics study rather than other detection methods, because it has structural identification capabilities and detection with MS is more sensitive and accurate. However, ion suppression due to coeluting compounds is the major limitation for LC–MS. The LC separation depends on the molecular properties of the analyte, which determines what type of stationary phase (column type) and mobile phase are to be used for a better separation. In tissue metabolomic studies, reversed phase (RP; C8/C18), normal phase (NP) and hydrophilic interaction chromatography (HILIC) are being used as common stationary phases depending on the specific class of compounds. Mostly published LC–MS-based nontargeted tissue metabolomics studies used RP silica-based columns with various particle sizes considering their sensitivity, lower detection limit and applicability to the majority of the compounds. The mixed use of RP with NP or HILIC has also been studied [60]. HILIC-based separations are well suited for hydrophilic compounds as with classical RP columns many polar compounds have poor retention, eluting near the void volume. In a recent study, Lv *et al.* compared eight different categories of column and found that RP pentafluorophenylpropyl showed better separation than RP C18, without the need for an ion-pairing reagent [61]. Usually the gradient for RP-based LC separation starts with high percentage of water and less organic solvent and HILIC based starts with mobile phase with high organic content with less aqueous modifiers. Almost all the RP, NP or HILIC-based tissue extract separations were following this gradient criteria using water as aqueous, and methanol, acetonitrile and isopropanol as organic solvents (see Table 1). The uses of different percentages of formic acid/acetic acid/ammonia have also been studied in order to increase metabolite ionization [10,57,58,62–65]. In all cases electrospray ionization (ESI) has been chosen as the ionization mode using only positive or both positive and negative mode. While preparing tissue extract for LC–MS study using RP stationary phases, either using methanol alone or a mixture of organic compounds, a single phase has been preferred in most cases [10,38,53,56,60,66–68]. For the combination of RP and HILIC or while focusing only on nonpolar compounds, biphasic separations and separated injections of each phase were undertaken [69,70]. LC–MS based studies usually include an tandem MS approach to characterize compounds from tissue extract [22]. The introduction of ultrahigh-pressure LC with smaller column particles, operating at high pressure, increases the efficiency by increasing both sensitivity and resolution with shorter analysis time. Ultrahigh-pressure LC–MS has also been applied in nontargeted tissue analysis resulting in wider metabolite coverage [38,69]. Want *et al.* have suggested a LC–MS-based workflow for the metabolic profiling of tissues [49]. A LC–MS-based nontargeted approach has been used to identify the molecular mechanism or diagnosis of different disease states including cancer, acute kidney injury, Alzheimer's, depression, HIV infection among others [23,56,58,66,71].

GC–MS

GC–MS is the suitable comprehensive analytical tool for identification and quantification of volatile and semivolatile organic compounds in complex mixtures, as it combines high-separation efficiency with selective and sensitive mass detection. Moreover it can be used to identify unknown organic compounds both by matching spectra with reference spectra and by *a priori* spectral interpretation. Moreover, the compound identification is quite straight forward owing to the extensive and reproducible fragmentation pattern obtained in full-scan mode. Unlike other separation techniques GC has few limitations. Only thermally stable compounds with high vapor pressures can be analyzed by GC–MS; however, as the samples contain a complex mixture, sample preparation steps are quite long and complicated in order to properly vaporize the analyte in the mixture. Nearly all the published articles regarding GC–MS based nontargeted tissue metabolomics used an electron ionization source and up to now, very few have used exact mass analyzers. A fused silica capillary column with a 5% phenyl group or 100% polydimethylsiloxane were used in most cases [36,72–74]. Along with this a short guard column was also used in most of the cases in order to increase the sensitivity. In order to vaporize the polar

