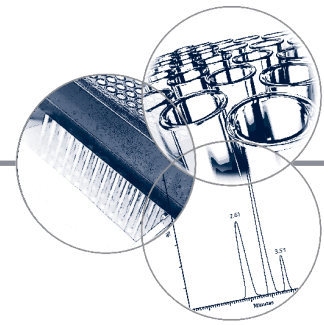

Monitoring free drug concentrations: challenges

Measurement of drug concentrations in biological samples is of utmost importance in many research areas. The information about the amount of drug in a biological sample can be given as either total concentration, which ignores the interaction of the drug with the sample matrix, or as free concentration, which shows the portion of molecules able to diffuse through membranes and exert biological activity. Although the historical trend has been towards determining total concentrations, measurement of free concentrations is becoming more important since it correlates better with pharmacological and toxicological effects. This review will discuss the most popular experimental approaches for monitoring free drug concentrations, based on the type of sample to be investigated and the kind of information to be collected. It is shown that the current challenges in measuring free concentrations are: convenience, accuracy, precision, wide applicability, availability of accurate and precise reference methods, ruggedness, and standardized sample conditions.

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Humans have always been interested in using chemical substances to cure diseases and improve on body functions. This interest has resulted in the development of several scientific disciplines, among which pharmacodynamics, **pharmacokinetics** and toxicology are the most important. All three disciplines depend on the ability to accurately measure drug concentrations in biological samples, both *in vitro* and *in vivo*.

Since most biological samples contain significant amounts of proteins that can reversibly bind the investigated drug (total concentration), some of the drug molecules in the sample will be free to diffuse through biological membranes (**free concentration**) while other molecules will be associated with protein molecules and unable to pass through membranes (bound concentration). Drug distribution throughout the body is mostly influenced by the flow of blood, which contains two main drug-binding proteins: albumin (HSA, ~662 µM) and α-1 acid glycoprotein (AGP, ~24 µM). Drug molecules that are not bound by plasmatic proteins are more easily available for excretion and metabolism (unidirectional processes) as well as for partitioning into tissues and binding to receptors (reversible processes). Accordingly, free drug concentrations are well known in pharmacology and toxicology to be more closely related to drug effect than total concentrations (Figure 1). However, most **therapeutic drug monitoring** decisions continue to be based on the sum of free plus bound drug concentrations [1]. This discrepancy is mainly caused by a historical bias towards measuring total concentrations, but also by the greater technical difficulty of accurately measuring free drug concentrations. Some authors claim that free concentrations do not need to be measured when there is a constant free drug fraction within and between individuals [2]. However, this is rarely the case: while the free fraction is fairly constant when drug concentrations are much lower than protein concentrations, the free fraction always depends on the concentration of binding protein which is seldom the same among individuals [3,4]. Furthermore, the binding characteristics of proteins can change dramatically in genetic or metabolic diseases, as well as in patients who suffer from burns or malnutrition. Also, several studies reported nonlinear protein binding even at low drug concentrations [5–8]. In such cases of unusual protein binding, the total drug concentration is not directly related to pharmacodynamic activity and the free drug concentration should be monitored instead.

This review paper will discuss the most popular experimental approaches for monitoring free drug concentrations along with their associated challenges.

Approaches for measuring free concentrations

Over the past few decades, several methods have been applied for analysis of free concentration of analytes, and most of them are based on fractionating the sample into free and bound portions followed by direct analysis of unbound molecules. Such approaches based on separation include affinity chromatography, dialysis (rapid or equilibrium), ultrafiltration, ultracentrifugation and electrophoresis. These

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Key Terms

Pharmacokinetics: Mathematical study of drug absorption, distribution, and elimination.

Free concentration: Concentration of analyte molecules that are not associated/bound to macromolecules in the sample.

Therapeutic drug monitoring: Optimization of drug therapy for minimizing adverse reactions and maximizing benefits.

Plasma-protein binding: Association of drugs with plasma proteins; considered important in therapeutics if more than 80% of the drug molecules in the bloodstream are bound to plasma proteins.

Binding constants: Equilibrium constant for the binding/association of two molecules into a complex; a high value indicates strong binding; most binding constants for association of drugs with plasma proteins range between 10^4 and 10^7 M^{-1}.

methods are generally lengthy, the investigated compounds can be irreversibly removed by the separation device (when used), and may be inaccurate if the binding equilibrium changes while the sample is fractionated. The mobile phase used by chromatographic methods is very different from the composition of biological fluids and can result in significant artefacts [9–11]. This is also the case for techniques based on mass spectrometers with atmospheric pressure ionization sources, which are applicable only when the samples are dissolved in suitable buffers, and produce useful data only if the ratio between binding macromolecule and analyte is 1:1. If the binding ratio between protein drug and is more than one, the mass spectra of the resulting complexes become too complicated and impractical.

While techniques based on immobilized proteins assume a quick shift between bound and unbound states (on the order of seconds), ultrafiltration and ultracentrifugation are applicable when the change between states is slow (tens of minutes), so the unbound molecules can be fractionated without influencing the molecules bound to the ligand. Flow-dialysis techniques are reliable, but have only been applied when the combination ratio is 1:1. CE has wide applicability and is suitable for any combination ratio between analyte and binding macromolecule, but the temperature inside the capillary can vary significantly during an experiment and the technique only works with certain compatible solvents.

One of the most recent sample preparation methods that has found applications for monitoring free concentrations is solid-phase microextraction (SPME) [4,12,13]. Although this approach based on partial separation has been used for more than two decades, it is rarely mentioned in review papers about free concentration determinations, probably because it is mainly used in research laboratories and has been applied only

