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Particle sizing methods for the detection of protein aggregates in biopharmaceuticals

Protein aggregation is a common biological phenomenon which is responsible for degenerative diseases and is problematic in the pharmaceutical industry. According to the rules provided by regulatory agencies, industry is supposed to assess the product quality regarding the presence of subvisible particles. Also, they should evaluate the technologies that are used to measure these particles. Therefore, US FDA and industry have been looking for methods capable of accurately characterizing the protein products. Four sizing techniques reviewed here are good candidates to be used for characterization of protein and their aggregates: dynamic light scattering, size-exclusion chromatography, electron microscopy and Taylor dispersion analysis. The first three are more established techniques while the last one is a more recent and growing technique.

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Proteins play essential roles in all life forms, including the human body. They can be major components of cell structure, catalyze biochemical reactions, act as receptors for signaling molecules and transport molecules within a cell [1]. They naturally require a 3D-folded structure to function effectively [2,3]. The fundamental forces that drive protein folding include van der Waals and hydrophobic interactions, hydrogen bonding, as well as charge–charge interaction, among others [4]. These types of interactions occur between amino acids, the building blocks of proteins, and are critical to maintaining structural integrity. However, these interactions not only occur between amino acids of the same protein, but also occur between amino acids of adjacent proteins. Interprotein interactions can result in aggregation if the newly synthesized protein does not fold correctly, or if certain chaperone molecules within the cell fail to initiate the degradation or refolding of the faulty protein. Consequently, protein aggregation is an inevitable phenomenon that occurs under certain conditions [2]; mutations, defects in protein biogenesis, environmental stress conditions and aging can all cause protein aggregation in cells [5]. This aggregation has been identified as the primary cause of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and Huntington's disease [6]. Protein aggregation is not only the cause of the diseases as mentioned earlier but also a major concern for pharmaceutical industries.

Pharmaceutical companies are becoming increasingly interested in proteins for the development of therapeutic drugs. There has been a remarkable increase in the development of protein-based therapeutics since the approval of insulin as the first recombinant

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protein-based therapeutic by the US FDA in 1982 [7]. Currently, about 250 protein-based products are commercially available, and they have played a critical role in improving human health in the last few decades. They have been successfully used to treat and control some debilitating diseases, such as diabetes and various forms of cancer [8,9]. The remarkable growth of protein-based drugs has been influenced by the advantages they offer over small molecule drugs, such as lower toxicity and higher specificity toward targets [10]. However, protein aggregation poses a challenge for the development of biological products. Aggregation can adversely affect product quality and efficacy, or potentially induce an immune response in the patient [11] Regulatory agencies such as the FDA exist for this reason; they certify the safety and efficacy of drugs before they are approved and allowed to enter the market [12].

Aggregation can occur during the manufacturing of protein-based therapeutics due to variation in solution conditions (pH, ionic strength and the presence of surfactants), temperature fluctuation or exposure to light [13]. Even if these variables are controlled, there is still a possibility that aggregates will form during production, storage, shipment or delivery to the patient [14]. In this way, the formation of aggregates under various conditions should be investigated to ensure the safety and stability of protein formulations. The early detection and characterization of protein aggregates, including size, morphology and interactions, is critical in therapeutic products [15]. Moreover, the *in vitro* and *in vivo* screening of protein aggregation can advance the understanding of which molecular mechanisms cause the protein aggregation associated with neurodegenerative disease [16].

The growth of the biotech industry has increased the demand for analytical techniques that can be used to study proteins and their aggregates. Sizing techniques are the workhorse for this field because changes in size are most noticeable when proteins move from monomer to oligomer and then to aggregates [17]. However, the unknown nature of aggregates, as well as their wide size range, from a few nanometers to a few millimeters, makes the analysis of protein aggregates challenging [18]. Each of the available sizing techniques covers a specific range of sizes, so the combination of several techniques is necessary to gain comprehensive knowledge about which types of particles are present in a sample. These techniques are based on different physical principles and hence generate different types of information about the sample [19].

Previous reviews have described various sizing techniques that can be applied to the study of protein aggregations but have focused on either a particular instrument or a specific particle size. Pryor *et al.* reviewed a variety of techniques used to study the aggregation of amyloid β protein, which plays a significant role in several diseases, including AD. They also compared the resolutions, sensitivities and costs of these techniques for the quantitative detection of aggregates with different sizes [20]. den Engelsman *et al.* published a commentary paper with some recommendations from biotech societies about which strategies should be implemented to prevent protein aggregation and therefore, unwanted immunogenicity [19]. Other authors have rather focused on one specific technique, such as dynamic light scattering (DLS), presenting its implications for a broad range of particle sizes [21]. In addition, some papers have discussed recent advances in analytical techniques with a focus on biotherapeutic proteins and antibodies [15]. A comprehensive understanding of the instruments, along with the applications and limitations of techniques, will help pharmaceutical researchers choose the optimal method for their studies with more finesse. In this review paper, we present four analytical techniques that can be used to study protein aggregation. In addition to a discussion about the applications of each technique, the underlying principles and technical concerns are also discussed. Figure 1 shows how frequently the four techniques presented in this paper have been used to study proteins and their aggregates over the past 20 years.

Techniques

DLS

DLS is a well-established method and is widely used to measure the size and size distribution of particles. The noninvasive nature of this technique makes it a good candidate for early stages of research that use valuable materials [19]. DLS is an ensemble method that measures all particles at once, as opposed to separation and counting methods [22]. It is used to measure the size of a variety of particles, including proteins, protein aggregates and polymers. DLS measures particles with a broad range of sizes; it is effective from the nanometer scale, for quantum dots and nanoparticles, to the micrometer scale, for polymers and grains [23–26]. Fast data acquisition, high sensitivity and the reproducibility of this technique have made it a favorable monitoring technique and attracted attention from many pharmaceutical companies [27].

DLS is based on the scattering of light from particles and their inherent Brownian motion. Scattering occurs when coherent monochromatic light with a wavelength of λ strikes a particle. If the size of the particle is considerably smaller than the wavelength of the incident light (typically less than 1/10 λ), then the scattering will be elastic (Rayleigh scattering) and the intensity of the scattered light is proportional

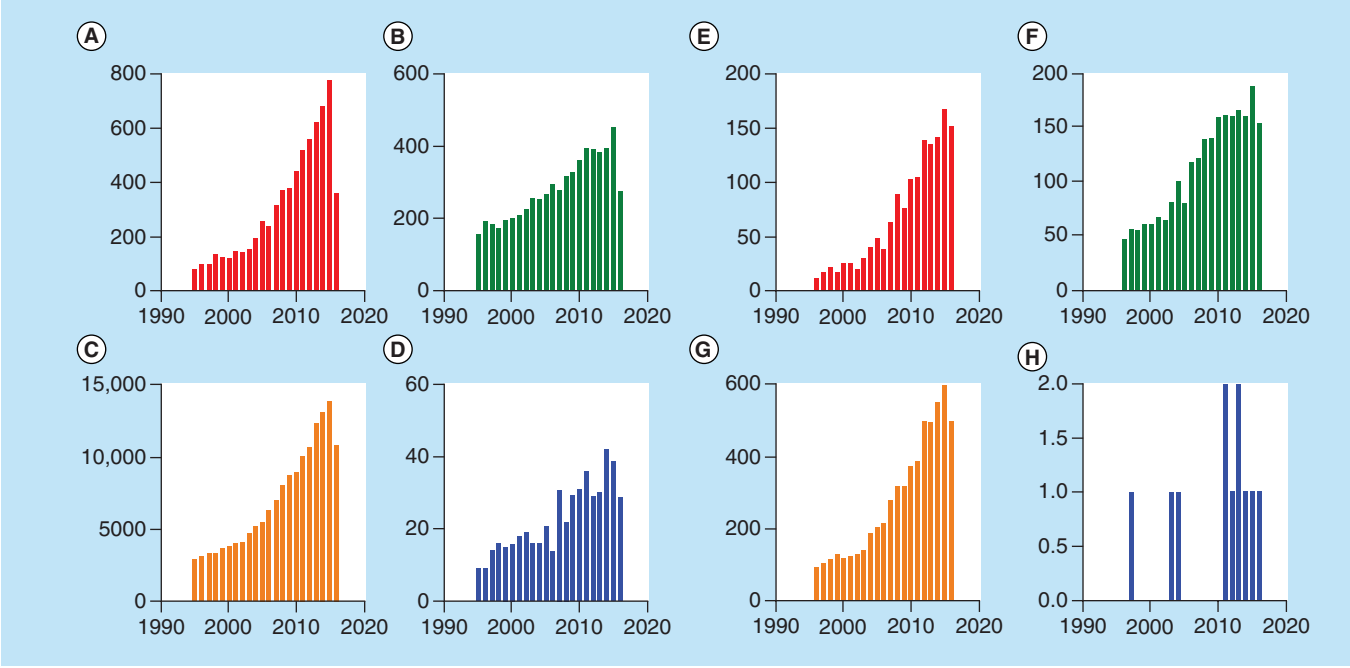

Figure 1. Sizing techniques published over the last 20 years. Annual number of articles featuring **(A)** dynamic light scattering, **(B)** size-exclusion chromatography, **(C)** electron microscopy and **(D)** Taylor dispersion analysis, in the title or abstract between 1995 and 2015, according to Science Direct. **(E–H)** are number of papers in **(A–D)** used specifically for studying proteins and protein aggregates.

to the sixth power of the particle's radius [28]. The intensity of the scattered light fluctuates over time due to the Brownian motion of the particles, which describes the random movement of particles in a fluid caused by interaction with surrounding molecules. The rate of this Brownian motion depends on the diffusion rate of the particles, which is affected by particle size, viscosity and temperature. Therefore, the intensity fluctuations recorded during DLS analysis provide time-scale information about the motion of the particles in the medium (diffusion coefficient). A larger particle will have a smaller diffusion rate, and therefore, a slower intensity fluctuation [29–31]. A time-domain analysis method, the autocorrelation function, is often used to extract quantitative information from the scattering intensity fluctuations. Hence, DLS is also referred to as photon correlation spectroscopy [30]. Figure 2 shows the intensity fluctuations of scattered light versus time for particles with two different sizes, as well as the subsequent correlation function plot. The correlation decreases over time, and this decay is representative of the diffusion coefficients of the investigated particles. For monodisperse, spherical particles undergoing Brownian diffusion, the autocorrelation function decays exponentially over the delay time T as follows:

$$g(\tau) = A.e^{-Dq^2\tau} + B$$

Equation 1

Where D is the translational diffusion coefficient and q is dependent on the scattering angle, the refractive index (RI) of the medium and the wavelength of the laser light [30].

For spherical particles, the hydrodynamic radius Rh can be obtained from the translational diffusion coefficient (D) using the Stokes–Einstein relationship:

$$D = \frac{k_B T}{6\pi\eta R_h}$$

Equation 2

Where k_B represents Boltzmann's constant, η is the solvent viscosity and T is the absolute temperature. If the particle is nonspherical, then Rh can be used to describe either the apparent hydrodynamic radius or equivalent sphere radius. DLS experiments use these relationships to transform the obtained data into particle size information [32].

The cumulants method is widely used to derive size distribution information for polydisperse samples. This method assesses the mean size and polydispersity index (PDI) and it reports the size in terms of intensity-weighed mean diameter (Z average) [30].

DLS measures hydrodynamic size, which is the diameter of a hard sphere that diffuses at the same rate as the particle being measured. The hydrodynamic size not only depends on the particle itself but also on the types of solvation forces that exist in solution,

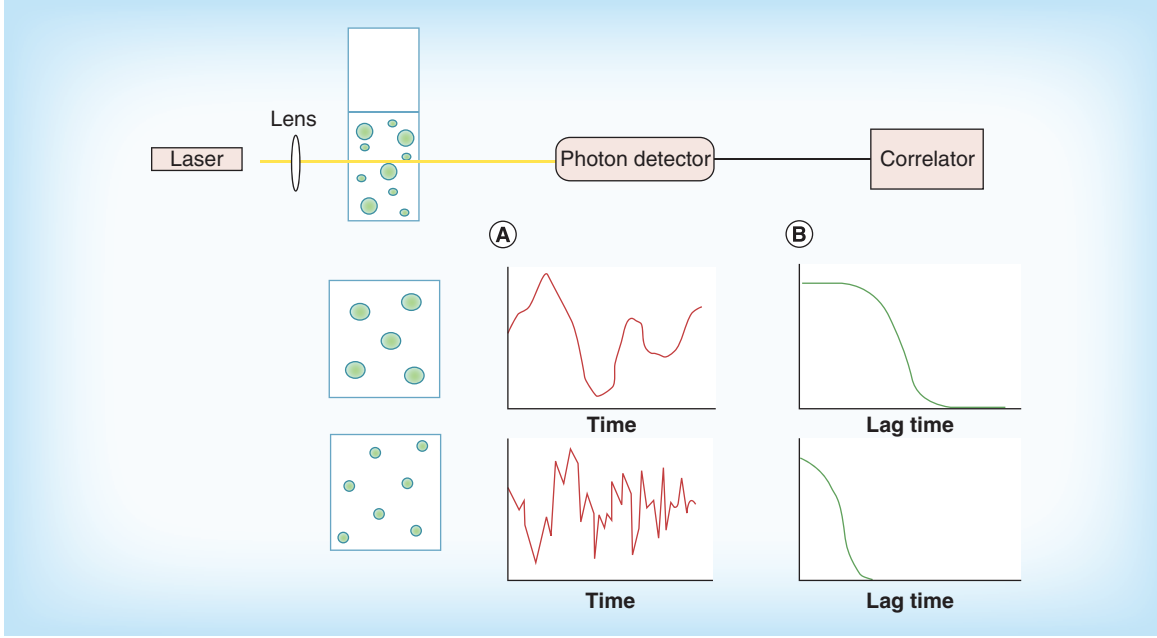

Figure 2. Schematic drawing of dynamic light scattering for two particles with different sizes. Dynamic light scattering instrumentation and **(A)** the fluctuation of intensity of scattered light versus time for different sized particles with **(B)** the subsequent correlation function.

along with surface adsorption [33]. For a nonspherical particle, DLS will give the diameter of a sphere that has a similar average translational diffusion coefficient as the particle of interest, but which also has a higher PDI [34].

The precision and repeatability of DLS depend on the measured parameter. For example, the calculation of Z-average is one of the most robust properties of DLS, as it is calculated directly from the decay rate of the intensity correlation function [35]. Since the calculation of the Z-average is mathematically stable, the result is insensitive to noise and therefore a preferred DLS size parameter [30].

In contrast to some other techniques that require specific temperature, pH and salt concentration conditions for analysis, DLS performs under normal operating conditions, which allow proteins to maintain their native structure. The fact that proteins maintain their native structure benefits the study of intrinsically disordered proteins (IDPs), which lack a 3D structure. IDPs play a vital role in the pathology of numerous diseases, including cancer, neurodegenerative diseases and infectious diseases [36]. The investigation of IDPs started during the 1990s, and their unusual behavior soon attracted the attention of many scientists and protein engineers [37]. Static light scattering is used along with DLS to characterize the size, molar mass and intermolecular interactions of IDPs [38].

The diversity of information provided by DLS makes it applicable to various areas of the protein industry. In addition to information about size, DLS can report the PDI, which is a good indicator of the homogeneity of a studied product. This parameter can play an important role in crystallography studies. A homogenous solution and pure macromolecules are required to grow the few large, high-quality and high-performance crystals that will be analyzed through x-ray diffraction [39]. DLS is one of the methods that are routinely used to evaluate protein homogeneity under various conditions and concentrations. The advent of highly-sensitive DLS instruments equipped with plate readers has made this technique easier, faster and more desirable for crystallography applications. Another advantage of this method is that the protein is easily recovered for other uses after the measurement [40].

In an ideal DLS measurement, the reported size is independent of the concentration as long as there is no interaction between individual solutes. However, there are certain restrictions on the concentration range. At low concentrations, there may not be enough dispersant to scatter the incident light, which will result in a very low S/N. At high concentrations, on the other hand, there is a risk of multiple scattering along with changes in viscosity and aggregation [41,42]. Furthermore, measurements of concentrated solutions include a possibility of interparticle and hydrodynamic interactions, both of which can affect the accuracy and precision of DLS results. However, the adverse effects of these interactions can be eliminated by extrapolating the apparent hydrodynamic radii to a concentration of zero. Takeuchi *et al.* used this method to evaluate the size of a set of globular proteins, and the accuracy

they reported was comparable to results obtained from more established methods, such as size-exclusion chromatography (SEC) [43].