Table 5. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (L).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Liver	GC–MS	Comparing the liver metabolome of specific pathogen-free and germ-free mice	Tissue homogenization with methanol, addition of water and chloroform, deproteinizing upper layer by centrifugal filtering, addition of IS, lyophilization and derivatization before analysis	[81]
Liver	GC–MS	To develop an optimized extraction method and comprehensive profiling technique for liver metabolites	Grinding liver tissue using mortar and pestle, addition of solvent containing IS, centrifugation, drying and derivatizing the supernatant before analysis	[82]
Liver	GC–MS	Metabolomics evaluation of the altered biochemical composition after exhaustive and endurance exercises in rats	Tissue homogenization in methanol, addition of water and chloroform, centrifugation, filtering aqueous layer, addition of IS, drying and derivatizing before analysis	[88]
Liver	GC–MS	Investigating abnormal metabolic process in both serum and liver tissue of liver transplanted rats	Tissue homogenization in sodium chloride, addition of acetone and IS, incubation, centrifugation, drying supernatant and derivatizing for analysis	[89]
Liver	ICR-FT-MS	To investigate the applicability of FT-ICR-MS based metabolomics on biopsy sample to a small but well-defined cohort of patients undergoing liver transplantation	Tissue homogenizing in 80% methanol, addition of chloroform and water, vortexing, incubation on ice, centrifugation, drying both the polar and nonpolar layers, and reconstituting before analysis	[95]
Liver	UHPLC–MS	To characterize the metabolic profile of steatosis in human tissue and to identify the potential disturbances in the hepatic metabolism of liver damage	Homogenizing frozen tissue in 80% methanol containing IS, metabolite extraction with chloroform and ethanol, centrifugation, separation of the layers and injection separately	[60]
Liver	UHPLC–MS and GC–MS	Metabolomic investigation of the effect of PON1 deficiency on histological alterations and hepatic metabolism in mice after high-fat high-cholesterol diet	Tissue homogenization, deproteinization with methanol, supernatant collection, drying, reconstitution in 0.1% formic acid and 6.5 mM ammonium bicarbonate for UHPLC. For GC–MS drying and derivatization before analysis	[23]
Liver	LC–MS	Evaluating of the metabolic characteristics of tumor tissue and the impact of tumors on surrounding tissue	Tissue homogenization in cold 80% methanol, ultrasonication, deproteinization, centrifugation, supernatant collection, freeze drying and reconstitution in 80% methanol before analysis	[71]
Liver	LC–MS	Identification of differential endogenous metabolites and their molecular mechanism in hepatitis-B-related hepatocellular carcinoma	Tissue homogenization in deionized water, ultrasonication, centrifugation, supernatant collection, deproteinization, centrifugation and filtering before injection	[103]
Liver	LC–MS	Identifying the gene expression related to hepatocellular carcinoma and metabolite profiling of nonalcoholic fatty liver disease	Liver tissue, homogenization in ice-cold methanol with 0.1% formic acid, centrifugation, supernatant collection and injection	[104]

FT: Fourier transform; ICR: Ion cyclotron resonance; IS: Internal standard; UHPLC: Ultrahigh-pressure liquid chromatography. .

Table 6. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (L).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Liver	LC–MS	Using data-driven procedures to improve metabolite extraction protocols for mammalian liver metabolomics analysis	Tissue homogenization in methanol or PBS, dividing supernatant in two parts after centrifugation, dilution with either 0.1 M formic acid or 0.1 N hydrochloric acid, recentrifugation, filtering twice, applying solid-phase extraction before analysis	[105]
Liver and lung	LC–MS	Integration of *in vivo* sampling during liver and lung transplantation, sample preparation and global extraction of metabolites using solid phase microextraction	Solid phase microextraction	[106]
Liver and muscle	GC–MS	To test how artificial selection for high mass-independent maximal aerobic metabolic rate affects the metabolite profiles in seven generations of rat	Tissue pulverizing under dry ice and liquid N_2, addition of cold methanol:chloroform, sonication, addition of chloroform:water and IS, centrifugation, processing of two separate phases for analysis	[90]
Liver and muscle	UHPLC–MS	Developing single metabolite extraction protocol for simultaneously performing targeted and nontargeted metabolomics as well as lipidomics	Crushing tissue using Qiagen tissue lyser, lyophilization, addition of methanol:methyl-ter-butyl-ether:water (two phase) for metabolite extraction, drying and reconstituting separately before injection	[69]
Liver and muscle	LC–MS and GC–MS	To obtain an unbiased map and understand the metabolic decline during aging-related diseases in mammals	Tissue homogenization, metabolite extraction with methanol containing IS, supernatant injection in LC–MS, drying and derivatizing before analysis in GC–MS	[91]
Liver, aorta, heart and muscle	CE–MS	Characterization of the metabolic imbalances of hypercholesterolemia in a Watanabe heritable hyperlipidemic rabbits model	Addition of IS containing methanol in tissue, homogenization, addition of water and chloroform, centrifugation, filtration, lyophilization, resuspend in water before analysis	[94]
Liver, kidney, heart, intestine and muscle	LC–MS	A broad profiling of hydrophilic metabolites from biological samples using a reversed-phase pentafluorophenylpropyl column	Tissue homogenizing in 50% ice-cold methanol, addition of chloroform, vortex-mixing, centrifugation, analyze the supernatant	[61]
Lung	LC–MS; GC–MS and CE–MS	Fingerprinting method validation and application on rat model of sepsis	Tissue homogenization in 50% methanol, metabolite extraction methyl-ter-butyl ether:methanol (one phase), centrifugation, injection of supernatant in LC, drying and derivatizing for GC Deproteinizing the homogenate with 0.1 M formic acid by 30 kDa millipore protein cut-off filter, drying and reconstituting in 0.1 M formic for injection	[10]