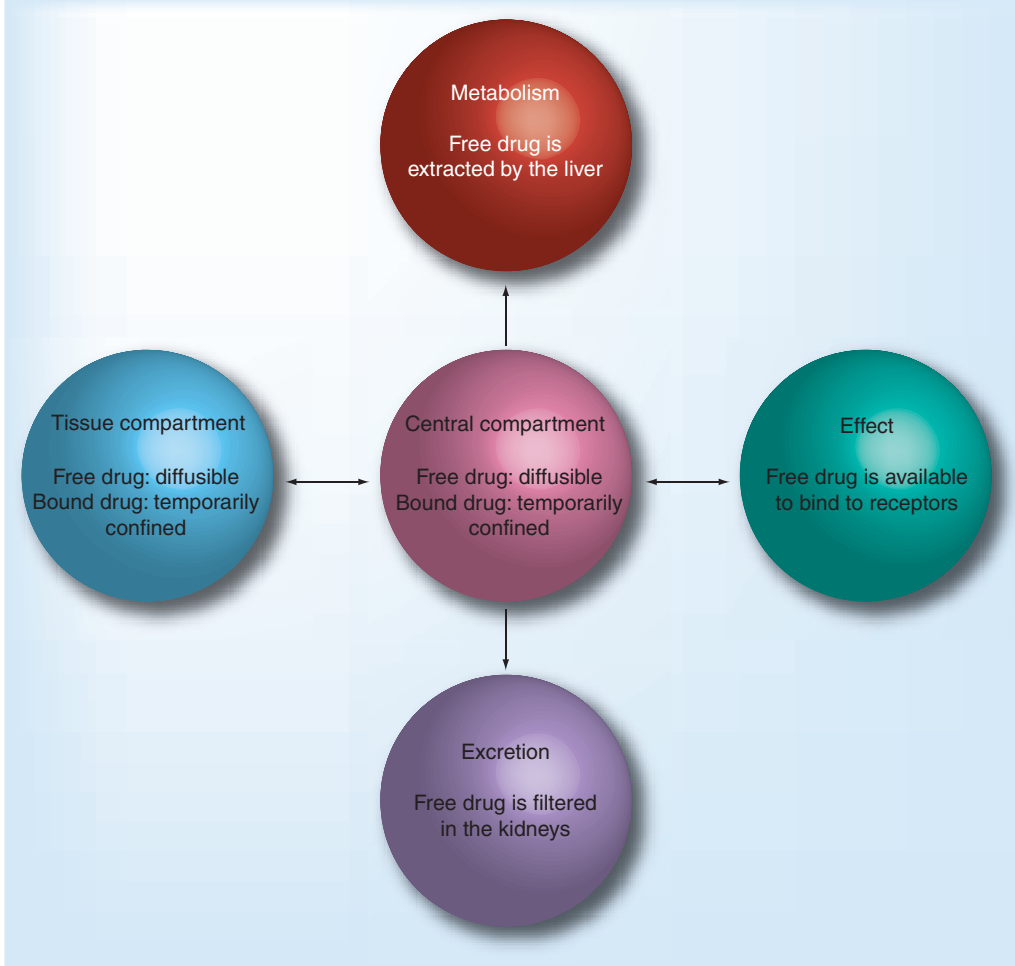

Figure 1. Drug distribution, elimination and effect are directly proportional to the free concentration that is available to diffuse through biological membranes.

recently for free concentration determinations. The main strengths of SPME are the reduced volume of the solid phase and the equilibrium-based sample fractionation, which permit the minimally invasive investigation of drug–protein association. Methods based on microextraction are applicable for complex biological samples and at a wide range of concentrations, as long as an appropriate extraction phase is chosen.

Methods that can measure free drug concentrations without separating the drug from the binding protein include surface plasmon resonance, calorimetry and spectroscopy. These methods interfere the least with the binding equilibrium, but are not suitable for complex biological samples.

An overview of selected applications based on measurement of free drug concentrations is presented in Table 1 and Table 2. All applications have clinical significance and are grouped by the sample type, analytical approach and investigated drug(s). In this paper, the free concentration assays are discussed based on their application to simple samples (isolated proteins) or complex mixtures (whole blood, plasma or serum).

Methods suitable for isolated protein samples

Investigation of the interaction of drugs with proteins is an important initial step in drug discovery, whether it is focused on the determination of **plasma-protein binding** or the interaction between drugs and receptors. While the value of plasma-protein binding is an important parameter in clinical practice, the drug–protein **binding constants** are much more important for investigating the interaction of ligands and receptors. In order to obtain accurate results, the binding constants are usually determined with purified proteins that are either attached to a solid support or dissolved in buffers that mimic biological conditions. The interactions between drugs and proteins are usually monitored based on changes in the properties of the free or attached drug molecules. Nevertheless, a few methods such as calorimetry and surface plasmon resonance depend on changes in the structure of the binding protein. A selection of the most accepted applications and methods for studying the interaction between drugs and isolated proteins is presented in Table 1.

Affinity chromatography

The greatest majority of investigations regarding binding of drugs to specific plasmatic proteins are based on affinity chromatography with the protein immobilized on column. With this approach, the investigated drug is injected as a small volume (zonal elution) or very large volume (frontal analysis) onto a column containing the protein and the profile of the eluate is monitored. The advantage of zonal elution is

Table 1. Selected applications based on measurement of free drug concentrations: methods suitable for isolated protein samples.

Analytes	Sample	Investigation	Analytical approach	Ref.
Carbamazepine	α-1 acid glycoprotein (immobilized on column)	Binding constants	Affinity chromatography (frontal analysis)	[9]
Lidocaine	α-1 acid glycoprotein and human serum albumin (immobilized on column)	Binding constants	Affinity chromatography (frontal analysis, zonal elution and competitive binding)	[15]
Amodiaquine, primaquine, tafenoquine, quinacrine, chloroquine	α-1 acid glycoprotein and human serum albumin (isolated)	Binding constants, number of binding sites	Affinity chromatography and induced circular dichroism	[10]
Phenytoin	Human serum albumin (immobilized on column)	Study of phenytoin binding to serum albumin	Affinity chromatography (frontal analysis and competitive binding zonal elution)	[14]
Propofol and halothane	Human serum albumin (isolated)	Binding constants, comparative binding	Affinity chromatography (zonal elution), isothermal titration calorimetry, hydrogen–tritium exchange and geometric analyses of high-resolution structures	[16,17]
Quinine, quinidine, naproxen, ciprofloxacin, haloperidol, paclitaxel, nortriptyline	Human serum albumin (isolated)	Protein binding study	Solid-phase microextraction and LC–UV/LC–fluorescence	[11]
Octylphenol	Bovine serum albumin, human serum albumin	Free concentration, exploration of matrix effects	Solid-phase microextraction and LC–MS/MS	[19]

Table 2. Selected applications based on measurement of free drug concentrations: methods suitable for complex biological samples.