The applicability of DLS to highly concentrated protein solutions appeals to pharmaceutical companies since it enables the analysis of high doses of drugs that are designed for subcutaneous administration. However, high protein concentrations lead to high-resolution viscosity, which can affect the production, processing and/or usage of the drug [44]. The rate of diffusion depends on the size of the particle as well as medium viscosity, and this relationship has extended the application of DLS to measuring the viscosity of high concentration protein solutions. The DLS-based results for viscosity are comparable to those obtained through the Cone and Plate method, which is commonly employed in the biopharmaceutical industry to measure the viscosity of protein solutions. DLS measures viscosity based on the light scattering signal from spherical polystyrene beads that are added to a protein solution. The size of beads is known and the viscosity is determined as an adjusting parameter to get the correct bead size. These beads are significantly larger than protein molecules, and the DLS signals originating from the beads can be easily separated from the signals originating from proteins by the decay time. The utilization of automated plate reader systems has made this method at least five-times faster than the Cone and Plate technique. Additionally, the DLS method requires small sample volumes, a characteristic that makes it especially useful for measuring viscosity during the early stages of biopharmaceutical development, a phase when there is often a limited amount of material available for analyses [41].

The aggregation of proteins poses a challenge for the manufacturing of biological products, but characterization of these aggregates can help control their formation. DLS has proven to be a reliable technique for monitoring and studying protein aggregation, and it can also provide information about the hydrodynamic dimensions of particles to ensure product consistency and help control aggregation. Yu *et al.* employed DLS to monitor inclusion body solubilization, protein refolding and aggregation during the production of recombinant protein-based vaccine candidates and investigated how urea and a reducing reagent affected the unfolding process of the proteins [45].

Moreover, DLS can be used to study the aggregation of proteins under various conditions, an insight that is crucial for explaining the different behaviors of proteins in the body. For example, Tomar *et al.* used DLS to study the decapacitation mechanism of the Con A binding fraction of human seminal plasma to better understand the human fertilization process. They monitored the degree to which Con A proteins aggregate when the pH or concentrations of salt, sugar and cholesterol change, hypothesizing that the aggregation of these proteins might be required to prevent premature capacitation [46].

DLS can also be coupled to other techniques to obtain more comprehensive information about biological systems. The nondestructive nature of DLS and its fast analysis enable it to connect to other sizing techniques or separation instruments. For instance, the coupling of Raman spectroscopy with DLS can provide enough information to determine if protein size has increased due to aggregation or unfolding. Raman spectroscopy provides details about the secondary and tertiary structures of proteins, whereas DLS gives information about the size and polydispersity of the sample. Both techniques apply to solutions with high concentrations of proteins, and enable the study of a protein's physical properties and behaviors in the formulation condition, rather than a diluted sample of the pharmaceutical product. Zhou *et al.* studied the structure, aggregation and heat stability of a high-concentration formulation of intravenous immunoglobulin using Raman and DLS. While Lewis *et al.* used the combined DLS and Raman approach to characterize the colloidal and conformational stability of proteins and study the mechanism of lysozyme aggregation as a function of both pH and concentration [47,48].

DLS has been coupled with fluorescence to investigate fibril formation in polyglutamine peptides. Thioflavin T fluorescence demonstrates β-sheet fibril content while DLS measures particle size distribution. The combination of these two techniques is used to study complex aggregation kinetics and reveal the multiple stages of amyloid fibril formation [49]. DLS has also been coupled to many additional techniques, such as SEC and Taylor dispersion analysis (TDA).

Although DLS is considered a popular technique for biopharmaceutical research, it suffers from certain limitations. The main disadvantage is that this technique is highly sensitive to large particles as the intensity of scattered light is proportional to particle size raised to the sixth power. This drawback causes DLS to be more susceptible to contaminants such as dust, requiring an efficient filtration of the solution before measurements [50].

The other constraint of this technique, which makes it less desirable for polydisperse samples, is its low resolution. DLS will not accurately characterize a polydisperse sample if the size difference is less than a factor of three [35]. Finally, the complicated data analysis procedure and the lack of quantitative results further hinder the wide application of this technique.

SEC

SEC is one of the primary analytical techniques that is used to characterize proteins and their aggregates and determine the size distribution of molecules in pharmaceutical products [18]. Molecules pass through the stationary phase of a column and are separated based on size [29]. The stationary phase often consists of heterosporous linked gels that are in equilibrium with a suitable mobile phase [51]. SEC is also called gel filtration chromatography when the mobile phase is aqueous and gel permeation chromatography when the mobile phase is an organic solvent [51]. SEC is often used to study large molecules, such as polymers as well as proteins and their aggregates [52,53], and it can also be applied to dendrimers, liposomes and lipid nanoparticles [54,55].

Various mechanisms describe the elution order of a heterogeneous mixture of molecules, but the prevalent mechanism underlying SEC is steric exclusion. This mechanism is based on the idea that beads with pores of a certain size within the gel matrix are available for molecules of different size [29]. Figure 3 illustrates how three particles of different sizes are separated according to steric exclusion theory.

The total volume of the column is divided into three parts:

$$V_t = V_g + V_i + V_0$$

Equation 3

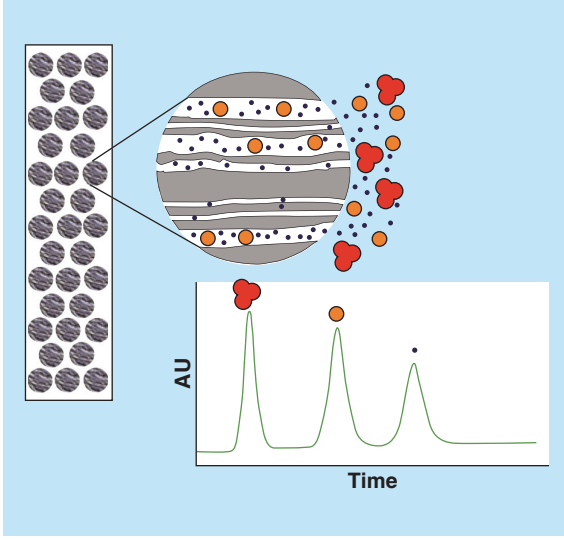

Figure 3. The steric exclusion mechanism for the separation of three molecules of different sizes in size-exclusion chromatography packed with porous beads. The larger molecules that are completely excluded from the pores move through space outside of the gel particles and elute first, whereas smaller molecules spend more time inside the bead pores and elute later.

Where V_g is the volume occupied by the solid matrix of the gel and V_i is the space between the beads of the gel matrix, referred to as the inclusion or internal volume and determined using very small molecules. V_0 is the volume outside of the beads and is determined by using a molecule that is larger than the exclusion range for the gel.

$$V_e = V_0 + K_d V_i$$

Equation 4

V_e is the volume required to elute intermediate size molecules and the fraction K_d describes the extent to which molecules can enter the interstices of the gel. In an ideal situation, where the molecules of interest do not interact with the column, this coefficient would be between one and zero [56]. The K_d of molecules that are larger than the pore size is zero and therefore, the elution volume is volume of the solvent outside of the pores. Small particles that can permeate into all pores are eluted with a volume of $V_0 + V_i$.

Ideally, the stationary phase would only minimally interact with the sample, which would retain biomolecular activity [57]. However, the nonspecific adsorption of proteins to the column matrix often affects the accuracy by abnormal elution positions and reduces recovery by the loss of proteins; it also results in an undesirable change in the peak shape and chromatographic resolution [58]. A common approach to reducing these interactions is modifications in the mobile phase. Arakawa *et al.* showed that the presence of salt in the mobile phase suppresses undesirable electrostatic interactions and an organic solvent reduces hydrophobic interactions [59].

SEC is commonly used during the manufacturing and formulation of pharmaceuticals due to its high speed, reproducibility and accuracy [60], and is widely used to study protein aggregates and their behaviors. Printz *et al.* used SEC to study protein aggregation under several conditions, such as the stress caused by pH changes, temperature changes, freezing and thawing, light and shaking. They showed that each stress factor led to different patterns in the size and degree of unfolding of aggregates. These types of experiments are valuable for biopharmaceutical companies since they provide information that can be used to control and minimize protein aggregation and therefore, increase the stability of the products [13]. Although SEC is mainly applied to so-called soluble aggregates, it can also be used to confirm the presence of large and insoluble aggregates. Barnard *et al.* used indirect SEC to study the formation of subvisible particles during freeze-thawing of an IgG_2 monoclonal antibody by comparing the loss of area in the treated sample versus the control sample [61].

Although SEC is a relatively fast and robust, high-throughput method, it is still considered a low-resolution technique. To improve the resolution, smaller particles are used in the packing of the stationary phase in the column. Fekete *et al.* evaluated how three different particles sizes for packing influence the ability of SEC to separate protein aggregates. They reported that, on average, sub-2 µm particles had two- to five-times lower plate height values than the 3- and 5-µm particles used for column packing. The lower plate height values of the sub-2 µm particles represent higher column efficiency. They also demonstrated that, in the practical plate number range, the use of sub-2-µm particles reduces the analysis time [62].

SEC is capable of detecting and characterizing a wide range of small aggregates, but sometimes even modifications of the stationary phase and the mobile phase cannot provide adequate resolution. To overcome this limitation, SEC can be combined with other techniques. For example, Moneeruddin *et al.* coupled SEC with native ESI–MS to characterize the commercial protein samples that were forming small aggregates. The ability of native ESI–MS to resolve different protein assemblies based on their masses helps to overcome the resolution constraint in SEC, and the separative power of SEC eliminates the need for purifying and desalinating the sample before analysis, along with reducing concerns about the co-elution of proteins. Thus, the combination of these two techniques provides a robust and powerful method for the analysis of biopharmaceutical products [63].

SEC can also be complemented by the addition of a variety of detectors, such as those for UV, fluorescence, light scattering and RI [60]. These detectors are used to draw more information from the sample. Printz *et al.* used SEC to separate monomers from oligomers, a UV detector for their quantification and fluorescence to observe structural changes in the proteins. Hence, combining SEC with UV and fluorescence detection helps to distinguish the different types of particles that form under different stress conditions [13]. SEC can also be coupled to DLS to perform absolute SEC, which provides rapid and direct measurements of protein size without the need for costly and laborious column calibration. This method can be further combined with multi-angle light scattering (MALS) to investigate the mechanisms of aggregation [64].

SEC-RI-MALS setup is used to determine the absolute molar mass and does not need any calibration. To have access to information on size, it requires the determination of the RI increment (dn/dc) which is a time-consuming step (prior dialysis of the sample against the eluent to operate at constant chemical potential).

SEC-RI-MALS-viscosimeter is a triple detection setup and evaluates the molar mass and size of particles which still requires the determination of the (dn/dc) increment.

In some cases, depending on the type of detector, calibration curve is needed to derive the absolute value for molar mass or the size. Standard and universal calibration methods are mostly used with SEC. Standard calibration which is obtained by the logarithm of the molecular weight versus the elution volume ($\log[M] = f[V_e]$) and it requires only a concentration sensitive detector such as RI or UV. This calibration curve is prepared by using standards of known molar mass which ideally should have similar chemical nature as the solute. However, the obtained elution volume obtained for the particle of interest is not accurate because the molecular size of different types of particles cannot simply be related to the molar mass [29]. Universal calibration is another method in which the logarithm of the hydrodynamic volume versus the elution volume is plotted ($\log[R_h] = [V_e]$). Since, SEC separation is governed by the size of the particles in dilute solutions, the hydrodynamic volumes of all species eluting at the same elution volume are assumed to be identical. Universal calibration is implemented on SEC-UV or SEC-RI setups and it requires standards of known size (R_h) in the same conditions. The universal calibration can also be used with a SEC-RI-viscosimeter equipment (with standards of known molar mass) by plotting $\log([\eta]M) = f(V_e)$ where $[\eta]$ is the intrinsic viscosity derived from the online viscosimeter. The universal calibration was first introduced by Benoit *et al.*, who plotted $\log([\eta]M)$ versus the elution volume and obtained the calibration plot for polymers of various chemical composition [65].

Electron microscopy

Electron microscopy (EM) is a powerful technique that provides direct visual information about the size, shape and aggregation state of a sample. Thus, it has a wide range of applications in the studies of protein therapeutics [66]. Electron microscopes are analogous to light microscopes in principle, but the irradiation source differs; the former uses a beam of electrons while the latter uses a light beam. Electrons have a shorter wavelength than visible light, and for this reason, electron microscopes can provide significantly higher-resolution images [67]. The instrument consists of an electron gun, an electromagnetic lens, which focuses the electron beam on the specimen, and a detection system, which is normally a fluorescent screen. The entire apparatus is contained in a vacuum to prevent any interactions between electrons and air molecules [68]. Figure 4 shows the setup of a conventional electron microscope.

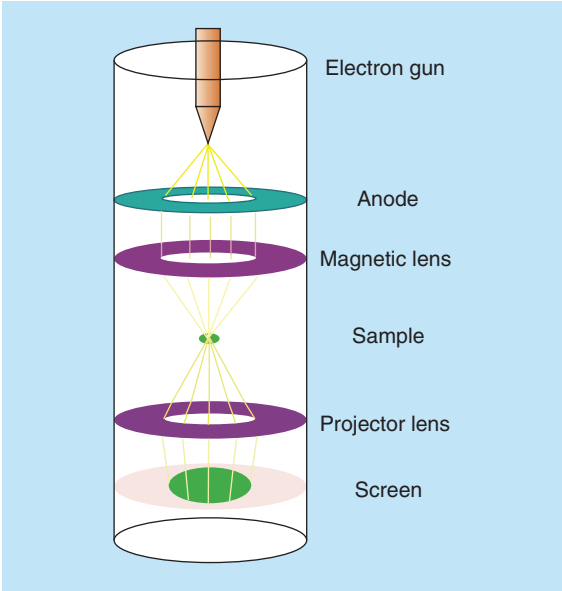

Figure 4. Electrons emitted from the electron gun are directed by the anode to the magnetic lens, which focuses the electron beam on the sample. From there, a projector lens projects the passing beam onto a fluorescent screen for detection.

The scanning electron microscope (SEM) and the transmission electron microscope (TEM) are two of the most commonly used electron microscopes. TEM was the first developed electron microscope, and it detects the transmitted electrons that pass through a thin sample slice. On the other hand, SEM uses an electron probe to scan the surface of an object, and the scattered electrons are detected to form the image. TEM offers greater magnification and higher resolution, while SEM provides more information about the 3D structure of a particle [69].

Electron microscopes can be used to investigate the structural change before and after a sample is fluorescently labeled. For example, the localization of amyloid β can be studied by fluorescence microscopy after they are labeled. TEM can answer the question of if labeling the proteins alters the structure of the protein. For this reason, Valeri *et al.* evaluated how fluorescent tags affect fibrils grown from both AD-associated peptides and Parkinson's disease associated proteins. Their results showed that, in most cases, the fluorescent tagged proteins are comparable with the native proteins of interest [70].

Electron microscopes are often used to study biological samples, such as proteins, cells and viruses, in their native state. However, the interaction between high-energy electrons and the studied molecules may disturb molecular structure, potentially leading to bond breakage, the formation of free radicals and loss of secondary structure [71]. Proper sample preparation is necessary to alleviate these adverse effects. Negative staining is a commonly used method during of which the particles are coated with a reagent containing heavy atoms. Although this approach prevents the interaction between electrons and the target organic molecule, it may cause other problems, such as the loss of the internal structure information and the presence of artifacts in the final image [72]. Cryo-EM is another sample preparation technique that can be used to prevent the adverse effects of high-energy electrons. In this method, the sample is studied at cryogenic temperatures, usually achieved with either liquid helium or nitrogen, which maintains the natural environment of the sample [73]

The rapid cooling in cryo-EM traps the macromolecules in their native state and therefore allows the elucidation of their natural conformational distribution and spatial arrangement [74].

Cryo-EM has attracted a lot of attention and has been widely used in biological studies. Many researchers have used this technique to obtain information about the size, morphology, and structure of proteins and their aggregates. The application of EM to studies of amyloid aggregates and the mechanism of their formation is leading the frontier for understanding diseases such as AD. For example, Wendler and Saibil found cryo-EM to be a promising technique for studying the structure of Hsp100 proteins, which are chaperone proteins that function to reverse the aggregation process, and they have reviewed the application of cryo-EM to studies of proteins responsible for reversing the aggregation process [75]. Joyce *et al.* used TEM to study the aggregation process of IgG under freeze-thaw stress, characterizing the size, morphology and distribution of aggregates, as well as the effect of surfactant on aggregate formation. They also compared the two sample preparation techniques for EM, negative staining and frozen-hydrated states [66]. Zhao *et al.* evaluated the shape, size, and structure of HIV-1 capsid protein using 8 Å resolution cryo-EM, which is the basis for research on capsid functions and their assemblies [76]. Cryo-EM also has applications to studies of drug delivery with liposomes or lipid nanoparticles [77]. Fox *et al.* used cryo-EM to explore the interaction between antigens and an anionic liposome, which forms flattened liposomes consisting of opposing bilayer disks, and they compared these results with those obtained from light scattering techniques [78].