IS: Internal standard; PBS: Phosphate buffered saline; UHPLC: Ultrahigh-pressure liquid chromatography.

metabolites with less thermal stability prior to GC analysis it is necessary to perform chemical derivatization, which is usually performed with an oximation reagent followed by silylation, or solely silylation with *N*-methyl-*N*(trimethylsilyl)-trifluroacetamide or *N,O*-bistrifluroacetamide with trimethylchlorosilane reagent for tissue metabolomics study [16,20,36,54,73,75–90]. Only one author used an alternative derivatization reagent with ethylchloroformate and the derivatization was done in two steps [55]. Many GC–MS applications alone or in combination with LC–MS have been applied to almost all kinds of tissue type in order to find out the differing

metabolites for cardiovascular disease, cancer, depression, age-related disease, as well as developing databases (see Table 1) [36,55,77,79,82,91,92]. Metabolite extractions were mainly focused on single phase using methanol or mixtures of other organic solvents with water. Separation of polar and nonpolar metabolites has also been performed and analyzed separately. A 2D GC technique has also been applied in several studies [19,37,76,77]. The advantage of GC×GC–MS over GC–MS has been studied by Mal *et al.* in a nontargeted study of colorectal cancer. The study found broad significant metabolic space coverage compared with GC–MS. Moreover, the clustering of quality control samples was better with GC×GC–MS [76].

CE–MS

A major number of metabolites belong to the group of polar and ionic compounds. CE is able to separate a wide range of analytes from inorganic ions to large proteins. CE has an advantage over GC or LC for the resolution of these ionic compounds and even their isomers because CE separates metabolites according to their charge to mass ratio. On the other hand, GC and LC require the interaction with a stationary phase for metabolite separation. This separation criteria makes CE a complementary tool to the more established chromatographic separation technique. CE has more advantages over the other separation techniques, such as the analysis is fast, has a high resolution, is low cost, requires low sample volume (a few nanoliters) and requires very easy sample preparation steps. Although it has many advantages, CE has not been used extensively in metabolomics studies owing to several constraints. One of the main reasons behind this is the interface to combine CE with MS, which is not an easy task due to the necessity of sheath liquid. Another factor is the low volume of sample injection. As the amount is very low, sensitivity is low too. The interphase ESI (with or without sheath flow) enables metabolites to change from liquid to gas phase and it is the most common interphase used for MS as it is easily adaptable with CE. However there are not many reports based on CE–MS nontargeted metabolomics and very few related to tissue metabolomics. A CE-ESI-MS based nontargeted approach has been applied in lung, adipose, heart, liver, aorta, muscle and in all rat organs [10,51,64,93,94]. Different homogenization and extraction steps were followed either using the monophasic mixture of methanol/chloroform/water or only methanol. In all cases the extracts were dried and reconstituted before injection to avoid any interruption with the CE current. Sugimoto *et al.* have applied several CE–MS-based nontargeted metabolomics approaches, following a similar extraction procedure for all tissue types and analytical conditions for analysis in positive and negative ionization modes [17]. Sample extracts were passed through a 5 kDa protein cut-off filter to get a clear solution for injection [51,64,93,94]. In our own study we described a multiplatform method validation based on lung tissue using a 30 kDa protein cut-off filter to remove proteins or other tissue debris from the extract without any analysis problems. Moreover the homogenization and extraction was very simple only with 50% methanol and later diluting it with equal volume of 0.1 M formic acid. Presently no ideal analytical platform exists that covers the entire metabolome [10]. Different techniques have distinct advantages to investigate different groups of metabolites. So the use of multiplatform approaches could better characterize the entire metabolome, hence CE–MS can be a perfect complementary tool.