Analytes	Sample	Investigation	Analytical approach	Ref.
Phenytoin	Human plasma	Free fraction, factors influencing protein binding	Ultrafiltration and fluorescence polarization immunoassay	[21]
Phenytoin	Human serum	Calculation of normalized concentrations in special populations	Ultrafiltration and fluorescence polarization immunoassay	[22]
Phenytoin	Human serum (pediatric)	*In vivo* binding in pediatric patients	Ultrafiltration and fluorescence polarization immunoassay	[23]
Phenytoin	Human serum (pediatric)	Influence of temperature on protein binding	Ultrafiltration and fluorescence polarization immunoassay	[20]
Phenytoin and valproic acid	Human serum	Free fraction, drug interactions	Ultrafiltration and fluorescence polarization immunoassay	[24]
Valproic acid	Human plasma	Influence of serum albumin on free fraction	Ultrafiltration and fluorescence polarization immunoassay	[25]
Valproic acid	Human plasma (pediatric)	Individualized dosing based on free concentration	Ultrafiltration and fluorescence polarization immunoassay	[26]
Valproic acid	Human plasma	Protein binding at transient high doses	Ultrafiltration and immunoassay	[27]
Valproic acid	Human serum	Usefulness of monitoring free concentrations	Ultrafiltration and fluorescence polarization immunoassay	[28]
Naproxen	Human plasma	Fraction unbound, number of binding sites	Ultrafiltration and LC–MS	[29,30]
Atazanavir	α-1 acid glycoprotein and human serum albumin (isolated and in human plasma)	Influence of protein concentrations on drug pharmacokinetics	Ultrafiltration and LC–MS/MS	[32]
Propofol, fatty acids, indomethacin, lidocaine	Human serum albumin (isolated) and human plasma	Relationship between free fraction and drug concentration	Ultrafiltration and GC–MS or LC–UV	[6,7]
Anthracyclines, taxanes	Human serum	Study of pharmacological effect	Ultrafiltration and CE	[33]
Roscovitine	α-1 acid glycoprotein, human serum albumin, blood and plasma	Bound fraction, binding protein	(a) Equilibrium dialysis and LC–MS/MS (b) Ultrafiltration and LC–MS/MS	[34]
Mycophenolic acid	Human serum albumin (isolated) and human plasma	Factors influencing the free fraction	(a) Equilibrium dialysis and scintillation counter (b) Ultrafiltration and scintillation counter	[35]
18 serotonin, dopamine transporter inhibitors	Rat brain	Relationship between free concentrations and brain receptor occupancy	Rapid equilibrium dialysis and LC–MS/MS	[37,38]
Chlorpromazine, chloroquine, propranolol, a proprietary compound	Human whole blood	New analytical approaches, prediction of non-linear pharmacokinetics	Rapid equilibrium dialysis and LC–MS/MS	[36]
Clarithromycin, levofloxacin, amiodarone, metoclopramide, gabapentin, meloxicam	Mouse plasma and adipose tissue	Free concentration in plasma vs adipose tissue	Equilibrium dialysis and LC–MS/MS	[39]
Gefitinib	α-1 acid glycoprotein and human serum albumin (isolated), as well as plasma from four species: human, rat, mouse and dog	Free fraction, binding constants	Equilibrium dialysis and LC–MS/MS	[40]

Table 2. Selected applications based on measurement of free drug concentrations: Methods suitable for complex biological samples (cont.).

Analytes	Sample	Investigation	Analytical approach	Ref.
Warfarin and phenprocoumon	Human serum albumin (isolated) and serum samples	Binding constants, influence of fatty acids	Equilibrium dialysis and spectrophotometry	[41,42]
Testosterone	Human serum	Free concentration, prediction based on equations	Isotope dilution equilibrium dialysis and scintillation counter	[43]
Ibuprofen, warfarin, verapamil, propranolol, caffeine	Human plasma	Plasma-protein binding	Solid-phase microextraction and LC–MS/MS	[4]
Diazepam, nordiazepam, warfarin, verapamil, loperamide	Human plasma	Plasma-protein binding	Solid-phase microextraction and LC–MS/MS	[46]
Vitamin D binding protein	Human serum	Association with lipoproteins	Gel-permeation, ultracentrifugation and immunonephelometry	[44]
Bilirubin	Cell culture containing human serum albumin	Cytotoxicity of free vs total bilirubin	Horseradish peroxidase assay	[45]

that it uses a small amount of sample but accuracy and precision can sometimes be low [14]. The large volume of sample used in frontal analysis allows saturation of the protein bound to the chromatographic column which results in better accuracy and precision at the disadvantage of having to use a larger quantity of drug; while this is not a problem with well-established drugs, it can prevent application of this method for newly developed compounds. Once an affinity chromatography column is prepared, it can be used numerous times for many compounds. The greatest challenges for this approach are to minimize nonspecific binding to the support and to prepare reproducible columns without altering the binding affinity and native configuration of the protein. This is usually done by using end-capped stationary phases tailored for certain compounds and by careful development of the protein immobilization technique.

A very active research group in the area of affinity chromatography from the University of Nebraska have published numerous papers describing the interaction of isolated drugs and proteins. The free concentration of carbamazepine in the presence of α-1 acid glycoprotein (AGP) was investigated using an immobilized AGP column under controlled temperature. The authors found low-affinity interactions with the chromatographic support and high-affinity ones with the protein. By correcting for the interaction with the support, the association equilibrium constant between carbamazepine and AGP at 37°C and pH 7.4 was accurately determined. Competition studies showed that these interactions were occurring at the same site that binds propranolol on AGP. Controlled-temperature studies indicated that the change in enthalpy was the main driving force for the binding of carbamazepine to AGP. Their results provided a much more complete picture of how carbamazepine binds to AGP in human serum [9]. In another study, the same research group studied binding of lidocaine to two serum proteins, HSA and AGP, using both frontal analysis and zonal elution. It was shown that lidocaine has strong binding to AGP and weak-to-moderate binding to HSA. Competitive experiments with site-selective probes showed that lidocaine interacts with Sudlow site II of HSA and the propranolol site of AGP. Their study demonstrates how affinity chromatography can be used to examine the binding of a drug with multiple serum proteins and provide detailed information on the interaction sites and

equilibrium constants that are involved in such processes [15]. A thorough investigation of the binding of antimalarial drugs to serum proteins was performed by Zsila *et al.* [10]. The researchers used multiple techniques, such as induced circular dichroism and affinity chromatography to study specific interactions between six antimalarial agents of quinoline and acridine types to AGP. Induced circular dichroism spectra showed binding of amodiaquine, primaquine, tafenoquine and quinacrine to AGP, the serum level of which greatly increases in *Plasmodium* infections. Association constant values of about 10^5–10^6 M^{-1} could be determined. Analysis of the UV spectra of the drug–AGP complexes suggested the inclusion of the ligands into the central hydrophobic cavity of the protein. Results of fluorescence experiments also supported the AGP binding of these drugs and provided further insights into binding details. Fluorescence and circular dichroism displacement experiments showed the high-affinity AGP binding of mefloquine (association constant of ~10^6 M^{-1}). HSA association constants estimated from affinity chromatography (10^3–10^5 M^{-1}) were found to lag behind those for AGP. In the case of chloroquine, no significant binding interaction was found either with AGP or HSA. The authors also discussed the pharmacological aspects of the results in great detail [10].

All three previous studies showed that chromatographic columns with immobilized AGP maintain the original binding affinity in solution and can be prepared reproducibly – which have been the main challenges when attaching AGP to solid supports.

A very carefully conducted study about the binding of phenytoin to immobilized HSA showed that the drug can interact with the protein at the warfarin–azapropazone, indole-benzodiazepine, tamoxifen and digitoxin sites. This was accomplished through frontal analysis and competitive binding zonal elution experiments, the latter of which used four probe compounds for the major and minor binding sites of HSA injected into the presence of mobile phases containing known concentrations of phenytoin. Both allosteric interactions and direct binding for phenytoin appear to take place at the warfarin–azapropazone and tamoxifen sites. It was concluded that this rather complex binding system indicates the importance of identifying the binding regions on HSA for specific drugs as a means of understanding their transport in blood and characterizing their potential for drug–drug interactions [14].