EM techniques are still considered the gold standard in the study of different types of materials. They cover a large size range, from nanometer scale to millimeter scale, and can provide high-resolution images for the chemical composition of a particle [19,74]. Although EM provides an enormous amount of information about specimen shape, atomic structure, composition and surface fea-

tures, as well as powerful magnification, this technique still has certain disadvantages that need to be considered. The most notable limitation is the price of the instrument and its maintenance. Furthermore, the microscope requires a large area that is protected from any vibration and sources of unintended influence for the electrons. The sample preparation is also time-consuming and laborious, not to mention that most of the techniques associated with EM require specialized training [79].

Taylor dispersion analysis

TDA is a rapid and absolute method for determining diffusion coefficients, from which the hydrodynamic radius of a molecule is calculated. The method was first developed by Taylor in 1953 and then modified by Aris in 1956 [80,81]. Taylor dispersion is achieved in a long, narrow capillary tube. For this reason, CE instruments, which use narrow bore tubes to separate molecules, are particularly suitable for performing TDA. In CE, only a few nanoliters of the sample is usually injected, which is advantageous for the analyses of samples with low availability, for example, therapeutic proteins or drug delivery systems [82,83]. As a result, TDA has recently attracted a lot of attention for studying valuable biological samples. In addition, in theory TDA does not require calibration as it is an absolute method, which makes it more appealing than other sizing techniques, such as SEC. This technique can be used to study a wide variety of particles, from small molecules such as amino acids, peptides, proteins, to macromolecules, polymers, nanoparticles and liposomes [84–86]. It also applies to nonaqueous solutions, which makes it suitable for the characterization of hydrophobic compounds [87]. Furthermore, TDA can measure a wide range of sizes, from an angstrom to a few hundred nanometers [82].

TDA is based on recording the concentration profile of a solute as a function of time at given positions. This describes the longitudinal dispersion of a small solute plug in a narrow, open tube under Poiseuille laminar flow as a combination of radial diffusion and axial convection. The longitudinal dispersion results in peak broadening, which can be quantified by fitting a Gaussian function to the concentration profile and measuring the temporal variance (σ^2). The diffusion coefficient is estimated using Equation 5.

$$D = \frac{R_c^2 t_0}{24\sigma_t^2}$$

Equation 5

The diffusion coefficient (D) can be accurately determined from measurements in a straight capillary under two conditions: first, longitudinal diffusion is negligible and second, the analyte is well mixed across the capillary of radius R_c during the time of flow [88]. Figure 5 depicts the longitudinal dispersion of a sample along the capillary column.

Since this technique is based on measurements of temporal variance of the elution profile, other factors that influence peak variance may affect the accuracy of the results. The use of a two detection window system is a good approach to tackle this problem [89].

The diffusion coefficient can then be used to calculate the hydrodynamic size according to the Einstein–Stokes equation (Equation 2). For mixtures, TDA gives the average of diffusion coefficients of its components. Cottet *et al.* evaluated the classical TDA approach for calculating the average diffusion coefficients. They showed the average diffusion coefficient value obtained during the experiments depends on the type of the detector use (mass concentration or molar concentration-sensitive detector). Equation 6 shows the weight-average hydrodynamic radius using TDA with a mass concentration-sensitive detector.

$$R_{h,\,avs}^{mass} = \frac{\sum_i N_i M_i R_{h,i}}{\sum_i N_i M_i}$$

Equation 6

Where N_i is the number of moles of macromolecules of molar mass M_i and hydrodynamic radius $R_{h,i}$ the in the injected sample [90].

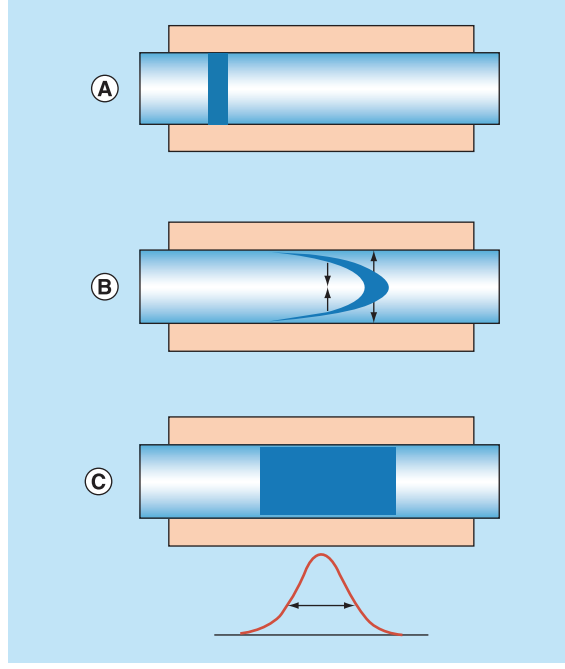

Figure 5. Taylor dispersion of a sample plug in an open tube capillary column. (A) Insert sample. **(B)** Pressure is applied which forms parabolic flow. **(C)** The combination of axial convection and radial diffusion leads to longitudinal dispersion.

Cottet *et al.* compared the weight average hydrodynamic radius with the z-average value obtained by DLS (Equation 7) for five generation of Dendrigraft poly-L-lysine.

$$R_{h,avs}^{DLS} = \frac{\sum_i N_i M_i^2}{\sum_i \frac{N_i M_i^2}{R_{h,i}}}$$

Equation 7

They demonstrated that the presence of aggregates affect the DLS results more significantly and therefore TDA is a better candidate for these types of studies [91].

Also, for complex mixtures, the possibility of the inline coupling of CE to TDA is investigated. This technique combines the powerful separation of CE with the size characterization of TDA and offers a promising method for protein analysis [92].

The diffusion of drug substances is an essential part of the drug absorption and elimination processes that occur within the human body. Over the past 10 years, numerous studies have assessed whether TDA can be used to characterize drug diffusivity, size and charge. Ye *et al.* studied the possibility of using TDA to evaluate the diffusion coefficients of three different drugs (lidocaine, ibuprofen and bupivacaine) in various pharmaceutical solvents. They showed that drug diffusivities decrease as solvent viscosity increases [93]. Likewise, Hulse *et al.* evaluated the use of TDA in measuring the sizes of therapeutic proteins and small molecules [94]. Jensen *et al.* used TDA to determine the apparent diffusion coefficient, size and self-association of insulin over a broad range of concentrations. They took the study one step further and investigated the release kinetics of insulin in agarose hydrogel, which mimics the subcutaneous tissue environment. They observed that the diffusivity and transport of insulin changes with pH and/or concentration [95].

TDA is also used to characterize the size and charge of drug delivery systems that carry small drugs or therapeutic proteins. These systems maintain a constant drug dose over a long period of time and achieve a controlled release of both hydrophilic and hydrophobic compounds once they reach the target [96]. Ibrahim *et al.* used TDA and CE to characterize the size and charge of nanogel based drug delivery systems containing the hydrophobic groups of vitamin E [89]. Oukacine *et al.* used TDA to measure the hydrodynamic radii of drug-loaded polymeric micelles [97]. TDA has also been applied for the sizing of biopharmaceutical microemulsions used for drug formulation. In this application, a UV or fluorescent marker is used to trace the nano-sized oil drops. Comparison of DLS and TDA in this study provides valuable information on the polydispersity of the microemulsions [98,99].

TDA has also been involved in the study of protein aggregates, which is critical for the batch-to-batch monitoring of pharmaceutical products [83]. In addition, the immunogenicity assessment of a therapeutic product, for example, evaluates whether aggregates are present at various points of the drug formulation. Lavoisier *et al.* reported that TDA can be used to monitor the presence of aggregates in a series of antibodies; they showed that this technique can identify different types of antibodies and aggregates, with applications for evaluations of the consistency of final products [100]. In another study, Hulse and Forbes heat stressed BSA proteins to induce aggregation and then measured the size change using TDA. They showed that this technique is capable of detecting both monomers and aggregates [83]. In further experiments, they monitored the aggregation process over time and compared the results with DLS [94]. The applicability of this method to both small particles and large molecules makes it a good candidate for evaluating the progress of aggregation when both monomers and oligomers are present in a solution. It can also provide quantitative information about the conversion rate of proteins to aggregates under different conditions. In this way, it is a promising method for monitoring a mixture of proteins and their aggregates. To draw even more information from a mixture, TDA can employ different algorithms to estimate the polydispersity of a solution. The Taylorgram obtained for polydisperse samples is the sum of individual Gaussian peaks, each representing a given diffusion coefficient.

The cumulant analysis can be used to extract information on the sample polydispersity by giving a PDI and a corresponding log-normal size distribution. Cipelletti *et al.* applied this method to analyze moderately polydisperse polymer samples and the bimodal mixtures of these samples [82]. They also investigated the use of another data processing method, constrained regularized linear inversion, to evaluate the polydispersity of the samples. Constrained regularized linear inversion approach allows determining the whole hydrodynamic radius distribution (in addition to the evaluation of the polydispersity of the sample that can already be determined by the cumulant approach) [101].

Conclusion

This review described four sizing techniques that can be used to study the size of proteins and their aggregates. The underlying principles, applications, advantages and disadvantages of each method are presented. Additionally, a schematic illustration of each technique is provided to further demonstrate how the method

works. It has also been highlighted that the combination of these techniques expands their size range, as well as the amount of extracted information.

Future perspective

The pharmaceutical industry is shifting toward protein-based therapeutics to battle an array of human diseases. However, the quality of these biopharmaceuticals can be affected by aggregation, which could compromise product safety. In an environment where regulatory agencies require comprehensive information about the safety and efficacy of new pharmaceuticals, sizing techniques are vital in the evaluation and characterization of the protein aggregates in a sample.

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Executive summary

- Protein-based therapeutic drugs are attracting more attention as they are proving to be more efficient in treating diseases due to their resemblance to natural proteins found in the body.
- Aggregation of proteins is a concern because it is not only the cause of some diseases but also the source of inconsistency in pharmaceutical products which may also lead to immunogenic responses in the patient.
- Selecting appropriate analytical techniques for studying of proteins and their aggregates is critical, and the reviewed sizing techniques are among the top candidates.
- Dynamic light scattering and Taylor dispersion analysis report the hydrodynamic size based on measurement of the diffusion coefficient.
- Size-exclusion chromatography reports size based on the elution time of molecules related to their hydrodynamic radius.
- Electron microscopy (EM) uses EM techniques and gives information about the morphology of the molecules and Cryo-EM is costly but provides most direct observation.

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• of interest; •• of considerable interest

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Methodology

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Bioanalysis

The application of capillary microsampling in GLP toxicology studies

Aim: Capillary microsampling (CMS) to collect microplasma volumes is gradually replacing traditional, larger volume sampling from rats in GLP toxicology studies. **Methodology:** About 32 µl of blood is collected with a capillary, processed to plasma and stored in a 10- or 4-µl capillary which is washed out further downstream in the laboratory. CMS has been standardized with respect to materials, assay validation experiments and application for sample analysis. **Conclusion:** The implementation of CMS has resulted in blood volume reductions in the rat from 300 to 32 µl per time point and the elimination of toxicokinetic satellite groups in the majority of the rat GLP toxicology studies. The technique has been successfully applied in 26 GLP studies for 12 different projects thus far.

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Keywords: assay validation • capillary microsampling • end-to-end capillaries • GLP • NC3Rs • rat studies • toxicokinetics • toxicology

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Since the introduction of dried blood spots (DBS) [1], about 8 years ago, as a novel tool for analyzing small blood volumes, the field of microsampling has rapidly evolved. Even though DBS has not been able to meet all the initial expectations, it has triggered research into alternative microsampling techniques. Capillary microsampling (CMS) [2], volumetric absorptive microsampling [3], accurate weighing and dilution-assisted plasma microsampling [4], solid-phase microextraction [5] or noncapillary devices for isolating plasma from small volumes of blood samples [6] are just a few examples of these alternatives. The ever increasing sensitivity of mass spectrometry instruments has of course facilitated the reduction of sample volumes needed in bioanalysis. This trend in sample volume downsizing is particularly beneficial for toxicokinetic (TK) evaluations in small lab animals and helps to achieve at least two of the three goals of NC3Rs (replacement, refinement and reduction of the use of animals in research) [7]. Smaller sample volumes result in a reduction of animal numbers in toxicology studies since samples can now be collected from the main study animals, and TK satellite groups can be removed or at least reduced to a large extent. This allows linking toxicological findings directly to the blood levels of parent drug or metabolites. Microsampling also leads to a refinement of the blood collection procedures (ease of sample collection, no or less warming of the animals before sampling) which assumingly results in less stress for the animals.

Some of the analytical challenges related to DBS or dried plasma spots [8–11] are inherent to the nature of the sample, being dried instead of liquid. Almost in parallel with DBS, CMS was introduced [12,13] as an alternative for sampling small blood volumes. CMS enables the collection of a liquid microsample which is more compatible with the techniques currently used in the bioanalytical laboratory. Second, it enables the genera-

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tion of a liquid plasma sample which still seems to be the matrix of choice for the toxicokineticist. This also facilitates switching from traditional sampling within an ongoing drug development program as pharmacokinetic (PK) data generated by CMS can be directly compared with data obtained from conventional plasma (macro)sampling. Last, CMS of plasma is less likely to trigger questions from regulators as it is basically no different from regular plasma sampling, apart from the scaled down volumes.

We have evaluated different microsampling techniques for many years, starting with the application in discovery studies. Initially, DBS was tested, but in our hands the benefits of this technique (ease of sample collection, transportation and storage) did not outweigh some of the scientific hurdles (hematocrit effect, sample homogeneity or compound specific extraction recovery changes upon spot ageing). Therefore, it was soon decided to direct our efforts more toward liquid plasma microsampling.

The next approach evaluated was plasma microsampling with adapted small collection devices such as the Sarstedt Microvette® (CB300; ref 16.444.100) (Nümbrecht, Germany) or the Kabe capillary blood collection device (Kabe Labortechnik GK 110, ref 076604) (Nümbrecht, Germany) [14]. One drawback of this methodology is the increased complexity and workload for the animal technicians. We opted for accurate plasma aliquoting and dilution at the *in vivo* site before freezing the sample and sending it off to the bioanalytical laboratory. One could, of course, also consider to perform the accurate pipetting at the bioanalytical laboratory but it can be challenging to take an accurate aliquot out of a small plasma volume after a freeze–thaw cycle. For a juvenile toxicology study, we used this approach and were able to validate an assay with a 5-µl plasma volume albeit with an adapted pipetting technique and special attention from laboratory technicians to guarantee accurate aliquoting.

Another issue we ran into for a number of analytes when using the Sarstedt Microvette tubes was poor recovery, and due to these drawbacks the next technique looked into was CMS. The major advantage of capillaries is the accurate sample aliquot, eliminating the need for plasma pipetting at the sample collection site or further downstream in the bioanalytical laboratory. Initial investigations were performed in discovery studies using whole blood as the matrix [15] but the preference for plasma over blood PK data soon directed us toward plasma collection after centrifugation of the capillaries. There are two main techniques described in the literature: Vitrex [2,12,13] or Drummond [16] capillaries. Our preference lies with the former technique and it is currently used for TK sampling in rat GLP studies. In this paper, we aim to report on our experience with this technique. Although the CMS approach is not novel and has been extensively described before, the number of publications focused on the application for GLP studies is still limited. We feel that there is an opportunity to elaborate in more detail on some of the regulatory aspects of assay validation and sample analysis using CMS in GLP toxicology studies. Hopefully, this can help in bringing down some of the hurdles which, in our opinion, still exist in applying microsampling on a larger scale in regulated studies.

Materials & methods

The process of CMS for plasma as we have implemented it in our laboratories is described in detail below.