ICR-FT-MS

ICR-FT-MS coupled to an ESI source could provide fine resolved ions of small molecules in metabolomics analysis. It enables high-throughput global analysis of compounds in a complex matrix with high mass accuracy and fast identification solely based on the mass to charge ratio of each peak. However, the current limitations of FT-ICR-MS include lower technical reproducibility, less quantitative analysis, higher detection limit and less information as the identification is based only on the mass to charge ratio without additional information such as retention. An ICR-FT-MS-based nontargeted approach has been applied in the liver, brain and heart tissue [25,50,95]. The extraction solvent was either methanol or a combination of methanol/chloroform/water to get one or two phases depending on the need of metabolite analysis.

Several studies including our own have described the utility of using multiplatform approaches [10,23,29,39,40,64,70,91,96]. We described a multiplatform nontargeted approach on lung tissue using LC–MS, GC–MS and CE–MS from method development, validation and successful application on sepsis and control [10]. The study provided high-metabolite coverage and even the application described significant differences whereas sepsis is not related to lung. Moreover the amount of tissue required was minimum compared with other studies. The study showed the same homogenization process and the very simple sample extraction protocol for all three platforms could be ideal for all tissue analysis.

Data treatment & pathway integration

Nontargeted metabolomics approach generates huge data sets that need to be handled with careful data handling and mining sense. Several statistical software

packages (univariate or multivariate) are available for data analysis. Multivariate algorithms can often present these data sets as predictions of class separation and it is very important that this prediction is carried out in a relevant way. There are several strategies that are already being used for validating a statistical model,

Table 7. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (L–P).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Lung	GC–MS	To identify a clinical biomarker for lung cancer	Tissue homogenization with methanol:water:choloroform (2.5:1:1), addition of IS, centrifugation, addition of water in the supernatant, centrifugation, freeze drying the supernatant and derivatizing before injection	[16]
Lung and prostate	CE–MS	To understand tissue-specific tumor microenvironments, in order to development of more effective and specific anticancer therapeutics	Addition of IS containing methanol in frozen tissue, homogenization, addition of water and chloroform (500, 200, 500 µl), centrifugation, filtration by 5 kDa millipore filter, lyophilization, resuspend in water before analysis	[75]
Muscle and liver	UHPLC–MS	To examine the metabolite alterations in liver and muscle tissues in mice after a high-fat diet supplemented with betaine	Grinding tissue samples with mortar and pestle, metabolite extraction with 90% methanol (100 mg of powdered tissue), centrifugation and filtering supernatant before injection	[107]
Pancreatic	GC–MS	Examining the metabolic changes for acute pancreatitis in cerulean- and arginine-induced pancreatitis mice model	Tissue homogenization in methanol:water:chloroform, addition of IS; centrifugation, addition of more chloroform and water in supernatant, centrifugation, lyophilizing and derivatizing the supernatant before injection	[83]
Pancreatic	LC and UHPLC–MS	Metabolic pathway identification in pancreatic ductal adenocarcinoma by integrating metabolomics and transcriptomics	Tissue homogenization, deproteinization with methanol, supernatant collection, drying, reconstitution in 0.1% formic acid and 6.5 mM ammonium bicarbonate for UHPLC. For conventional LC in 0.1% formic acid (in 10% methanol) before analysis	[38]
Pancreatic	LC–MS and GC–MS	Identification of the metabolic pathways that are perturbed in pancreatic ductal adenocarcinoma	Homogenization, addition of IS, protein precipitation, supernatant collection, vacuum drying, resuspend in acidic solvent for LC–MS analysis and derivatization for GC–MS	[96]
Placenta	GC–MS	To determine whether the altered O_2 tension affects the composition of the placental metabolome	Tissue homogenizing with cold 50% methanol and PBS, centrifugation and supernatant collection, repeating process twice, addition of IS, lyophilization and derivatization before analysis	[84]
Placenta	UHPLC–MS and GC–MS	To describe methodologies used to interrogate data acquired from a wide range of complex metabolomes	Placental tissue cultured for 96 h in a serum-based growth medium, suspending in the biomass pellets methanol, freezing in liquid N_2 and thaw on ice, centrifugation, repeating three-times. Repeating the extraction again with the pellet, lyophilize the combined supernatant and reconstitution in water for analysis	[92]

IS: Internal standard; PBS: Phosphate buffered saline; UHPLC: Ultrahigh-pressure liquid chromatography.