Anesthetics are a class of drugs that have to be particularly well monitored, given the high risk they pose. Since numerous investigations showed that their effect is better associated with free concentrations, numerous researchers are investigating drug–drug interactions by measuring the binding constants between anesthetics and HSA. For example, propofol and halothane are clinically used general anesthetics which are transported primarily by HSA in the blood. Researchers from the University of Pennsylvania Medical Center characterized anaesthetic–HSA interactions in solution using elution chromatography, isothermal titration calorimetry, hydrogen-exchange experiments, and geometric analyses of high-resolution structures. They found that propofol has a much higher binding affinity for HSA than halothane. The binding stoichiometry of the two drugs was also considerably different, with propofol binding to albumin in a 2:1 ratio as compared with 7:1 for halothane. Hydrogen-exchange studies in isolated recombinant domains of HSA showed that propofol-binding sites are primarily found in domain III, whereas halothane sites are more widely distributed. In addition to pharmacokinetic implications of propofol displacing halothane from some HSA sites, their data suggest that HSA might be a suitable platform for further characterization of the general anesthetic structure–activity relationship [16]. The first author of the paper continued to publish several papers on this topic. Recently, the group has published an interesting commentary about the lack of competition between bilirubin and propofol for binding sites on HSA, based on the fact that binding of these two compounds occurs at different sites [17].

Solid-phase microextraction

Solid-phase microextraction is a rapid sample preparation technique in which a small amount of extracting phase is put in contact with a sample for a controlled period of time. Because of the partial extraction approach, the method can be used to determine free drug concentrations in either negligible or non-negligible mode [18]. However, the partial extraction mode also makes the outcome of the method more susceptible to variations in temperature, time and contaminants. Also, the extraction phase must be carefully selected so that it does not adsorb (or absorb, in the case of liquid extraction phases) the binding protein. Nevertheless, although method development

must be carefully addressed, SPME allows for significant flexibility in the choice of extraction phase and analytical method, which is evidenced by the large number of publications. A researcher from Aristotle University applied this method to study the binding of seven drug compounds to human serum albumin. The preferred extraction mode was 'negligible depletion' when the concentration of drug in the sample remains almost the same after extraction. This way, the bound drug–free drug equilibria are not disturbed. Calibration curves were constructed for each drug by HPLC–fluorescence and HPLC–UV analysis. Binding of each drug to human serum albumin was studied independently. The author found his experimental results to be in agreement with literature data and ultrafiltration experiments performed in parallel, indicating the feasibility of the method for such bioanalytical purposes [11]. Since matrix effects are crucial when measuring free concentrations, Heringa *et al.* investigated the influence of proteins on the kinetics of microextraction. It was found that there is a large effect of protein presence on the kinetics of octylphenol uptake and fouling of the microextraction phase appears to occur. However, the amount of protein extracted on the probe was low and did not reduce or enhance the measured uptake of octylphenol. Based on measurement of free octylphenol, the authors successfully determined the apparent affinity constant for bovine serum albumin [19].

Methods suitable for complex biological samples

Measurement of free drug concentrations in complex biological samples is performed either to determine the extent of overall plasma-protein binding (without necessarily identifying a specific binding protein) or to assure therapeutic efficiency for drugs with low therapeutic index and high binding to plasma proteins. A selection of excellent applications on this topic is presented in Table 2.

Determining the overall degree of drug binding to macromolecules is an important part of drug development, both in the discovery and clinical phases. Although the main drug-binding proteins are HSA and AGP, plasma contains many other proteins; accordingly, most drug compounds will have a certain degree of association with plasma components. In order to investigate this interaction, a drug–plasma binding assay is mandatory.

Ultrafiltration

Perhaps the most popular separation method for analysis of free drug concentrations in complex biological samples is ultrafiltration, and this can easily be seen from Table 2. Ultrafiltration is usually the method of choice especially in clinical laboratories due to its simplicity and speed. The biological sample is usually centrifuged in tubes with semipermeable membranes and the free drug is measured in the ultrafiltrate.

Two of the most challenging problems with using ultrafiltration in clinical practice are the temperature at which the process is carried out and the specificity and sensitivity of the analytical method. Although it is now well known that changes in temperature significantly affect the extent of drug binding to proteins, most clinical laboratories continue to measure free concentrations by ultrafiltration at room temperature (25°C). In a comprehensive study, Kodama *et al.* measured the free phenytoin concentration by ultrafiltration at body temperature and room temperature and discovered a difference of 44% in binding affinity, which clearly shows the importance of controlling the temperature for such measurements [20].

Another issue is the fact that many clinical assays are based on antibodies that have cross-reactivity to drug metabolites and sometimes even to endogenous compounds. While this may not be a problem when total drug concentrations are measured, it can definitely lead to poor accuracy when the metabolites are less bound to plasma proteins than the parent drug. In this case, the ratio of drug to metabolite concentration in the ultrafiltrate can increase in an unpredictable manner, as a function of the metabolic rate and time of sample collection. Depending on the extent of metabolite crossreactivity, errors can be as high as 100%. In such cases, analytical methods based on chromatography should be used. Unfortunately, because of speed and convenience, immunoassays continue to be the analytical method of choice in many clinical laboratories.

In addition, some ultrafiltration-based assays suffer from nonspecific binding of drugs to the ultrafiltration device. However, this can easily be tested by filtering protein-free buffer solutions of known drug concentration and measuring the concentration in the ultrafiltrate. Nonspecific binding can then be determined by comparing the measured concentration with the initial one. Finally, there is a lot of variability between studies regarding the molecular mass cutoff of

the membrane used for ultrafiltration, ranging from 3 to 50 kDa. Currently, there is no consensus regarding the best experimental approach and more research is needed.

Some of the most commonly monitored free drug concentrations in clinical practice are from the class of antiepileptics. Of these, phenytoin and valproic acid are well-known drugs that were extensively studied. Iwamoto *et al.* evaluated the relationship between free phenytoin concentrations and clinical responses, as well as the factors influencing protein binding of phenytoin. Their results show that the free phenytoin concentration is more useful than the total concentration for monitoring antiepileptic effects in patients receiving phenytoin monotherapy. In addition, they found that the free phenytoin fraction was significantly influenced by aging, creatinine clearance and serum albumin levels [21]. Other researchers used the same analytical approach to validate a new equation for calculating normalized phenytoin levels for patients with abnormal serum albumin levels [22]. A team from the Miyazaki Medical College Hospital has published several papers regarding the free concentration of phenytoin in pediatric patients. Binding parameters of phenytoin to pediatric serum proteins were compared with *in vivo* and *in vitro* binding parameters in adult subjects reported by other investigators. Their results suggest that although the number of binding sites is the same, there are some differences in binding constants between pediatric and adult subjects, with adult albumin having an affinity approximately 1.2-times higher [23]. Mamiya *et al.* investigated drug-drug interactions between phenytoin and valproic acid in patients with severe motor and intellectual disabilities with epilepsy and found that hypoalbuminemia and valproate coadministratation with phenytoin increased the free fraction [24].