Blood (32 µl) was collected in 1.15 mm (internal diameter) × 1.55 mm (outer diameter) × 75 mm (length) EDTA-coated microhematocrit tubes (Vitrex Medical, ref 164113) (Herlev, Denmark). The end of the capillary which was used to collect the sample was plugged with wax two-times or three-times (sealing wax plate, Hirschmann, ref 9120101) (Eberstadt, Germany) put in an individually labeled secondary tube (e.g., Falcon, ref 352052 [12 × 75 mm, a 5-ml polystyrene round bottom test tube]) and placed in an ice bath awaiting centrifugation. The capillaries were centrifuged for 10 min at 1500–1900 × *g* (in a precooled centrifuge at 4°C to prevent the wax from melting and the sample being lost). After centrifugation, the capillary was scored and snapped just above the blood cell layer and one aliquot of an exact plasma volume was collected using a 10-µl end-to-end capillary (Vitrex Medical, ref 174313) or 4-µl capillary (Vitrex Medical, ref 174213) (Herlev, Denmark) in case of insufficient plasma volume. The capillaries for storing the resulting plasma were manipulated using a pipette holder (Brand ref 708605) (Wetheim, Germany) to avoid contamination. The plasma capillary was put in a labeled storage tube (1.0 ml, 96-Well Format Sample Storage Tube with External Thread Screw Cap, FluidX®, ref 68-1000-11) and stored in the freezer until shipment to the bioanalysis laboratory.

Upon analysis, capillaries (study samples and quality control [QC] samples) were thawed in the storage tube and washed out by the addition of 100 µl (or 40 µl in case of a 4-µl capillary) of a 0.05 M phosphate buffer solution, containing 2% bovine serum albumin (BSA, Merck, Albumin Fraction V for Biochemistry; Darmstadt, Germany). The capillaries were washed out by shaking the storage tubes horizontally for 10 min on an orbital shaker (IKA KS 260) (Staufen, Germany). Before shaking, the

tubes were firmly tapped to prevent capillaries from sticking to the storage tube walls and to ensure that the capillaries were fully immersed in the washout solution. From the resulting 110-µl sample, 22 µl (one-fifth of total volume, corresponding to 2 µl of plasma) was aliquoted into 96-well plates for analysis. QC samples were prepared in bulk in blank plasma, from which 10-µl capillaries were filled. The capillaries were then put in individually labeled FluidX tubes (Nether Alderley, UK) and stored in the freezer. Calibration standards were spiked fresh each day, directly into 96-well plates to 22-µl aliquots of blank plasma+buffer containing 2% BSA 1+10 (v/v) from DMSO spiking solutions (no capillaries). Internal standard was added to all 22-µl subaliquots (study samples, QCs and standards), and further processing was done in a similar way as with any traditional plasma assay.

To test the appropriateness of the procedure described above, a number of experiments were performed. To assess the recovery of analyte during washout of the capillary in the storage tube, diluted human plasma containing four proprietary compounds at 200 ng/ml was shaken in three tubes and cap combinations for 5 or 60 min. To maximize the interaction with the tube cap, the tubes were placed upside down during this experiment. The following materials were tested: micronic tubes (ref MP22504) with a thermoplastic elastomer push cap (ref MP53066) or with ethyl vinyl acetate push caps (ref MP82605) (Lelystad, The Netherlands) and FluidX tubes with a polypropylene screw cap with external thread (ref 68-1000-11). The concentrations of the four analytes were quantified by an LC–MS/MS assay and the results compared with the nominal concentrations.

The accuracy and precision of the volumes delivered by the end-to-end capillaries were also tested. To this end, two different types of experiments were conducted. In the first test, the volumes of the 4- and 10-µl capillaries were determined gravimetrically by filling the capillaries with water or human plasma (purchased from Bioreclamation) (Hicksville, NY, USA) using a microbalance (Sartorius MC5) (Goettingen, Germany). For the 10-µl capillaries, two different lots were evaluated with six replicates each for water and plasma. For the 4-µl capillaries one lot was tested but experiments were performed by two different laboratory technicians; again six replicates each for water and plasma. Further assessment of the accuracy and precision of the capillaries in comparison with traditional pipetting was also done indirectly by aliquoting human blank plasma spiked with the same 4 proprietary compounds at 200 ng/ml with the 2 capillary types (16 replicates each with 10- and 4-µl capillaries) or a pipette (10 µl, Eppendorf® Reference®; 8 replicates) (Hamburg, Germany). All aliquots were processed with the standardized procedure (4-µl capillaries were washed out with 40 µl of buffer instead of 100 µl; plasma aliquoted by the pipette was diluted with buffer) and subsequently analyzed by LC–MS/MS to quantify the four analytes.

Results & discussions

Selection of capillaries, storage tubes & washout solution

We collect only one single plasma aliquot from each 32-µl blood capillary with the use of a 10-µl uncoated end-to-end capillary. In case of insufficient plasma volume, a 4-µl capillary is filled. Other groups [13,17] have selected 8-µl and 4-µl capillaries but given that these have an identical length (15 mm as opposed to 29 mm for the 10-µl size); we feel this is prone to errors at the animal testing site or the bioanalytical laboratory. Moreover, the 20% extra volume collected with the 10-µl capillary may be valuable in case of a reassay or metabolite analysis.

The 10-µl plasma aliquot in a capillary is stored frozen and shipped to the bioanalytical laboratoty. In contrast to non-CMS no plasma pipetting is required for the animal technician or BA lab technician. Upon sample analysis, this sample in a capillary is further diluted with 100 µl of buffer containing 2% of BSA producing a diluted sample. The reason for this dilution is to increase the sample size to a volume that is more compatible with current laboratory techniques. It enables aliquoting a subsample large enough to allow accurate pipetting and performing reassays in case of a failing run, concentrations above the validated calibration range or assessment of incurred sample reproducibility (ISR). This dilution step is feasible because of the high doses routinely administered in toxicology studies and the high sensitivity of current LC–MS/MS instruments. However, the dilution with an aqueous solution lowers the protein content and adsorption effects could surface which may not be an issue with undiluted plasma samples. That is why we selected a buffer solution containing 2% of BSA as a first choice washout solvent. This diluent is also used with low protein samples (e.g., urine) containing highly lipophilic compounds where BSA is added to prevent adsorption. We have experienced that 2% of BSA is effectively preventing adsorption for most of the compounds we evaluated, although we have recently worked on one particular analyte with an exceptionally high logP (>7) for which this buffer was not able to produce acceptable recoveries. In this case, acetonitrile was used to wash out the capillaries which resulted in accuracies within acceptance criteria.

We have experienced that the materials of the storage tube and cap can also affect recovery. Indeed, when the capillaries are washed out by shaking the storage tubes horizontally, the analyte will come into contact with the tube and the tube cap, potentially leading to adsorptive losses. Figure 1 displays the results of the experiment to assess the recovery of four analytes during washout of the capillaries. The micronic tubes with a thermoplastic elastomer push cap result in a reduced recovery for three of the four compounds, already within 5 min, while for the same tubes with ethyl vinyl acetate push caps the recovery was closer to 100%. The loss of recovery seems both analyte and contact time dependent. For the FluidX tube no losses were observed for any of the four analytes tested, even after 1 h. The latter tubes were therefore selected as the default storage tubes in CMS. An additional benefit of tubes with screw caps is that opening and closing can be automated (e.g., using a fluidX Aperio), reducing the risk of cross sample contamination.

Finally, an important aspect to highlight is that capillaries, with a cost of around 30 cents per piece, are less expensive than some of the alternatives used for microsampling.

Considerations on the amount of blank plasma volumes used during the bioanalytical phase

The goal of microsampling is to reduce the sample volume and consequently the number of animals needed for toxicological testing. This reduction should also be taken into consideration in the bioanalytical laboratory during assay validation and sample analysis. Therefore, the amount of blank animal plasma consumed in the laboratory (and the number of animals sacrificed to produce this blank plasma) should be restricted to the absolute minimum. In the assay validation, QCs for the assessment of accuracy and precision of the assay are prepared in capillaries to reflect real study samples. The low and high QCs are also used for stability assessment. Other validation experiments, for example, the evaluation of selectivity, matrix effect or hemolysis, are performed in diluted blank plasma without capillaries. The typical volumes of blank plasma required to perform an assay validation are listed in Table 1. As mentioned previously, calibration standards are not prepared in capillaries. Instead, calibration standards from solvent-based dilutions are spiked into diluted plasma on the day of analysis. This limits the required volume of blank animal plasma. This approach will also identify loss of the analyte due to adsorption to the glass capillaries or the storage container as it would result in a systematic negative bias for the QCs. In this workflow, only 2 µl of blank plasma per calibration point is required. It goes without saying that blank plasma should not be used to wash out the capillaries. Instead, a suitable diluent is used, 2% BSA in phosphate buffer in our case, minimizing the risk of recovery loss in a low protein-content matrix.

Accuracy & precision of the volumes delivered by end-to-end capillaries

The Vitrex capillaries are produced according to very tight acceptance criteria with a tolerance of ±0.50% for accuracy and 0.75% CV for precision for volumes from 0.5 to 10 µl. As part of an assessment by the liquid microsampling team within EBF [18] a number of member companies including ourselves performed experiments to investigate the accuracy and precision of the volumes delivered by 1-, 2-, 4- and 8-µl end-to-end capillaries. These results have been posted on the EBF website [19]. Since we standardized on plasma volumes of 10 µl, we performed some additional tests with 10-µl capillaries. The results from the weighing experiment are displayed in Table 2. The table displays the average results from six replicates. These data along with the data produced under the umbrella of EBF demonstrate that the accuracy and precision of the volumes delivered by capillaries are at least as good as what can be achieved by pipettes (internal acceptance criteria for bias ±5% for volumes below 20 µl) and are operator independent. The results are slightly outside of the specifications listed by the supplier but well within our internal acceptance criteria for pipettes listed above. It should also be mentioned that weighing these small volumes of liquid contained in a capillary is not straightforward.

Further assessment of the accuracy and precision of volumes delivered by the 10- and 4-µl capillaries in comparison with traditional pipetting was also performed indirectly by aliquoting human blank plasma spiked with four analytes (using the same compounds as presented in Figure 1) at 200 ng/ml with two capillary types or a pipette and quantifying the analytes in the aliquots with an LC–MS/MS assay. The accuracy and precision are presented in Figure 2. All accuracies fell within a 98–104% range for all analytes, which is well within the acceptance criteria for a bioanalytical assay. However, for the four analytes tested, the 4-µl capillaries systematically generated the highest bias (+1.5 to +4.0%), while for the 10-µl capillaries, the bias was at the lower end (-2.0 to -1.2%). The results for the 10-µl pipette were in between those for the two capillary types (-0.7 to +1.8%). Also the variability was highest for the 4-µl capillaries (5.2–9.3%), while for the 10-µl capillaries the variation was somewhat lower (2.6–4.8%), and the pipette shows the lowest variation (1.8–3.1%). This small difference between the two capillary sizes cannot be explained by a difference

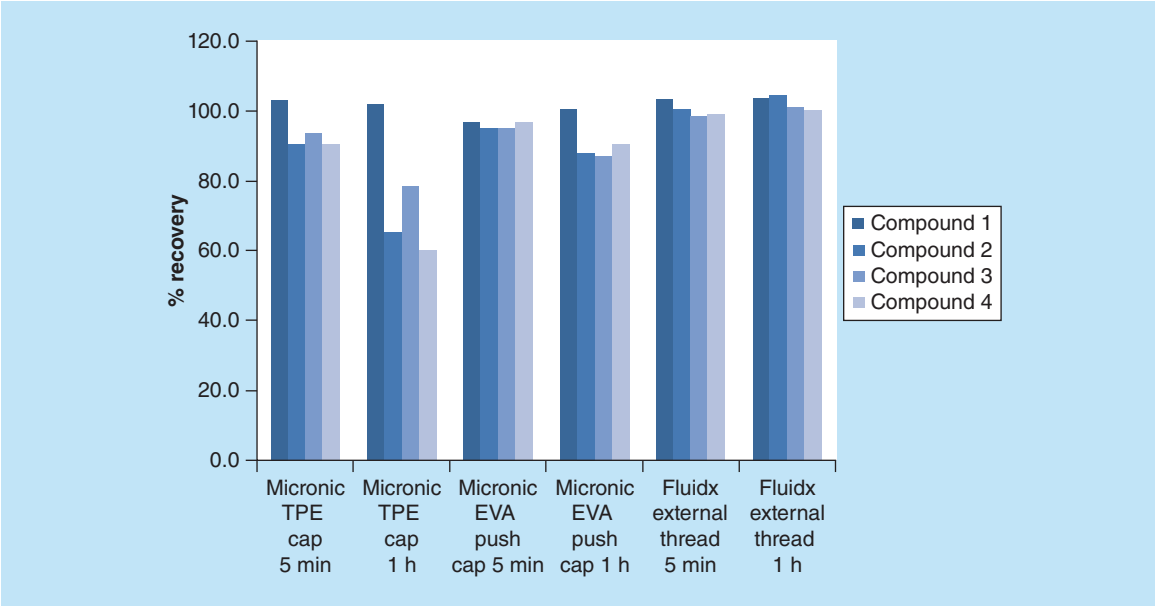

Figure 1. Recovery of four different analytes as a function of shaking time and sample storage tube during capillary washout. Plasma was spiked at a concentration of 200 ng/ml and washed out with ten parts of 2% BSA in phosphate buffer.
BSA: Bovine serum albumin; EVA: Ethyl vinyl acetate; TPE: Thermoplastic elastomer.

in accuracy and precision of the volumes looking at the results from Table 2. Also the results from the pipette cannot be explained since tests in our laboratory (data not shown) have demonstrated that the accuracy and precision of the volumes delivered by a 10-µl capillary are slightly better than those for a pipette. Of course, one should take into account that the extra washout step for the capillaries will generate an additional random error contributing to the overall error.

As demonstrated above, one of the major benefits of CMS resides in the accurate and reproducible volume delivered by the capillary. This, however, only holds true if the capillary is completely filled and does not contain any air bubbles. It is therefore very important that the technicians involved in sample collection are adequately trained to guarantee the integrity of the samples. It is possible that the plasma volume after centrifugation is insufficient to completely fill a 10-µl capillary. In that case, we have chosen to collect a 4-µl plasma aliquot. It should be clear from the study documentation that samples have been collected in 4-µl capillaries as, despite their difference in length, it is not easy to discriminate from a 10-µl to a 4-µl capillary sitting inside a labeled storage tube, and could result in reporting of inaccurate concentrations.

As discussed before, QCs are prepared in bulk in plasma and subsequently aliquoted into capillaries. During this process, the capillaries should preferentially only touch the plasma surface. When the capillaries are dipped too deeply into the plasma, this results in a positive bias. Figure 3 shows the effect of the dipping depth on the accuracy of the QCs. For this experiment, the same four analytes at 200 ng/ml plasma were evaluated. In each test, eight capillaries were analyzed using an LC–MS/MS assay. It can be concluded that dipping the capillary deeper into the plasma pool leads to an increase in bias. An alternative procedure to prepare the QCs in capillaries is to take up the plasma in a 64-µl capillary and subsequently to fill the 10-µl capillaries from this larger capillary. This procedure generates the lowest concentrations as can be seen from Figure 3. This way of filling the capillaries seems the most recommendable as it mimics the process for study samples and avoids adding an excess of plasma to the capillaries.

Assay validation

CMS assays are intrinsically very similar to more traditional larger volume plasma assays. Basically, the plasma comes into the laboratory prealiquoted as an accurate 10-µl (or 4-µl) sample in a capillary. With traditional plasma sampling, the accuracy of the sample volume is determined by the pipette used to aliquot the sample and the QCs. The pipette is regularly tested to ensure that it delivers an accurate volume. In the case of CMS, however, one has to rely on the accuracy and precision of the capillaries, and the QCs cannot serve to monitor the accuracy of the volume delivered since samples and QCs are aliquoted at different locations and by different technicians (*in vivo* site vs BA lab). As the tests in the previous section demonstrate,

Table 1. Typical blank plasma volumes consumed during a validation of a rat plasma capillary microsampling assay.

Experimental test	Volume required	Comment
QC low	1000 µl	Also used for stability experiments
QC medium	1000 µl	
QC high	1000 µl	Also used for stability experiments
QC LLOQ	500 µl	
QC over the curve	500 µl	
Calibration standards	120 µl	12 standards × 5 runs × 2 µl
Hemolysis	490 µl of plasma +10 µl of blood	
Selectivity	10 µl/source × 6	
Extraction recovery	30 µl	
Matrix effect	10 µl of plasma/source × 6	
Total	4760 µl	

QC: Quality control.

the accuracy and precision of the volumes delivered by these capillaries is very good and comparable to the performance of pipettes. Moreover, a pipette does contain moving parts which are prone to wear and tear which justifies regular testing. The latter of course does not apply to glass capillaries.