Table 8. Mass spectrometry-based application of nontargeted metabolite analysis to different tissue types along with the study purpose and sample pretreatment: tissue types in alphabetical order (P–W).

Tissue type	Techniques	Purpose of the study	Sample preparation	Ref.
Prostate	LC–MS and GC–MS	Focusing on metabolomics signature of prostate cancer in tumor tissue	Tissue homogenization in deionized water, metabolite extraction with 80% methanol, centrifugation, supernatant collection, direct injection in LC–MS, derivatizing before injecting in GC–MS	[73]
Prostate	LC–MS and GC–MS	The initial focus was directed towards understanding the tissue metabolomic profiles as they exhibited more robust alterations	Tissue extractions were performed in four steps using solvents (ethyl acetate:ethyl alcohol; methanol; methanol:water and dichloromethane:methanol) containing IS. Combining all the supernatant, drying and reconstituting 10% methanol and 0.1% formic acid for LC and derivatization for GC analysis	[108]
Rat (all organs)	CE–MS	Developing Mouse Multiple Tissue Metabolome database	Addition of IS containing methanol in frozen adipose tissue, homogenization, addition of water and chloroform, centrifugation, filtration, lyophilization, resuspension in water before analysis	[17]
Renal	GC–MS	To characterize the key metabolic features of renal cell carcinoma	Tissue homogenizing, centrifugation, drying the supernatant and derivatization prior to analysis	[85]
Sarcoma	LC–MS	To demonstrate the analysis of polar metabolites extracted directly from formalin-fixed, paraffin-embedded specimens	Addition of methanol in tissue, incubation, centrifugation, drying the supernatant, resuspending in HPLC grade water before analysis	[109]
Skin	GC–MS	Explore the metabolic perturbation associated with ionizing radiation	Tissues homogenization in ammonium bicarbonate solution, addition of prechilled chloroform:methanol, centrifugation, drying and derivatizing the upper layer before analysis	[86]
Skin	GC–MS	Analyzing the volatile metabolic signature of a malignant melanoma	Solid phase microextraction	[87]
Spinal cord	UHPLC–MS and GC–MS	To examine a novel dietary strategy to provide significant antinociceptive benefits in rat pain model	Tissue homogenizing in water, protein precipitation with methanol containing IS, centrifugation, drying and reconstituting the supernatant for UHPLC and derivatizing for GC analysis	[39]
Whole insect	LC–MS	Metabolomic analysis of the genus *Metarhizium and Beauveria* (biological pesticides)	Separation of medium from tissues, snap freezing in liquid N_2, deproteinizing with acetonitrile, injecting the supernatant	[110]
Whole mussel	UHPLC–MS	To investigate the sex-specific differences in the mussel metabolome to understand their reproductive physiology	Tissue homogenization in methanol, centrifugation, addition of 5% methanol in sodium acetate buffer in supernatant, purifying, addition of IS, drying, reconstituting in 50% methanol and filtering prior to analysis	[102]

IS: Internal standard; UHPLC: Ultrahigh-pressure liquid chromatography.

which we are not going to describe as this is beond the scope of this review. Generally, normalization of data from animal tissues is based on tissue weight. However, some organs are heterogeneous and may differ in water content among samples, levels of DNA, proteins or even one specific protein can be used for normaliza-

tion. Data analysis always ends up with one or more statistically significant compound, which can give an insight into the progress or pathogenesis of specific conditions. These metabolites need to be integrated to the particular pathway in order to find out more about its relationship with a specific disease, hence identifying a disease-specific biomarker for clinical diagnosis and so on. Along with data mining software several databases are available online in order to identify the significantly different metabolites (e.g., METLIN, CEU mass mediator, LIPID MAPS). It is necessary to validate biomarker with a target specific analytical method, validating analytical method, proper reference material and applying proficiency test.