Valproic acid is a widely prescribed anticonvulsant and mood stabilizer. Although free concentration monitoring for valproic acid is not as well established as for phenytoin, numerous investigations point to its utility. All these investigations were performed by ultrafiltration and immunoassay. Recently, Alvarez *et al.* confirmed that the measurement of free fractions is complicated and developed an equation for predicting free valproic acid concentration as a function of total concentration and albumin concentration. The new equation was validated against experimental data obtained by ultrafiltration. Their study showed that there are significant differences between total valproate and free valproate which further confirms the need to measure or predict the free fraction [25]. Ueshima *et al.* from the Okayama University Hospital investigated the relationship between free valproic acid concentrations and efficacy in the case of intractable epileptic children. An increased unbound serum concentration of valproic acid observed in such high-dose therapy is likely to frequently cause toxicity, but the unbound concentration is rarely monitored in therapeutic drug monitoring activity, and the total valproic acid concentration is commonly determined instead. The authors showed that the unbound concentration non-linearly increased as the total concentration increased, and that unbound valproic acid concentrations in infants are generally higher and vary more widely than those in adult patients. They concluded by saying that unbound concentrations in neonates and infants should be closely monitored and used to individualize dosage regimens of valproic acid in intractable epileptic children [26]. The free concentration of valproic acid can also vary unexpectedly following rapid intravenous administration. Scientists from Abbott Laboratories and the University of Alabama characterized valproate protein binding in patients with epilepsy who achieve transient high (>150 mg/l) total plasma concentrations following rapid infusion at very high doses. This was done by measuring both total and unbound valproic acid concentrations. One and two binding-site models were explored in a nonlinear mixed effects population analysis framework. Because of the rapid administration of high doses, the authors found unbound valproate concentrations much higher than previous studies, further highlighting the importance of monitoring free concentrations [27]. Sproule *et al.* also acknowledge that interpreting total valproate concentrations can be challenging. The authors recommend monitoring unbound valproate concentrations in order to simplify interpretation of drug levels, particularly with dosage changes at higher concentrations and in elderly patients, even if albumin concentrations are within the normal range [28].

The determination of free concentrations for phenytoin and valproic acid are some of the best-known applications of monitoring free concentrations in clinical practice.

Several publications that challenge the current understanding about the calculation and measurement of free drug concentrations were written by a researcher from Genentech [5,29–31]. Many

publications show that the unbound drug fraction is concentration dependent, which should be taken into account in the interpretation of drug pharmacokinetics as well as in modeling. Naproxen binding to proteins in human plasma is considered as an illustration of the method. It is shown that the assumption that all binding sites of the protein have the same affinity yields the slowest possible concentration increase of the unbound drug fraction, while the assumption that a drug binds to a single binding site yields the highest possible value of the unbound fraction for a given drug concentration. Free drug concentrations were obtained by ultrafiltration after the drug was incubated with human plasma for 1 h. The ultrafiltrate was analyzed by LC–MS. No nonspecific binding of naproxen to the centrifugal filter devices was observed by comparison of the naproxen concentrations in plasma water and in the ultrafiltrate obtained after its centrifugation [29,30].

A thorough study of atazanavir pharmacokinetics was performed by Barrail-Tran *et al.* The authors determined the *in vitro* binding characteristics of atazanavir and also evaluated whether plasma-protein binding to HSA and AGP influences the pharmacokinetics of atazanavir in HIV-infected patients. For the *in vitro* study, atazanavir protein binding characteristics were determined in AGP- and HSA-containing purified solutions. Atazanavir was found to bind to AGP on a high-affinity saturable site and to HSA on a low-affinity nonsaturable site. For the *in vivo* study, blood samples from 51 patients were drawn prior to drug intake at week six. Atazanavir concentrations were assayed by LC–MS/MS, with free concentrations being separated by ultrafiltration. HSA concentrations, AGP concentrations and phenotypes were also measured in these patients. Their results indicate that atazanavir pharmacokinetics are moderately influenced by its protein binding, especially to AAG, without expected clinical consequences [32]. This is not unexpected, since atazanavir is less than 90% bound to plasma proteins.

Dawidowicz *et al.* recently published two papers about the anomalous relationship between the free fraction of a drug and its total concentration in drug-protein systems. Their reports are surprising, indicating that the free fraction of some drugs increases with the decrease in total concentration. They used both isolated albumin and human plasma to study the binding of propofol, fatty acids, indomethacin and lidocaine to proteins. Free drug molecules were separated by ultrafiltration through a 10 kDa molecular mass cutoff membrane and measured by LC. The experiments carried out in this study show that ligand hydrophobicity affects the dependence between the free ligand fraction and its total concentration, and that similar anomalous changes of the free drug fraction are observed not only for drugs interacting with different binding sites on HSA, but also for basic local anesthetics that bind to AGP. Their results expanded the present knowledge about drug–protein binding and are encouraging further research [6,7]. Ultrafiltration has also been combined with CE for measuring free concentrations. In a recent poster presented at Pittcon 2011, this approach was used to study the pharmacological effect of anthracyclines and taxanes. The kinetics of nonspecific binding of anthracyclines in the presence of taxane and *vice versa* was investigated. For quantitation, a novel sweeping method was developed and showed improved limits of detection for both drugs compared with previously reported methods. The proposed method was successfully applied to serum samples. The main advantages were short separation time, high peak efficiencies, and applicability for the separation of different combinations of anthracyclines and taxanes [33].

Equilibrium dialysis

While ultrafiltration is the most popular method, especially in clinical settings, equilibrium dialysis is still considered the gold standard for monitoring free drug concentrations and continues to be used in many research settings. The method is based on drug diffusion across a semipermeable membrane that separates the sample to be investigated from a buffer solution. The membrane should be permeable to the drug and not to the protein. The device is incubated until equilibrium is reached and the free drug is measured in the buffer solution. The main challenges with this method are long equilibration times (up to 2 days), difficulties for compounds with low solubility in water, volume shifts due to differences in osmotic pressure, artifacts created by electrically charged drugs or proteins, and nonspecific adsorption to the device. Although rapid equilibrium dialysis devices have been introduced, the equilibration time is still long, up to 6 h. Furthermore, this quick equilibration approach requires stirring and mixing, which may result in sample loss and/or leakage through the membrane. Since equilibrium dialysis is

mostly used in research settings, analysis of the free fraction is usually done by chromatography and MS, and not immunoassays as is the case for ultrafiltration.

Several publications report using both equilibrium dialysis and ultrafiltration to determine free drug concentrations, for cross-validation purposes. For example, Vita *et al.* used both methods to investigate plasma-protein binding of roscovitine at 25 and 37°C. Binding of roscovitine to HSA was constant (~90%) within the concentration range studied while binding to AGP decreased with increasing drug concentration, indicating that albumin is more important in clinical settings. Protein binding was higher at 25°C compared with 37°C. The results obtained by equilibrium dialysis were found to be in good agreement with those obtained by ultrafiltration [34]. In a highly cited article, Nowak *et al.* used equilibrium dialysis to validate results for free mycophenolic acid measurements obtained by ultrafiltration. HSA, high concentrations of the primary glucuronide metabolite, and sodium salicylate were found to significantly affect mycophenolic acid binding to proteins. The conclusion of the study was that the pharmacological activity of the drug is a function of unbound concentration [35].