The other major difference to traditional assays is that in the first step in the bioanalytical laboratory, the sample is further diluted with an accurate volume of an aqueous solution to increase the sample size. This diluted sample is then considered the starting material for further aliquoting and processing.

The similarity to traditional plasma assays is also reflected in the validation, with the majority of the experiments being identical, although some extra validation tests are needed. For the evaluation of accuracy and precision, QCs in capillaries are analyzed in three separate runs in sixfold with an individual capillary for each replicate (i.e., not pipetting replicate aliquots from one washout QC sample). For validating the dilution of study samples, over the curve QCs are analyzed in sixfold after dilution. In this experiment, six individual QCs in capillaries are washed out and subsequently diluted to the desired ratio by adding blank diluted plasma. Currently, no separate validation experiments are performed to assess the accuracy and precision with 4-µl capillaries. In the case of 4-µl capillaries, 40 µl of buffer containing 2% BSA is added as washout diluent and 22 µl of the resulting sample is taken through the analysis. The results from Figure 2 demonstrate that the accuracy and precision data from 4-µl capillaries are very comparable with those from 10-µl capillaries. In our experience, 4-µl capillaries are only exceptionally collected in rat studies.

For the stability experiments, it can be debated whether it is really a requirement to perform the testing in capillaries. One might argue that stability is determined by the matrix and that it does not really matter if plasma is stored in a glass capillary or in some other kind of recipient. Currently, we do perform these experiments in capillaries. The 2001 US FDA Guidance [20] states that "drug stability in a biological fluid is a function of the drug, the matrix and the container system" and in this case the capillary could be considered as the primary container system. One might also question the need to validate the effect of multiple freeze–thaws in the capillary since the plasma in the capillary will theoretically only be thawed once and then immediately washed out.

Due to the initial dilution step, besides the stability experiments performed in plasma, stability also needs to be assessed in the washed out sample. In order to streamline the procedures in the laboratory, we decided to keep the number of freeze–thaw cycles and the different stability tests (bench top, long-term frozen stability) identical for the diluted plasma and the plasma in the capillary. All stability assessments are performed in triplicate with each replicate starting from an individual capillary or washed out capillary. From the data generated so far, long-term freezer storage of capillaries or washed out capillaries can be demonstrated for an extended period of time. The longest stability period that has been tested so far was 175 days and was within the acceptance limits. This confirms that even for these microsamples, a volume reduction due to freeze–drying effects (sublimation of water) is under control, at least for the period tested. This alleviates the concern that the less favor-

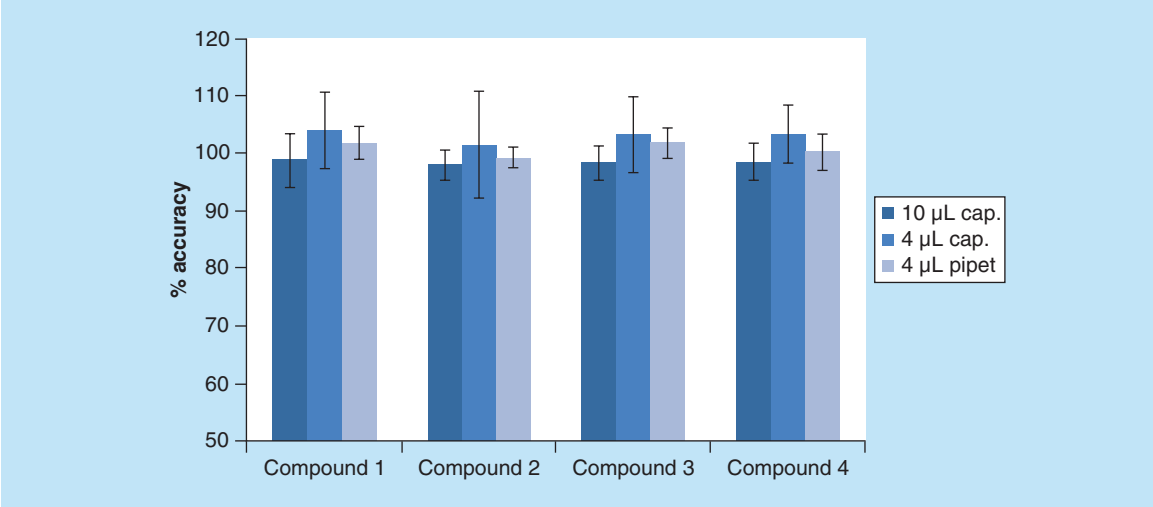

Figure 2. Accuracy and precision for quantification of four analytes spiked at 200 ng/ml in plasma in a 10-µl volume aliquoted with capillaries (n = 16) or pipette (n = 8) or in a 4-µl volume aliquoted with capillaries (n = 16).
Error bars represent 1 SD.

able volume/surface ratio for microsamples could impact the long-term freezer stability [18].

As mentioned previously, all other validation experiments (selectivity, matrix effect and hemolysis) are performed in diluted plasma without capillaries to limit the amount of blank plasma. These experiments are not different from the tests run for a traditional validation. To date, 12 assays (all in rat plasma) have been validated without major issues.

Sample analysis

In sample analysis, six Individual QCs (at three concentration levels in duplicate) for batch acceptance are washed out from six separate capillaries. In case of an eventual reanalysis or ISR experiment, a new aliquot is taken from the washed out study sample. For the QCs in ISR or reanalysis runs however we decided to use a fresh set of washed out capillary QCs. We realize that in this case the QCs are treated differently from the study samples but it avoids discussion as from which run the washed out QCs should be selected in case of reanalysis or ISR. The effect of freeze–thawing and frozen storage of the washed out sample is assessed during the validation of the assay. As ISR is executed on another aliquot of the washed out plasma sample and not on the original plasma sample, the reproducibility of the initial dilution step cannot be assessed. Alternatively two 4-µl plasma capillaries could be collected from each blood sample, instead of one 10-µl capillary, but this will generate double the amount of samples

Table 2. Accuracy and precision (% CV) of volumes delivered by Vitrex end-to-end 10- and 4-µl capillaries.

Repl.	Vitrex Lot # 1906556, 10 µl		Vitrex Lot # 1812543, 10 µl		Analyst #1, 4 µl		Analyst #2, 4 µl	
	Water	Plasma	Water	Plasma	Water	Plasma	Water	Plasma
1	9.95	9.89	9.79	10.1	3.89	4.02	4.01	4.06
2	10.0	9.87	9.86	9.81	3.98	4.10	3.97	4.02
3	9.85	9.90	9.82	9.87	3.99	4.17	4.04	4.01
4	10.1	10.1	10.3	9.95	4.03	4.07	4.05	4.04
5	9.87	10.0	9.96	10.1	3.99	4.08	4.07	3.97
6	10.1	9.97	9.82	10.1	4.01	4.06	3.99	4.02
Mean	9.99	9.96	9.92	9.99	3.98	4.08	4.02	4.02
Acc	99.9	99.6	99.2	99.9	99.5	102	101	100
Prec	1.18	0.89	1.91	1.35	1.21	1.28	0.949	0.738

Volumes (µl) determined through weighing using the density of water or plasma and corrected for temperature. The table displays the calculated volumes in microliters (µl).
Acc: Accuracy; Prec: Precission.

and make the sample collection process logistically more complicated and time consuming. Therefore, we argue that performing ISR on the washed out sample should be sufficient to demonstrate the reproducibility of the assay.

Because the technique is relatively new, and many of the GLP studies are outsourced, we have experienced the situation where the animal testing site was inexperienced in the use of CMS and the samples could not be collected in capillaries while the bioanalytical lab only had a validated CMS assay available. In this scenario, the bioanalytical laboratory aliquoted 10 µl of the received plasma with capillaries or with a regular pipette and used the validated assay with QCs in capillaries for batch acceptance. Both workarounds are not ideal but this can be considered as growing pains and will hopefully be resolved when the technique gains momentum and most sites are trained in performing CMS.

Experience in GLP studies to date

Our experience with CMS in the GLP environment so far is mainly in rat general toxicity studies. We are also currently implementing the technique in reproductive and juvenile toxicity studies as well as in other species such as mouse, rabbit and mini pig. The use of CMS is driven by bioanalytical and toxicological considerations [21]. For instance, the presence of unstable metabolites could preclude CMS, although there may be workarounds like washing out the capillary with a solution containing a stabilizer at the site of collection. The request for an assay with a lower quantification limit in the pg/ml range may be another showstopper for microsampling.

For projects that are already further advanced in development, the consideration is made on a case-by-case basis to make the switch to CMS as it does require a new assay validation. If the switch is made within a program from macro- to micro-sampling using CMS, we feel that a cross-validation is not required; both assays are validated according to regulatory acceptance criteria, and the matrix, plasma, is not changed. As already mentioned, CMS is simply the next step in scaling down the volume required for the bioanalytical assay.

So far, 26 rat GLP studies have been conducted for 12 different projects. Assay validations and sample analysis have been conducted in the internal laboratory and at three different CROs with in-life sampling in the internal toxicology group as well as at two different CROs. The feedback from animal technicians is generally that the sampling is relatively easy and because no warming or anesthesia is required, it results in reduced stress for the animals. In these studies, there were no issues with sampling due to coagulation of the blood in the capillaries.

The introduction of CMS has reduced the blood volume per TK time point in the rat from typically 300 µl down to 32 µl. This enables sampling from the main study animals, eliminating the TK satellite group. For a typical 1-month rat study this reduces the number of study animals from 104 to 80. As the TK sampling is now performed on all study animals, there is a direct link between exposure data and toxicological findings in individual animals. Composite sampling is currently performed with each rat sampled at a limited number of timepoints, in line with the recommendations to keep the maximal sampling volumes over a 24-h collection below 200 µl [22]. Currently, the sampling scheme is also being further optimized by taking statistical considerations into account. It should be noted, however, that satellite animals are currently not removed from all study types. For Irwin studies, for instance, there

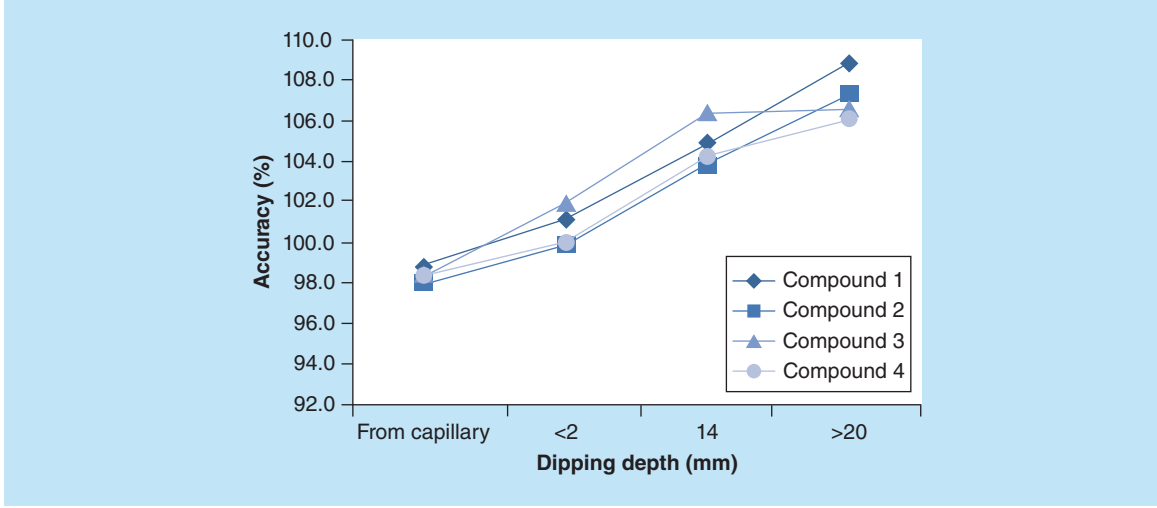

Figure 3. Effect of dipping depth when filling a 10-µl capillary on accuracy of the result.

is still a concern that sampling from main study animals could interfere with the outcome of the study by manipulations of the animals for blood sampling.

Conclusion

CMS has been implemented in the majority of rat GLP studies for new compounds in development at our company since June 2015. The technique allows the reduction of the blood volume collected at each timepoint from 300 to 32 µl and has eliminated the need for TK satellite groups in some studies or reduced the size of the satellite groups in others. CMS delivers an accurate volume of a liquid sample, compatible with current technology in the bioanalytical laboratory. Apart from some additional stability evaluations in washed out samples, the validation experiments are identical to traditional, larger volume plasma assays. Aspects like analyte adsorption in washed out samples or amount of blank plasma consumed in the bioanalytical laboratory should be taken into account. Blood volumes could be further reduced if TK were to be determined in blood instead of plasma.

Future perspective

The use of CMS will be extended to GLP studies in other species in the near future. We have already gained a lot of experience with CMS in mouse studies in a discovery setting, and the technique will soon be implemented in GLP mouse studies. Also for juvenile toxicology studies, the technique is hugely advantageous; rat pups, for instance, can be sampled from an age of 8 days without impact on growth or development. For the larger animals (e.g., rabbit and mini pig), reduction of the stress levels during sampling is the primary driver, and CMS is also considered in future studies.

We have used CMS in three clinical studies so far: in healthy volunteers, pediatric subjects and neonates. In these studies, sampling was done via finger or heel prick, and 15 µl of blood was sampled with EDTA-coated capillaries, but blood was the matrix of choice for PK assessment. Choosing blood instead of plasma as the matrix for PK assessment reduces the complexity of the sampling procedure. Specifically in a multisite clinical environment, it can be a challenge to train personnel in processing a blood capillary sample into a plasma capillary. For the application in clinical studies, the real benefit from CMS can only be obtained if blood is selected as the matrix for PK. But also for toxicology studies, the sample volumes could further be reduced if blood were to be selected as the matrix of choice for studying the TK of the compound.

Further developments of devices that enable the collection of small blood or plasma samples with limited manipulations could stimulate the breakthrough of microsampling in those clinical studies where the sample volume is critical or for at home sampling for therapeutic drug monitoring.

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.

No writing assistance was utilized in the production of this manuscript.

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

- Capillary microsampling for collecting an accurate 10- or 4-µl aliquot of liquid plasma with an end-to-end capillary has been implemented as the standard technique for toxicokinetic sampling in rat GLP studies.
- This implementation has resulted in the reduction of the blood volume from 300 to 32 µl per timepoint and the elimination of toxicokinetic satellite groups.
- Attention should be paid to the storage container + cap and the washout solution to avoid recovery losses in the low protein diluted sample.
- The amount of blank plasma used in the bioanalytical laboratory during assay validation and sample analysis should be scrutinized.
- The accuracy and precision of the volume delivered by the end-to-end capillaries are at least as good as what can be achieved by a pipette.
- Validation experiments for capillary microsampling assays are very comparable with traditional plasma assays although stability evaluations require some extra testing and considerations.
- For sample analysis some practical considerations for dealing with quality controls, reassays and ISR are proposed.

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•• of considerable interest

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Research Article

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Bioanalysis

Development and validation of a UPLC–MS/MS method for simultaneous determination of fotagliptin and its two major metabolites in human plasma and urine

Aim: Fotagliptin is a novel dipeptidyl peptidase IV inhibitor under clinical development for the treatment of Type II diabetes mellitus. The objective of this study was to develop and validate a specific and sensitive ultra-performance liquid chromatography (UPLC)–MS/MS method for simultaneous determination of fotagliptin and its two major metabolites in human plasma and urine. **Methodology & results:** After being pretreated using an automatized procedure, the plasma and urine samples were separated and detected using a UPLC-ESI–MS/MS method, which was validated following the international guidelines. **Conclusion:** A selective and sensitive UPLC–MS/MS method was first developed and validated for quantifying fotagliptin and its metabolite in human plasma and urine. The method was successfully applied to support the clinical study of fotagliptin in Chinese healthy subjects.

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Keywords: DPP-4 inhibitor • fotagliptin • human plasma • human urine • UPLC–MS/MS

The prevalence of diabetes mellitus (DM) has dramatically increased over the past decades, and the total number of people with DM was projected to be more than 360 million by the year 2030 [1–3]. Type II DM accounts for 90–95% of all cases of DM [4]. Even though there are 12 classes of antihyperglycemic agents available in market at present, optimal glucose control is still difficult to be achieved [5,6]. With the characteristics of low-risk hypoglycemia, neutral effect on bodyweight, inhibition of appetite, slowing gastric emptying and rare side effects, dipeptidyl peptidase IV (DPP-4) inhibitors is a new agent to control hyperglycemic [7].