Application on different biological tissues & findings

Compared with biofluid, tissue homeostasis is principally intracellular rather than extracellular. The physiological state of a complex tissue is reflected in the full complement of various metabolites by its constituent cells. Moreover, biomarkers derived from tumor tissues may provide higher sensitivity and specificity than those from biofluids, although obtaining tissue samples is always invasive (endoscopy or biopsy) [20]. Hence, not only biofluids but also tissue samples or biopsies are moving more and more into research focus including a nontargeted metabolomics approach. The number of publications indexed by the terms nontargeted tissue metabolomics and tissue fingerprinting in a PubMed search is growing exponentially. Figure 2 demonstrates the percentage of publications based on different tissue types from the number of publications returned with an 'un/nontargeted metabolomics, fingerprinting, human and animal tissue, mass spectrometry' keywords search of NCBI PubMed. For clinical diagnosis purposes, minimally invasive or noninvasive biofluids are preferred and in some cases biopsy samples. But in any altered/diseased condition the changes in metabolism are initiated at tissue level and later on the metabolites are excreted/transferred in biofluids. Thus nontargeted tissue metabolomics offers the opportunity to understand the site-specific molecular mechanism involved in any disease conditions. This also helps in the identification of potential biomarkers for the early diagnosis in biofluids and effective treatment. Until now, most of the tissue metabolomics works have been applied in order to understand the mechanism behind cancer. Application of nontargeted approaches on tumourous and nontumourous tissue from liver, breast, prostate, colon, esophageal, bladder and gastric cancer revealed almost similar altered metabolic pathways related to glycolysis, amino acid metabolism, tricarboxylic acid cycle and fatty acid metabolism [53,73,74,97–100]. However, advanced steps on biomarker development based on the findings are still lacking. Some of the studies found very strong relationships for certain metabolites, suggesting they need to be studied further in a targeted manner in order to obtain clinical markers. For example, the ratio of betaine:propionylcarnitine has been found to be significant when separating hepatocellular carcinoma patients from hepatitis and cirrhosis patients; palmitoleic acid has also been found in pancreatic cancer diagnosis and the metabolite ratio of cytidine-5-monophosphate:pentadecanoic acid has been found in breast cancer [61,98]. Tissue metabolomics has also been applied to several other diseases. The purpose of several nontargeted tissue metabolomics applications with the sample preparation strategy have been described in Tables 1–8.

Future perspective

By the use of such highly sensitive and reliable MS-based nontargeted metabolomics approaches, an improved ability to understand the site-specific mechanism of any disease state can be possible in complex biological specimens. However, the success depends on several factors, such as overcoming the challenges related to sample pretreatment, spectral acquisition of metabolites, correlating the significant metabolites with biochemical pathways and validation of the identified metabolites in another set of samples applying both qualitative and quantitative approaches. Moreover, the identification of metabolites in nontargeted metabolomics studies is solely based on online databases that are not yet 100% complete. Reproducible spectra fragmentation is very useful for identifying compounds in GC–MS. Moreover, with exact mass analyzers the potentiality could be exciting, but exact mass spectral libraries are not commercially available yet. As a result of all of this, many of the metabolites that could have strong relationships with specific conditions remain unknown. Therefore, another challenge in the nontargeted approach is to develop methodologies that will allow the fast identification of these 'unknowns'. Although, until now, several applications have been published based on nontargeted tissue metabolomics, none of them have moved forward in order to provide a target-specific marker, which requires analyzing a large set of samples as well as validating the strong differentiating metabolites to gain reliable biomarkers.

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Executive summary

Targeted tissue for study

- A mass spectrometry (MS)-based nontargeted metabolomics approach has been applied to almost all kinds of tissue type although mostly to the liver.

Homogenization & extraction

- Mechanical disruption of tissue was carried out using methanol and water as the homogenization solvent.
- 100% methanol and a monophasic or biphasic mixture of methanol/chloroform/water was the most used extraction solvent.

Multiplatform study

- More analytical metabolite coverage was obtained using a combination of liquid chromatography-MS, gass chromatography-MS and capillary electrophoresis-MS rather than a single analytical tool.

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