A very practical method for measuring free drug concentrations in whole blood using rapid equilibrium dialysis was developed by Chen *et al.* Chlorpromazine, chloroquine, propranolol and a proprietary compound of different erythrocyte partitioning properties were tested to determine the free concentration in whole blood. Extra precautions were taken in each step to avoid the hemolysis of erythrocytes. The compound concentrations in blood and isotonic buffer were quantified by LC–MS/MS. Their results show that equilibrium can be reached within 6 h with proper mixing and stirring. Preliminary data demonstrated method suitability for drugs with low, medium and high erythrocyte partitioning. Furthermore, these experimental results can be used to explain nonlinear pharmacokinetic profiles and to predict effect and doses across species [36].

It is a commonly accepted hypothesis that central nervous system activity of a drug is determined by the unbound brain drug concentration. However, limited experimental data are available in the literature to support this hypothesis. Liu *et al.* thoroughly tested this hypothesis by examining the relationship between *in vitro* binding affinity and *in vivo* activity quantified as the drug concentration occupying 50% of the transporters for 18 serotonin and dopamine transporter inhibitors. The unbound brain concentrations were calculated from total brain concentrations and the unbound brain fraction, which was determined by the brain homogenate method. The *in vitro* unbound fraction in brain homogenate and plasma for each compound was determined using a 48-well rapid equilibrium dialysis device. The results of this research were very interesting, showing that prediction of the biophase drug concentration by using the unbound brain concentration rather than the total brain concentration results in an approximately 100-fold improvement in accuracy. A tenfold improvement was also observed by using the unbound plasma concentration rather than the total plasma concentration to predict the biophase concentration in the brain. These studies strongly support the hypothesis that drug activity in the brain is much more accurately determined by the unbound brain drug concentration [37,38].

As in the above studies, more and more researchers are focusing on measuring free drug concentrations in the target organs and not in blood. The unbound drug in the tissue is the most important parameter for pharmacokinetic and pharmacodynamic modeling. However, this is not an easy task, and many studies are still conducted by collecting tissue samples and measuring total concentrations.

Hsueh *et al.* evaluated the *in vitro* and *in vivo* relationship between free drug concentrations in plasma and in adipose tissue for six commercial compounds with diverse structures and a wide range of plasma-protein binding values. The biological samples (plasma and homogenized diluted adipose tissue) were dialyzed against buffer for 4 h in a 96-well equilibrium dialysis apparatus to determine the free drug concentration. Of particular interest was the procedure for measuring the unbound concentration in the adipose tissue. Since the undiluted homogenized tissue could not be analyzed directly, a wide range of dilutions were prepared. The free drug fraction was determined in these diluted samples. Finally, the fraction unbound in undiluted adipose tissue was determined by extrapolating from the unbound fraction in diluted tissue versus dilution factor. The overall conclusion of the study was that the free drug concentration in adipose tissue is similar to the free concentration in plasma within a threefold error range [39]. Although this error can

be too high when accurate studies are pursued, the authors suggest that free drug concentrations in plasma could be used as a surrogate for free concentrations in tissues especially in a resource-limited environment.

Gefitinib, an inhibitor of epidermal growth factor receptor-tyrosine kinase, exhibits wide intersubject pharmacokinetic variability which may contribute to differences in treatment outcome. Since unbound drug concentrations are more relevant to pharmacological and toxicological responses, gefitinib binding in plasma and factors affecting this process were studied both *in vitro* and in cancer patients. An equilibrium dialysis method using 96-well microdialysis plates was optimized and validated for determining the unbound fraction of gefitinib in plasma. It was found that gefitinib was extensively bound in human, rat, mouse and dog plasma with mean f_u values of 3.4, 3.8, 5.1 and 6.0%, respectively. Accordingly, the variable plasma protein concentrations observed in cancer patients will affect gefitinib unbound fraction with implications for inter-subject variation in drug toxicity and response, warranting the need to monitor the levels of free drug [40]. Similar investigations were performed for anticoagulants such as warfarin and phenprocoumon [41,42].

Significant challenges in measuring free concentrations are encountered for compounds with high binding that are active in low concentrations. Furthermore, some compounds have significantly different binding depending on patient gender, age and disease state. For these complicated cases, the gold standard method for measuring free concentrations is isotope dilution equilibrium dialysis. This approach was used by Hackbarth *et al.* to investigate the accuracy of calculated free testosterone levels. Although total testosterone is generally believed to be sufficient for diagnosing significant androgen excess or deficiency, the free (bioactive) testosterone is of superior diagnostic value. Such cases are encountered in hyper- or hypothyroidism, liver cirrhosis, obesity, or exogenous sex hormone use, especially estrogen treatments. Serum concentrations of the globulin also increase with age, often affecting free testosterone levels disproportionately to total testosterone concentrations. The authors of the study compared three established and two new equations that differed only by their testosterone association constants with isotope dilution equilibrium dialysis in two patient groups with different gender distributions. Regardless of the equation, ≥25% of samples showed unacceptable deviation from isotope dilution equilibrium dialysis. The authors concluded that application of many free testosterone equations to wider populations will frequently yield results that differ substantially from isotope dilution equilibrium dialysis [43]. Obviously, the best approach in this case would be to always measure the free testosterone concentration.

Other methods

Many other methods have been developed to measure free drug concentrations, but their applicability is currently rather limited. A very interesting study that extends the concept of free concentrations to larger molecules such as proteins was conducted by Speeckaert *et al.* in order to investigate the potential association of vitamin D binding protein with lipoproteins. The presence of vitamin D binding protein in lipoprotein fractions was examined using precipitation, gel permeation chromatography, and ultracentrifugation. Total and actin-free protein concentrations were assessed by immunonephelometry and enzyme-linked immunosorbent assay. The study revealed that the lipid-bound vitamin D binding protein fraction is of greater clinical importance than initially thought [44]. In order to test 'the free bilirubin hypothesis', Calligaris *et al.* tested *in vitro* cytotoxicity in four cell lines exposed to different free bilirubin concentrations obtained by varying total bilirubin/albumin ratio, using serum albumins with different binding affinities, and/or displacing unconjugated bilirubin from albumin with a sulphonamide. Free bilirubin was assessed by a modified, minimally diluted peroxidase method. These experiments clearly showed that bilirubin-induced cytotoxicity in a given cell line can accurately be predicted by free bilirubin irrespective of the source and concentration of albumin, or total bilirubin level [45].

One of the methods that is increasingly being used for free concentration assays in research settings is SPME. Although the method has been mainly used for studies with isolated proteins (mentioned previously), several publications report its application for complex biological samples. The ability of SPME to measure protein binding for drugs with very high affinity is particularly important, since this is difficult to perform with popular methods such as equilibrium dialysis and ultrafiltration. In order to investigate such drugs, extraction phases with high affinity for the investigated compound should be selected. The applicability

of this method for determining drug binding to proteins in human plasma was established several years ago when it was tested on five well-known drugs: ibuprofen, warfarin, verapamil, propranolol and caffeine, with high, intermediate and low binding properties [4]. In another publication, biocompatible microextraction coatings that can be utilized in direct contact with biological matrices were developed and applied for fast drug analysis and assay of drug plasma-protein binding [46].