Fotagliptin benzoate is a novel DPP-4 inhibitor which was developed by Chongqing Fochon Pharma (Chongqing, China) using the strategy of structure-based drug design [8]. Preclinical pharmacological studies showed that fotagliptin could inhibit DPP-4 with IC_{50} of 2.27 nM. The no observed adverse effect level of fotagliptin on rat and dog were 33 and 110 mg/kg, which were 100-times higher than the effective dose nine [Unpublished Data]. Based on these efficacy and safety advantages, fotagliptin was approved to proceed with clinical trials by China FDA in 2013 [8].

Preclinical studies suggested that fotagliptin was extensively metabolized. Two major metabolites (M1 and M2–1) were identified. Plasma exposure of the two metabolites was more than 1% of that of fotagliptin in rat and dog. Thus simultaneous quantification of the two major metabolites could be better cognitive the pharmacokinetic characterize the pharmacokinetic characteristic of fotagliptin in human subjects. Besides, in order to acquire excretion characters of fotagliptin, drug level in urine over time also needs to be detected. In this manuscript, we report the development and validation of a sensitive and reliable ultra-performance liquid chromatography (UPLC)–MS/MS method for simultaneous determination of fotagliptin and its two major metabolites in human plasma and

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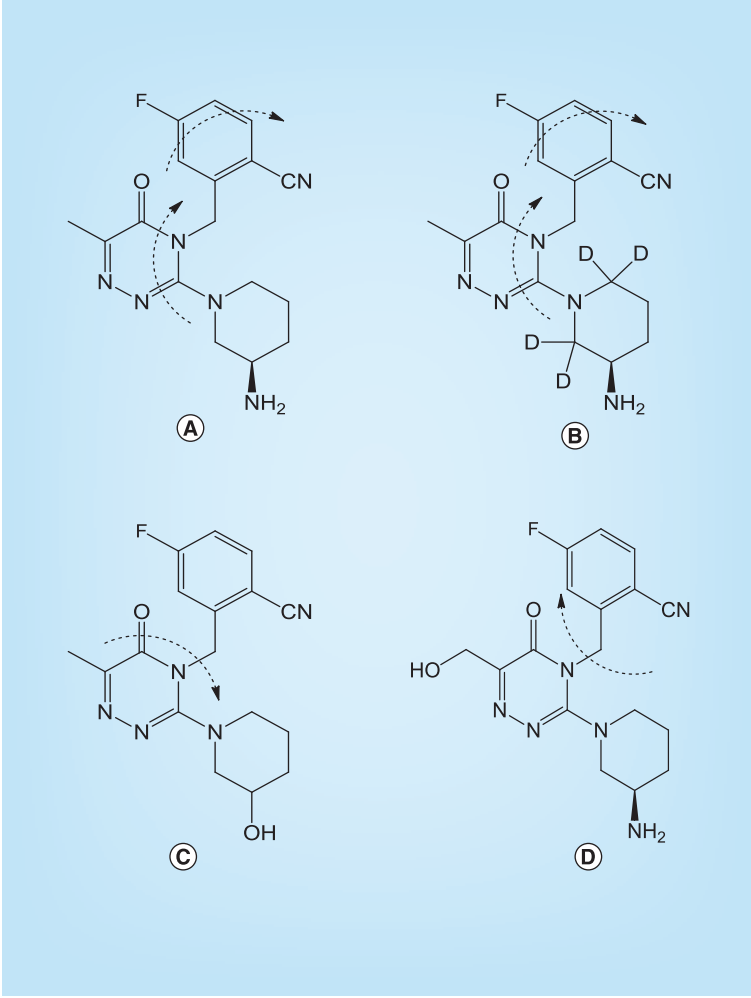

Figure 1. Chemical structures of fotagliptin (A), IS (B), M1 (C) and M2–1 (D). Arrows indicate the proposed fragmentation pathways leading to the product ions monitored in the assay: fotagliptin **(A)** *m/z* 343.3→217.1; IS **(B)** *m/z* 347.4→221.0; M1 **(C)** *m/z* 344.2→193.1; M2–1 **(D)** *m/z* 359.3→133.9. IS: Internal standard.

urine. To the best of our knowledge, this is the first study regarding determination of fotagliptin and its metabolites in biological matrices.

Experimental

Materials & reagents

The standard substance (Figure 1), including fotagliptin (purity 99.70%), its metabolites M1 (purity 99.90%) and M2–1 (purity 99.50%) and internal standard (IS) [$^2H_4$] fotagliptin (purity 99.22%), were provided by Shenzhen Salubris Pharmaceuticals (Shenzhen, China). HPLC-grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (NJ, USA). Formic acid and trifluoroacetic acid were obtained from Sigma-Aldrich (MO, USA) and J&K Scientific (Beijing, China), respectively. Aqueous ammonia and ammonium acetate were obtained from Sinopharm Chemical Reagent (Beijing, China). Deionized water was freshly prepared by a Millipore's Milli-Q system (MA, USA). Drug-free human plasma with heparin lithium as anticoagulant and urine were acquired from six healthy volunteers who had been drug free for 2 weeks.

Instrumentation

Samples were analyzed on UPLC–MS/MS system. The Acquity UPLC system (Waters Co., MA, USA) consisted of an autosampler, a column oven and a binary solvent delivery manager. The data were acquired on an API 5500 triple quadrupole mass spectrometer (Applied Biosystems, CA, USA) with analyst software v1.4.2.

Liquid chromatographic & mass spectrometric conditions

Gradient elution was carried out on a Waters Acquity BEH C_{18} column (2.1 × 50 mm id, 1.7 mm; Waters Co.) maintained at 40°C with the autosampler maintained at 15°C. The mobile phase consisted of A (5 mM ammonium acetate in formic acid-acetonitrile-water (1:50:1000, v/v/v)) and B (acetonitrile, 100%) with linear gradient elution at a flow rate of 0.25 ml/min. The elution started with 89.5% of Phase A until 1 min. At 1.01 min, Phase A was switched to 73.7% and kept to 3.00 min, then switched to 10.5% at 3.01 min and kept until 3.50 min. The total running time was 3.50 min. To eliminate carryover, the injection needle was washed with 2 ml strong needle wash solution (trifluoroacetic acid-acetonitrile [3:1000, v/v]) and 2.5 ml weak needle wash solution (formic acid-acetonitrile-water (2:20:80, v/v/v) after each injection.

The optimized source/gas parameters were as follows: curtain gas, 35 psi; collision gas, 8 psi; ion spray voltage, 5000 V; temperature, 600°C; ion source gas 1 (nebulizer gas), 40 psi and ion source gas 2 (turbo gas), 60 psi. Other mass-dependent parameters such as declustering potential, collision energy, collision cell exit potential and entrance potential for each analyte were optimized in positive mode using standard solutions. Finally, quantification was achieved using multiple reaction monitoring of the transitions of *m/z* 343.3→217.1, 344.2→193.1, 359.3→133.9 and 347.4→221.0 (Figure 1) with the collision energy 37, 35, 55 and 37 eV for fotagliptin, M1, M2–1 and IS, respectively. Dwell time for each ion was 100 ms.

Preparation of CS & QC samples

The standard stock solutions of fotagliptin/M1/M2–1 for calibration standards (CS) and quality control (QC) were prepared in acetonitrile at the concentration of 1 mg/ml separately. The corresponding working solutions were obtained by further diluting standard

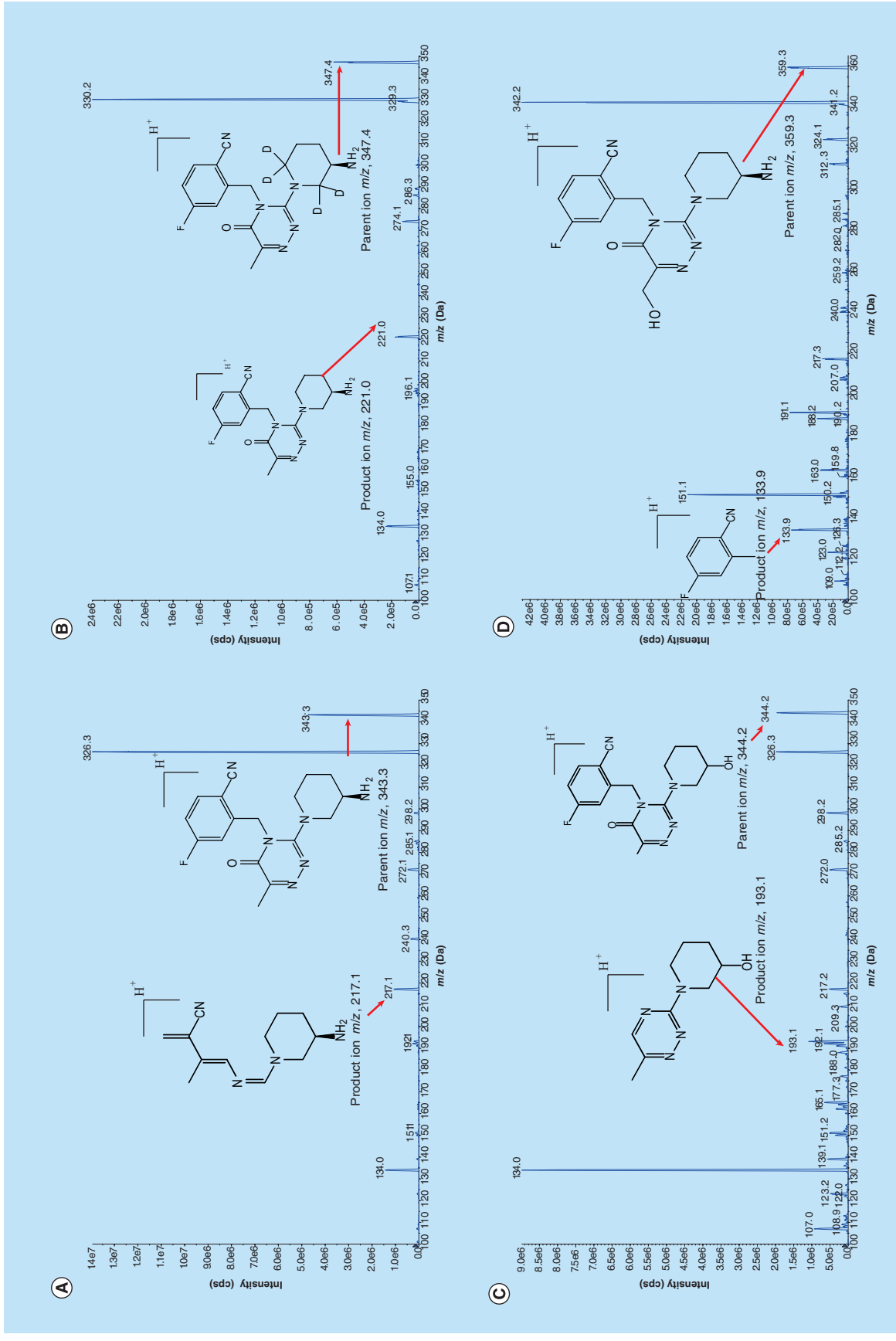

Figure 2. Production spectrum of fotagliptin (A), IS (B), M1 (C) and M2–1 (D).
IS: Internal standard.

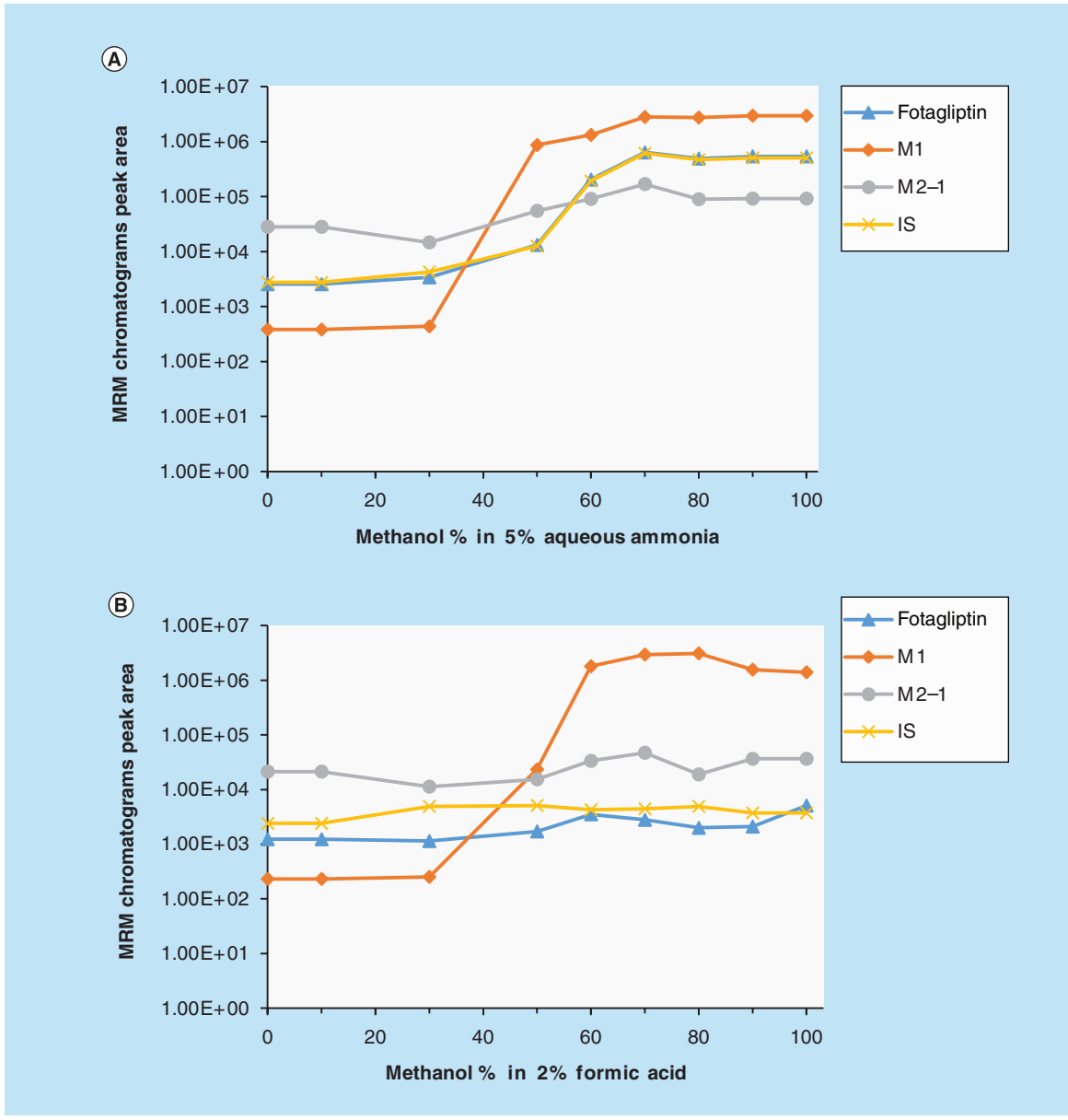

Figure 3. The peak area of fotagliptin (triangle), IS (cross), M1 (rhombus) and M2–1 (circle) for methanol% in 5% aqueous ammonia (A) and for methanol% in 2% formic acid (B).
IS: Internal standard.

stock solutions using acetonitrile-water (1:1, v/v). CS and QC samples were obtained by diluting corresponding working solutions with a dilution factor of 50-times using human plasma or urine. The fotagliptin/M1/M2–1 concentration levels of CS in plasma and urine were from 0.200/0.200/0.200 to 1000/160/1000 ng/ml and from 20.0/20.0/20.0 to 20,000/10,000/10,000 ng/ml, respectively. The fotagliptin/M1/M2–1 concentration levels of QC samples were 0.500/0.500/0.500, 20.0/5.00/20.0, 800/120/800 ng/ml in plasma and 40.0/40.0/40.0, 800/400/400, 16,000/8000/8000 ng/ml in urine, respectively. Stock solution of IS was prepared in acetonitrile-water (1:1, v/v) at a concentration of 1 mg/ml. The dilution quality control of fotagliptin/M1/M2–1 for plasma and urine were 8000/1200/8000 and 40,000/20,000/20,000 ng/ml with the dilution factor of ten-times and 50-times, which were prepared by diluting corresponding working solutions with human plasma and urine, respectively. IS working solution was prepared with water-formic acid (50:1, v/v) at a concentration of 10 ng/ml for plasma and 200 ng/ml for urine.