An interesting extension of the concept of free concentrations to large molecules refers to measurement of free antibody concentrations in the bloodstream. Therapeutic antibodies may exist in free, partially bound, and fully bound forms in the bloodstream. The choice of which form(s) to measure and how to measure them is gaining much attention with the increase in the number of soluble therapeutic targets [47]. A thorough analysis of the theoretical and practical aspects of quantifying 'total' and 'free' therapeutic antibodies and their targets has recently been published by Lee *et al.* [48].

Choosing an appropriate technique

When deciding on a method for measuring free drug concentrations, the sample type should be considered first, followed by the desired throughput.

Two general types of samples are usually monitored for free drug concentrations: isolated proteins and raw biological samples (FIGURE 2). When the binding of drugs to isolated proteins is investigated, more convenient methods can be built if the protein can be immobilized on a solid surface. In this case, affinity chromatography and surface plasmon resonance can be used. Both methods are fast, allow the investigation of binding constants and number of binding sites, and usually use low amounts of protein and sample. Unfortunately, the process of protein

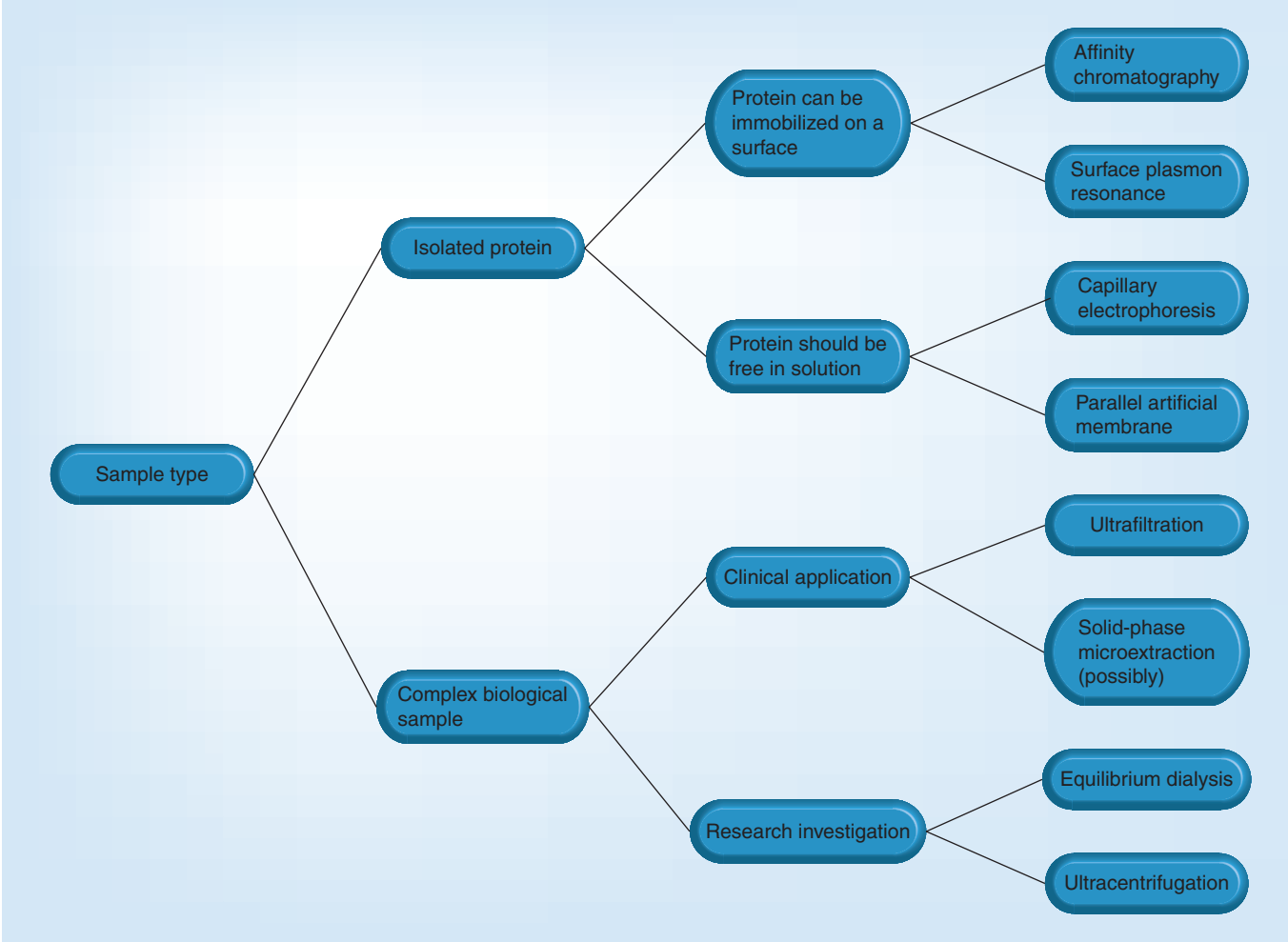

Figure 2. Selection of the most appropriate method for measuring free drug concentrations based on the sample type.

immobilization can be lengthy and can denature the protein. When the protein is preferred to be free in solution, the free concentrations can be monitored by CE or parallel artificial membrane assay. These methods are also fast and simple, but may not offer enough sensitivity for some drugs. Also, temperature control inside the capillary can be difficult and some compounds adsorb strongly on the walls of the device. An alternative method that can overcome some of these issues is dual-polarization interferometry, which can be used to measure changes in protein structure in real time.

Methods for complex biological samples can be divided into those that are mostly used in research laboratories and those with clinical applicability. Ultrafiltration is by far the preferred method in clinical settings due to speed and simplicity, but may suffer from nonspecific binding and lack of temperature control (it is still used at room temperature in clinical laboratories). SPME is a new potential candidate in this area; it is also a fast and simple approach, but method development can be lengthy. Equilibrium dialysis continues to be the gold standard for research investigations, but it is time consuming and suffers from numerous problems (mentioned above). Ultracentrifugation is also useful in research and appears to be an excellent method since it is simple and all investigated compounds remain in the same solution. However, the required instrumentation is expensive and various drugs sediment differently depending on their molecular weight, which introduces bias.