Sample preparation

800 µl of IS working solution were added into 50.0 µl aliquot of plasma samples. After being sufficiently mixed,

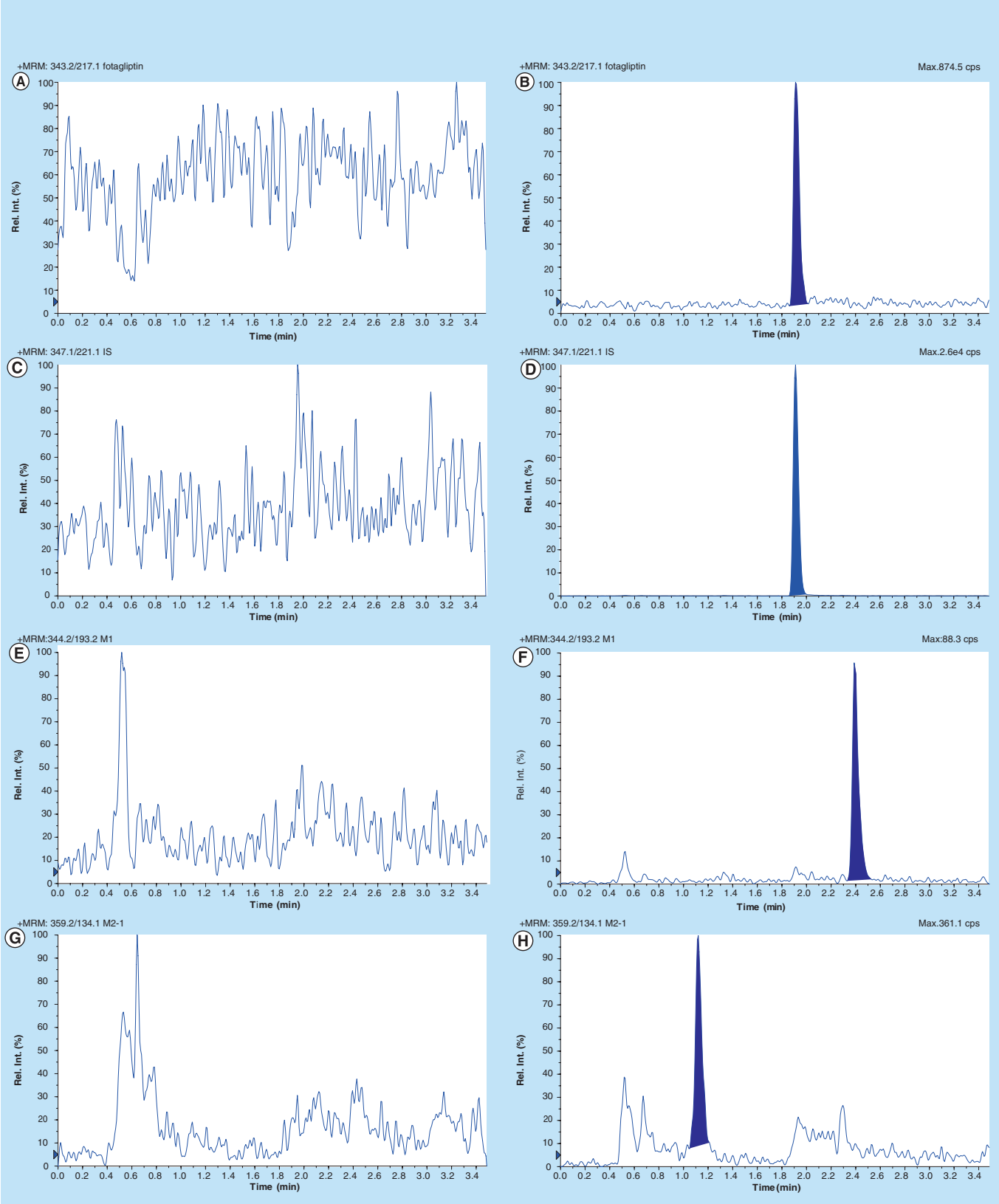

Figure 4. Representative multiple reaction monitoring chromatograms of fotagliptin, IS, M1 and M2–1 in blank plasma (A, B, C & D, respectively) and LLOQ (E, F, G and H, respectively).
IS: Internal standard; MRM: multiple reaction monitoring.

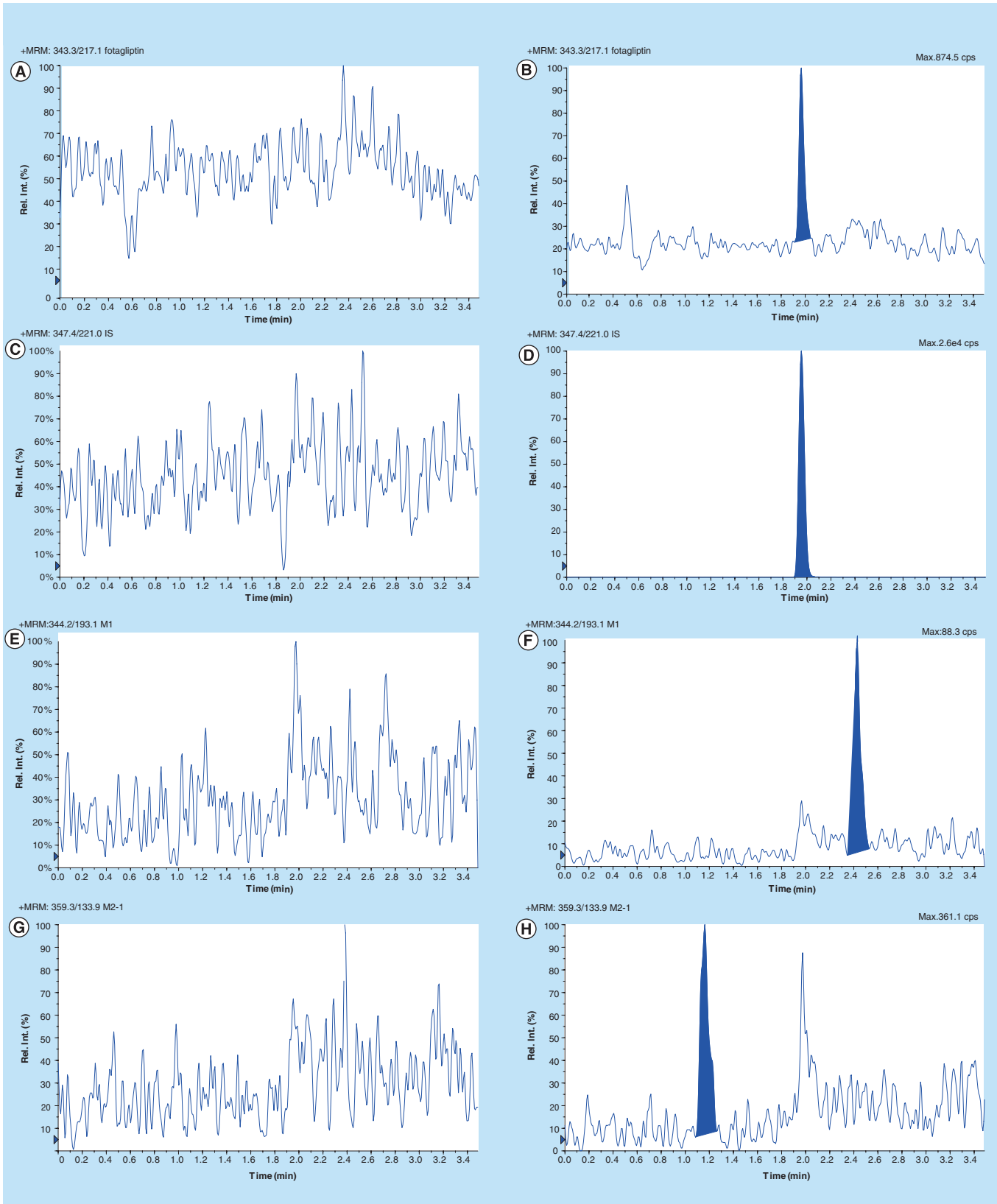

Figure 5. Representative multiple reaction monitoring chromatograms of fotagliptin, IS, M1 and M2–1 in blank urine (A, B, C and D, respectively) and LLOQ (E, F, G and H, respectively).
IS: Internal standard; MRM: Multiple reaction monitoring.

Table 1. Summary of intra-batch and inter-batch precision and accuracy of fotagliptin, M1 and M2–1 in human plasma.

Precision and accuracy	Fotagliptin			M1			M2–1		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Intra-batch (n = 5)									
Mean	0.503	19.2	780	0.489	4.46	126	0.512	18.4	828
SD%	8.4	3.7	4.8	7.0	3.1	5.1	9.8	3.8	6.8
RE%	100.6	96	97.5	97.8	89.2	105	102.4	92	103.5
Inter-batch (n = 15)									
Mean	0.469	19.5	773	0.510	4.89	127	0.524	20.4	855
SD%	9.7	2.5	2.9	7.4	7.1	4.1	9.9	7.7	5.8
RE%	93.8	97.5	96.6	102	97.8	105.8	104.8	102	106.9

HQC: High quality control; LQC: Low quality control; MQC: Mid quality control; RE: Relative error; SD: Standard deviation.

the mixed samples were purified using SPE plate (Oasis™ MCX, Waters® Co., MA, USA) on a Tomtec™Quadra 4 workstation (CT, USA). After conditioning, the SPE plate with 800 µl methanol first and 800 µl water-formic acid (50:1, v/v) second, the mixed samples were loaded to the SPE plate. Then SPE columns were washed with 800 µl methanol-water-formic acid (40:60:2, v/v/v) and methanol-water (40:60, v/v) sequentially. Then the columns were vacuumed to dryness and eluted with 700 µl methanol-aqueous ammonia (20:1, v/v). The elution was collected and evaporated to dryness under nitrogen stream at 40°C and then reconstituted with 200 µl acetonitrile-water (1:9, v/v).

Human urine samples were prepared by direct dilution. 990 µl of urine IS working solution were added into 100 µl urine samples followed by mixing. Then the mixed samples were diluted 100-times using acetonitrile-water (1:9, v/v) by the Tomtec Quadra 4 workstation automatically.

Method validation

Sensitivity, specificity, precision, accuracy, recovery, matrix effect and stability of the method were validated according to the US FDA, EMA and Chinese State FDA guidelines [9–11]. Briefly, the specificity was evaluated by analyzing interferences of human blank plasma and urine samples from six different individuals. Each blank sample was tested for interference, and compared with spiked plasma and urine sample that contained the analytes at LLOQ level. The acceptance criteria is that the deviations of the measured concentration is less than 20% of the nominal LLOQ values.

Accuracy, precision and repeatability of the method were determined from three batches. The precision was expressed as the percent coefficient of RSD (RSD%), and the accuracy was expressed as the percent of recovery% (relative error, RE%) from nominal concentration. The carryover was investigated by ana-

Table 2. Summary of intra-batch and inter-batch precision and accuracy of fotagliptin, M1 and M2–1 in human urine.

Precision and accuracy	Fotagliptin			M1			M2–1		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Intra-batch (n = 5)									
Mean	39.9	810	15,400	39.1	408	7550	36.6	376	9040
SD%	5.3	2.9	1.3	5.9	2.9	1.2	14.2	2.4	1.1
RE%	99.7	101.3	96.2	97.7	102.0	94.4	91.5	94.0	113.0
Inter-batch (n = 15)									
Mean	40.2	803	14,400	41.6	407	7320	37.9	379	8950
SD%	4.9	2.1	3.1	6.9	2.8	5.5	11.0	3.5	2.8
RE%	100.5	100.4	93.7	104.0	101.8	91.5	94.7	94.7	111.9

HQC: High quality control; LQC: Low quality control; MQC: Mid quality control; RE: Relative error; SD: Standard deviation.

Table 3. Summary of extraction recovery and matrix effect of fotagliptin, M1 and M2–1 in human plasma and urine (n = 6).

Recovery and matrix	Fotagliptin			M1			M2–1		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Plasma									
Recovery (%)	54.2	48.5	55.1	71.1	86.1	86.9	42.4	40.0	36.5
SD (%)	2.8	2.2	2.6	9.0	8.9	5.3	1.8	2.9	2.8
Matrix (%)	100.4	99.5	100.3	89.9	93.2	94.7	97.2	95.4	99.5
SD (%)	4.8	2.0	1.8	6.5	2.1	1.0	13.6	8.9	2.4
Urine									
Matrix (%)	104.2	101.2	105.0	103.7	99.9	106.7	108.3	109.3	107.3
SD%	5.7	2.3	1.2	3.9	1.9	1.9	9.9	2.0	1.1

HQC: High quality control; LQC: Low quality control; MQC: Mid quality control; SD: Standard deviation.

lyzing the double blank sample following the ULOQ sample. The peak area of each analyte in the double blank sample should be less than 20% of the LLOQ sample.

Recovery was determined by comparing the concentration of each analyte spiked before extraction with those spiked after extraction at three QC concentration levels. Matrix effects were investigated by comparing the peak area of each analyte spiked after extraction with the peak area of standards in neat solutions at three QC concentration levels.

The stability of each analyte in biological matrix and in reconstituted solution at different storage conditions were evaluated. The stability of extracted

Table 4. Summary of stability data of fotagliptin, M1 and M2–1 in human plasma.

Stability conditions	Fotaglipitin			M1			M2–1		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Autosampler stability									
Mean	0.467	19.5	753	0.519	5.02	125	0.517	20.9	876
SD (%)	8.4	0.3	1.3	3.1	1.3	1.6	14.0	1.7	1.9
RE (%)	93.4	97.7	94.1	103.8	100.3	104.5	103.5	104.5	109.5
Room temperature stability									
Mean	0.462	19.4	748	0.536	5.15	121	0.538	21.9	841
SD (%)	7.3	1.3	1.9	5.6	2.2	5.1	10.9	2.7	5.0
RE (%)	92.4	97.1	93.5	107.2	102.9	100.7	107.5	109.5	105.2
Refrigerator stability									
Mean	0.501	19.9	775	0.524	4.67	123	0.537	19.9	901
SD (%)	5.6	1.4	2.0	5.7	2.0	2.5	3.4	1.1	2.0
RE (%)	100.2	99.5	96.8	104.7	93.3	102.2	107.4	99.6	112.7
Freeze–thaw stability									
Mean	0.524	19.8	781	0.469	4.76	134	0.525	19.4	905
SD (%)	5.3	0.8	1.5	5.1	1.3	8.4	8.1	1.9	9.6
RE (%)	104.8	98.8	97.6	93.7	95.2	112.0	105.1	97.1	113.1
Long-term stability									
Mean	0.541	20.1	774	0.51	4.82	125	0.502	19.1	872
SD (%)	7.9	2.1	0.4	3.6	2.6	2.2	6.0	3.1	1.1
RE (%)	108.2	100.5	96.7	102.6	96.4	103.8	100.4	95.7	109

HQC: High quality control; LQC: Low quality control; MQC: Mid quality control; RE: Relative error; SD: Standard deviation.

Table 5. Summary of stability data of fotagliptin, M1 and M2–1 in human urine.

Stability conditions	Fotaglipitin			M1			M2–1		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Autosampler stability									
Mean	40.6	776	15,500	39.1	386	8320	41.1	371	8290
SD (%)	2.0	0.7	0.8	8.1	0.8	0.8	7.5	2.3	0.9
RE (%)	101.4	97.0	97.0	97.8	96.5	104.0	102.8	92.8	103.6
Room temperature stability									
Mean	39.9	798	14,400	40.2	411	7150	37.8	376	8740
SD (%)	6.6	1.1	1.1	6.2	2.2	0.9	13.0	4.2	1.5
RE (%)	99.7	99.7	90.0	100.5	102.7	89.4	94.4	94.0	109.3
Refrigerator stability									
Mean	38.8	792	15,660	41.1	395	7920	43.7	392	7970
SD (%)	2.7	1.8	0.7	4.8	0.8	1.6	1.9	3.8	1.6
RE (%)	97.1	99.0	97.9	102.8	98.8	99.0	109.3	97.9	99.7
Freeze–thaw stability									
Mean	40.3	778	15,600	39.3	379	8280	39.6	367	8200
SD (%)	2.7	1.1	0.5	1.2	2.1	1.2	3.9	3.4	1.1
RE (%)	100.7	97.2	97.5	98.2	94.7	103.5	99.0	91.8	102.5
Long-term stability									
Mean	39.5	802	15,700	39.1	398	7930	44.6	388	7920
SD (%)	4.3	2.2	0.7	3.9	2.7	0.8	4.1	1.7	2.2
RE (%)	98.8	100.3	97.9	97.7	99.5	99.1	111.5	97.1	99.0

HQC: High quality control; LQC: Low quality control; MQC: Mid quality control; RE: Relative error; SD: Standard deviation.

sample in autosampler at 15°C for 24 h and in refrigerator at -30°C for 7 days was assessed. The stability of fotagliptin and its two metabolites in plasma and urine sample at three QC concentration levels were examined after three cycles of freezing (-80°C) and thawing, at room temperature for 6 h, and at -80°C for 10 months in plasma and for 5 months in urine. Dilution reliability was demonstrated by analyzing accuracy of samples which were prepared by diluting from samples above the ULOQ with blank plasma or urine.