Another important decision when choosing a method is the desired throughput (FIGURE 3). For drug-discovery projects, methods with high throughput are needed. Depending on the amount of drug that is available, there are methods that need volumes of sample from low nl(CE) to low µl (zonal elution affinity chromatography), and methods that require

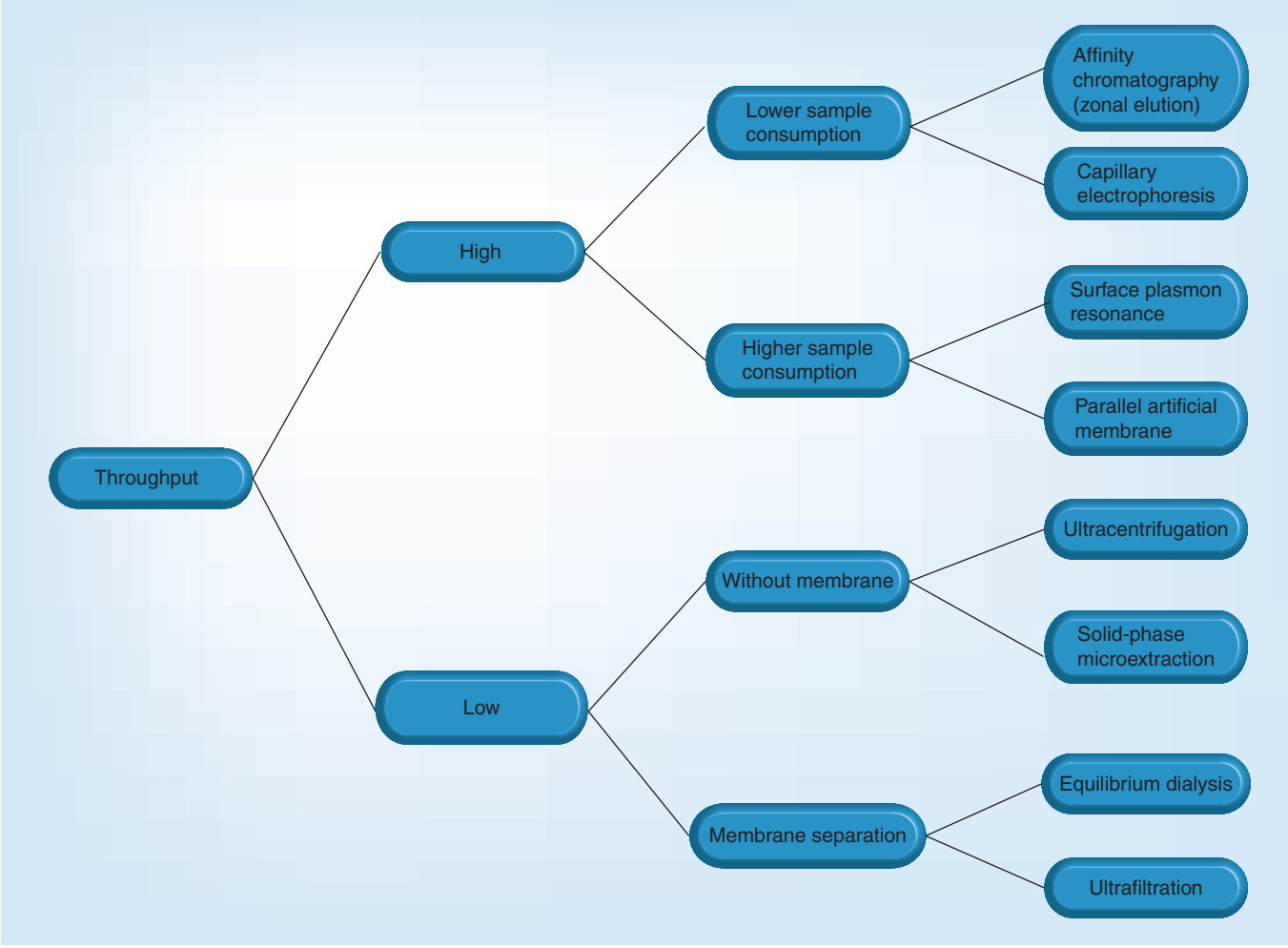

Figure 3. Selection of analytical approach based on method throughput.

volumes up to 500 µl, such as surface plasmon resonance and parallel artificial membrane. A method that requires a specifically high volume of sample, in the order of several ml, is affinity chromatography with frontal analysis. If on the other hand drug–protein binding is investigated for just a few compounds, usually drugs that are already on the market, methods with low throughput are sufficient. These can be methods without membranes, such as ultracentrifugation and SPME, which have the advantage of avoiding membrane artifacts, and methods with membranes that separate the sample from the solution where the free molecules are collected, such as equilibrium dialysis and ultrafiltration.

Future perspective

The current methods for measuring the free fraction of drugs suffer from numerous disadvantages. Although free drug concentrations are well recognized as being more useful for therapeutic decisions and research than total concentrations, they will not be regularly used in clinical practice unless the following challenges are solved by analytical chemists:

- Convenience: free concentration monitoring is currently more expensive and labor-intensive than total concentration monitoring.
- Accuracy and precision: the performance of current methods is poorer than those for total concentrations.
- Applicability for all drugs: these methods are difficult to apply for the drugs that need it the most – compounds present in low concentrations and with high protein binding; in such cases, the best methods are based on isotope dilution equilibrium dialysis.
- Availability of genuine gold standard methods: while equilibrium dialysis and isotope dilution equilibrium dialysis are currently considered the gold standard methods and are most accurate, the extended duration of analysis and the multiple technical difficulties usually result in poor precision; a further disadvantage of isotope dilution is the need to use radioactive tracers that are costly and potentially hazardous.
- Ruggedness: many clinical chemists are complaining about unacceptably high rates of device failure (in the case of ultrafiltration and equilibrium dialysis).
- Standardized sample conditions: some physicochemical properties change after biological sample collection; unless all samples have the same temperature, pH and CO_2 content, free concentrations values cannot be reproducible and meaningful.

Executive summary

Free drug concentration

- Concentration of freely dissolved drug molecules that can pass cell membranes and thus be effective in organisms.
- Can be calculated as the difference between total concentration and bound concentration (molecules bound to the sample matrix, usually proteins).
- Correlates very well with pharmacological and toxicological effects.
- Regrettably, most therapeutic drug monitoring decisions continue to be based on total concentrations.
- The discrepancy is mainly caused by a historical bias towards measuring total concentrations, but also by the greater technical difficulty of accurately measuring free drug concentrations.

Methods for purified protein samples

- Used to obtain binding constants and number of binding sites.
- The most popular method is affinity chromatography based on columns with immobilized protein; a single column can be used to investigate numerous drugs.
- Solid-phase microextraction is useful for studying drug–protein interactions especially in the case of hydrophobic drugs; in negligible extraction mode, the binding equilibrium between drug and protein is minimally disturbed.

Methods for complex biological samples

- Used to determine the overall binding to plasma proteins or to assure therapeutic efficiency for drugs with low therapeutic window and high binding to plasma proteins.
- Ultrafiltration, based on centrifugation in tubes with semipermeable membranes, is the method of choice in clinical laboratories; care must be taken to control the temperature during centrifugation and to investigate nonspecific binding to the device.
- Equilibrium dialysis is considered the gold standard for free concentration measurements, but is used mainly in research settings.

As an alternative, when free drug concentrations are too difficult to measure or the procedure is too expensive, the total drug concentration can be normalized by using various equations, such as those proposed by Sheiner, Winter, and Tozer for phenytoin [1,22,49] or those proposed by Hackbarth for testosterone [43]. If reliable and affordable methods for monitoring free concentrations are not developed, development of such equations may increase. However, it was shown numerous times that equations are never as good as actually measuring free concentrations [22,25,50,51].

Therefore, as bioanalytical methods become more sensitive, accurate and precise, we will certainly witness an increase in monitoring of free drug concentrations, which represent the 'active' fraction of the drug.

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