Results & discussion

Method development

In order to optimize the MS, a systematic screening and optimization strategy was used [12,13]. Both positive and negative ionization modes with ESI were tested for the detection of each analyte and the result showed that the positive ionization mode had better response. Acetonitrile produced a higher mass spectrometric response and lower background noise than methanol and thus acetonitrile was chosen as the organic solvent of mobile phase. In order to obtain the highest ion abundance, the source/gas and compound parameters were also optimized by infusing the standard solutions of each analyte with a concentration of 100 ng/ml in acetonitrile-water (50:50, v/v) into the mass spectrometer via a syringe pump with a continuous flow rate of 7 µl/min. The production scan spectrums of the compounds were depicted in Figure 2.

UPLC was used on account of better resolving power, faster analysis and higher throughput [12–14]. Isocratic mobile phases with different proportions of methanol or acetonitrile could introduce peak broadening and peak tailing. Further studies indicated that better peak shape could be obtained if the aqueous mobile phase contained 0.1% formic acid and 5 mM ammonium acetate when the column temperature was kept at 40°C using linearity gradient elution at a flow rate of 0.25 ml/min. Besides, adding 5% acetonitrile in the aqueous mobile phase could inhibit the growth of microorganisms, which was important to reduce the background. In order to eliminate carryover, weak needle wash and strong needle wash which are an additional measure equipped by Acquity UPLC system were optimized. During the initial stage of method development, carryover was obvious. Then it was founded that strong needle wash added with 3% trifluoroacetic acid and weak needle wash added with

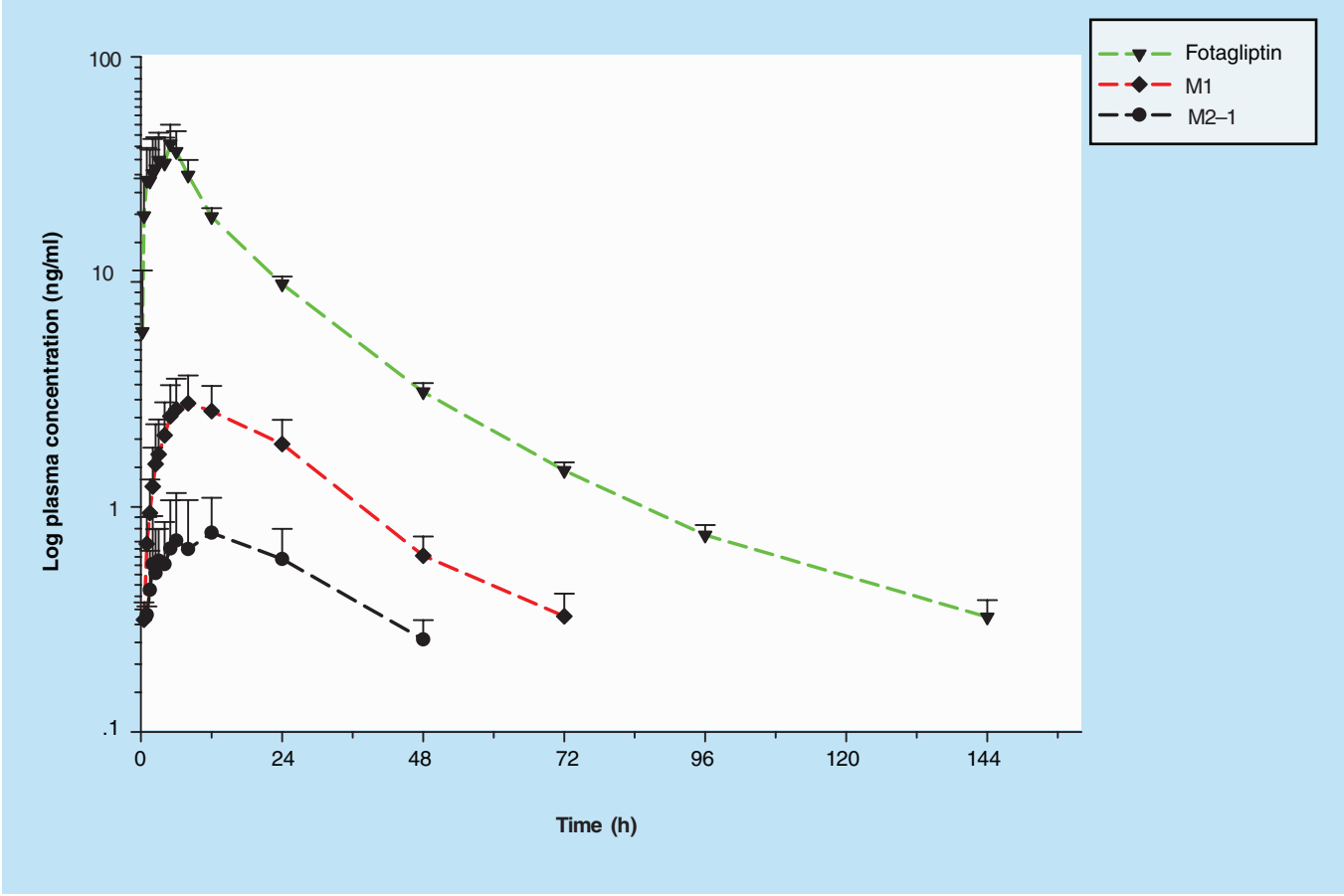

Figure 6. Mean plasma concentration–time curves of fotagliptin (triangle), M1 (rhombus) and M2–1 (circle) in six subjects after a single-dose oral administration of 12.5 mg fotagliptin benzoate capsule (mean ± standard deviation).

2% formic acid were able to eliminate the carryover favorably.

In order to get cleaner injection solution, SPE was chosen as the sample preparation method for human plasma since SPE was proved to be the most efficient method to remove endogenous compounds [15,16]. Rapid batch sample preparation for plasma could be conducted on a Tomtec Quadra 4 workstation with Oasis™ SPE family. Oasis™ MCX (30 mg, 2 ml) is a mixed-mode, reversed-phase/strong cation-exchange, water-wettable polymer which is selective for bases and stable in organic solvents. SPE condition was optimized using different proportions of methanol and water to reduce matrix effect and decrease the volume required for samples. The result showed that methanol-water (40:60, v/v) with or without 2% formic acid was the optimal wash solution, and methanol-aqueous ammonia (20:1, v/v) was the best final elution solution (Figure 3).

In order to prepare urine samples, direct dilution for 1000-times was used since urine samples are much cleaner compared with plasma. The validation results evidenced that the direct dilution method was appropriate and could meet the requirement of pharmacokinetic study.

Method validation

Linearity, sensitivity & specificity

The calibration standard was found to be linear from 0.200/0.200/0.200 to 1000/160/1000 ng/ml for fotagliptin/M1/M2–1 in plasma and from 20.0/20.0/20.0 to 20,000/10,000/10,000 ng/ml for fotagliptin/M1/M2–1 in urine. All calibration curves were evaluated using quadratic equation with a weighting factor of $1/x^2$. The correlation coefficient of all calibration curves was found to be above 0.99 and the observed concentration of calibration curve samples ranged 85–115% of the theoretical value. No significant carryover was observed for all analysis. No significant interfering peaks were found at the retention time of each analyte, which is approximately 1.91, 2.40, 1.12 and 1.91 min for fotagliptin, M1, M2–1 and IS, respectively, which suggested that the assay had good specificity. Representative mass chromatograms of blank plasma sample and LLOQ plasma sample are shown in Figure 4 and representative mass chromato-

grams of blank urine sample and LLOQ urine sample are shown in Figure 5.

Precision & accuracy

For all analytes both in plasma and urine, the intra-batch precision and accuracy ranged from 1.1 to 9.8% and 92.0 to 113.0%, respectively. The inter-batch precision and accuracy ranged from 2.1 to 11.0% and 93.8 to 111.9%, respectively. These data confirmed that the present method had satisfactory accuracy, precision and reproducibility. The precision and accuracy results for plasma and urine are summarized in Tables 1 & 2, respectively.

Recovery & matrix effect

The observed recovery for plasma samples ranged 45.8–55.1% for fotagliptin, 71.1–86.9% for M-1 and 36.5–42.4% for M2–1 at three QC concentration levels. The extraction efficiency was not concentration dependent and satisfactory for quantitative analysis. No significant matrix effect was observed in plasma or urine sample, as observed concentration of each analyte ranged within 85.0–115% of the theoretical value. This suggested that the analytical method could keep analytes free from impacts of endogenous substance in human plasma and urine. The results are presented in Table 3.

Stability

The stability tests were designed to cover the anticipated conditions the samples may experience. The stability results of each analyte in human plasma and urine are presented in Tables 4 & 5, respectively. Briefly, processed samples were allowed to stand in an autosampler (15°C) for 24 h and in refrigerator (-30°C) for 7 days for plasma samples, and in autosampler (15°C) for 36 h and in refrigerator (-30°C) for 7 days for urine samples prior to analysis. Three freeze–thaw cycles had no significant effect on the stability of each analyte in plasma or urine. Analytes were also proved to be stable at room temperature for 6 h and at -80°C for 10 months (long-term stability) in plasma, and at room temperature for 6 h and at -80°C for 5 months (long-term stability) in urine. The accuracy and precision of dilution reliability of fotagliptin/M1/M2–1 were 0.9/0.7/1.8 and 95.7/100.3/109.7 in plasma, and were 1.2/3.0/3.4 and 96.5/102.2/93.4 in urine, which indicated that ULOQ samples diluted ten-times for plasma and 50-times for urine would not affect the accuracy and precision of the method.

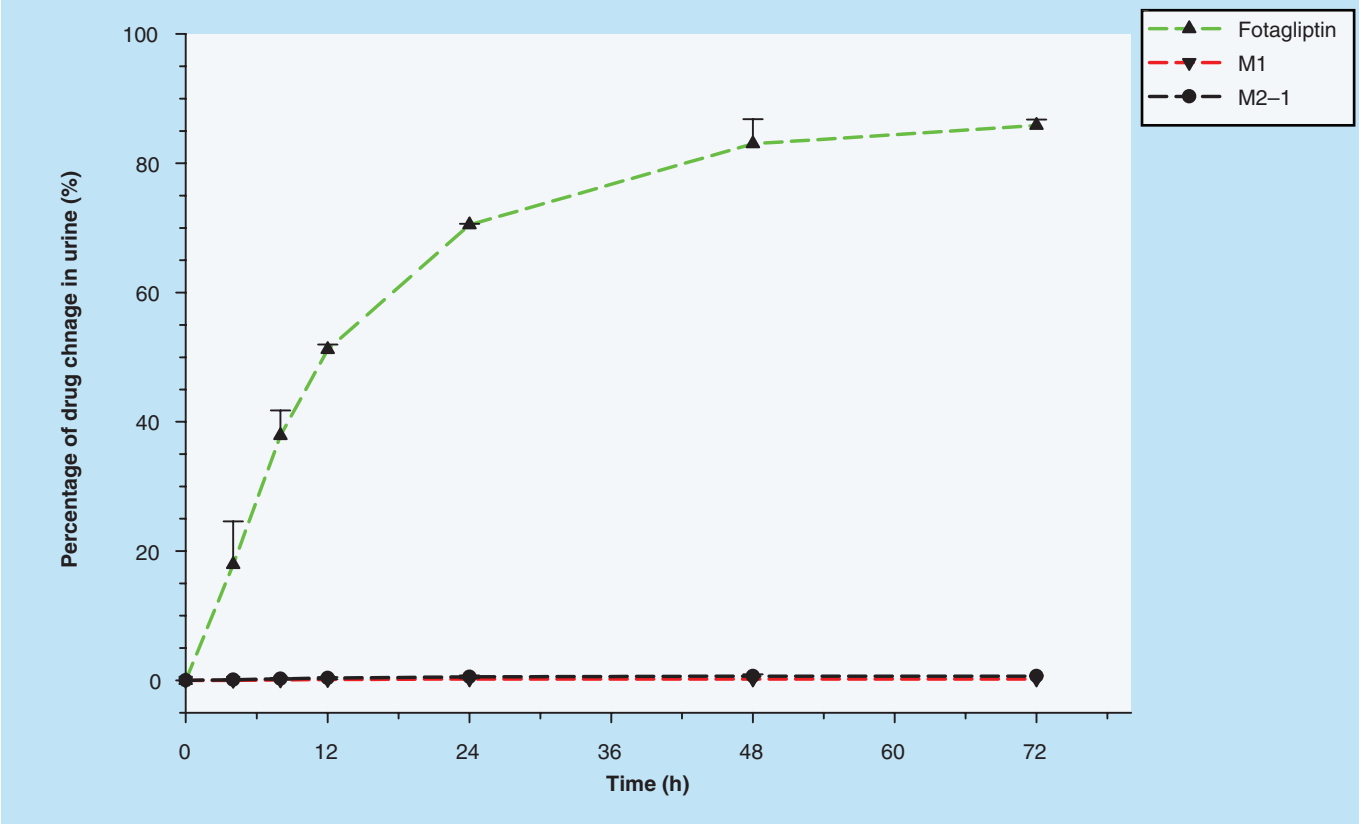

Figure 7. The mean percentage of drug unchanged in urine time curves of fotagliptin (triangle), M1 (rhombus) and M2–1 (circle) in six subjects after a single-dose oral administration of 12.5 mg fotagliptin benzoate capsule (mean ± standard deviation).

Method application

The developed method was successfully applied to investigate the plasma and urine pharmacokinetic profiles of fotagliptin, M1 and M2–1 in the first-in-human study of fotagliptin benzoate. 56 healthy Chinese subjects were enrolled in this randomized, double-blind, placebo-controlled, dose-escalation and single-dose study. The study was approved by the Ethics Committee of Peking Union Medical College Hospital, and written informed consent forms were obtained from all subjects before the study. In this study, 840 plasma samples and 390 urine samples including incurred sample reanalysis were analyzed successfully in 25 batches. No significant problems such as pressure ascending, shift in retention times and interferences were observed during the whole analysis period, which certified that the method was robust. The mean plasma concentration-time curve and percentage of drug excreted in unchanged form through urine over time for the 12.5 mg single dose of fotagliptin were shown in Figures 6 & 7, respectively.

Conclusion

A robust, selective and sensitive UPLC–MS/MS method for quantification of fotagliptin and its two major metabolites in human plasma and urine was first developed and validated. The procedure was fully validated following the FDA, EMA and Chinese State Food and Drug Administration guidelines for bioanalysis. The method has short analysis time (3.5 min) with automated pretreatment processing and adequate sensitivity (LLOQ, 0.2 ng/ml for plasma and 20 ng/ml for urine). A small sample volume (50 µl for plasma and 100 µl for urine) requirement makes this method highly suitable for clinical study. The method was successfully applied to the determination of fotagliptin and its two major metabolites in human plasma and urine to support the clinical study of fotagliptin benzoate in Chinese healthy subjects.

Future perspective

The simultaneous determination of new drug and its metabolites in human plasma and urine is essential for supporting the first-in-human study of novel drug. Therefore, a quantitative method that can be rapidly developed is always required. With the advances in technology, including both UPLC–MS/MS and automated pretreatment workstation, method development and validation have become easier. As described in this paper, our method development approach provides an example to achieve high-throughput bioanalytical assay.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles

Executive summary

Background

- The aim of this study was to develop and validate a selective and sensitive ultra-performance liquid chromatography (UPLC)–MS/MS method for simultaneous determination of fotagliptin and its two major metabolites in human plasma and urine.

Methods

- A systematic method screening and optimization approach was used to accelerate the speed of method development.
- Clinical plasma samples were pretreated using SPE and urine samples were prepared by direct dilution on an automated pretreatment workstation.
- The UPLC–MS/MS method was validated according to the international guidelines for industry bioanalytical method validation.

Results

- This method was fully validated following international guidelines. Sensitivity, specificity, precision, accuracy, recovery, matrix effect and stability were evaluated.
- The method was successfully applied to the determination of fotagliptin and its two major metabolites in human plasma and urine to support first-in-human study of fotagliptin benzoate.

Conclusion

- A selective and sensitive UPLC–MS/MS method for simultaneous determination of fotagliptin and its two major metabolites in human plasma and urine was first developed and validated. The method was successfully applied to support the clinical study of fotagliptin benzoate.

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.

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