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Handling difficult samples in bio-analytical chemical analysis

Analysing difficult samples is fraught with problems. Consideration of the thought processes, prior to picking up a pipette, will help to circumnavigate some of the pitfalls.

Jun 01, 2008
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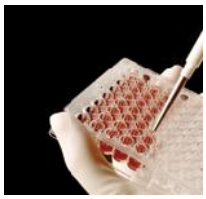
Pharmaceutical Technology Europe



There are two ways in which 'difficult' samples are usually categorized: either by the problems posed by the physical nature of the post-*vivo* sample matrix containing the chemical entity to be analysed, or by taking into account the known reactivity, interaction and stability of the chemical entity or analyte contained within the matrix and sample tube. An assessment of all physicochemical contributions will be made before an opinion is given regarding the difficulty of analysis.

On many occasions, biologically derived samples are submitted for analysis with insufficient thought given to the post-sample integrity of the chemical entities of interest. The samples are often frozen and stored at either –20 or –80 °C eventually, with the assumption that both the matrix and chemical entities of interest are of the same composition as when sampled. This view can be seriously flawed and changes to the chemical content of the sample can occur during the sampling and storage processes. Contributions from the storage containers also complicate future determination of the analytes of interest: either through adsorption onto the containers or addition of manufactured products from the container to the solution of sample matrix.

In light of such complexities and analytical challenges, the tasks of developing and validating robust analytical methods are often outsourced to provide more cost-effective and strategic solutions.

Usually, the solution is the quantitative or qualitative determination of chemical substances found in various matrices of biological origin. This article will refer to these as 'analytes' and will propose solutions to overcoming some of the challenges to their accurate determination.

Sample preparation

The overall analytical process from sample collection to analysis can be summarized into four stages:

Sample collection, transportation and storage.
Homogeneous sampling and measurement.
Isolation of the analytes from the matrix.
Preparation of the extracts for analysis.

It is useful if the analyst(s) is/are involved in toxicological and clinical studies from the conception and planning stages so that he or she understands the physicochemical nature and fate of the drug(s) after administration. Any measures proposed to safeguard the integrity of the parent drug and metabolites of interest must also be considered and put into place. At each stage, the physicochemical contributions of endogenous and exogenous entities in the sample should be considered, along with the controlled environmental conditions.

Assessing physical problems

Analytical methods are often required to determine concentrations or identify 'parent' compounds and metabolites from samples derived from toxicological or clinical studies.

Biologically derived samples containing analytes to be determined can be obtained from various sources including, 'whole' blood, plasma, serum, breast milk, saliva, urine, faeces and tissues from various organs ranging from brain through to colon.

On the go...

Treatment for some of these matrices, such as plasma, serum and saliva, after initial workup, can be generalized and grouped as being similar for extraction purposes. Tears and nasal fluid require minor modifications to the group analytical procedure.

Most matrices will contain different proportions of lipids, carbohydrates, proteins, lipoproteins, nucleic acids, salts and cellular debris, and various strategies have been employed to reduce the impact of these constituents in the determination of the targeted analytes. A possible solution for the removal of particulates is to induce precipitates using filtration or centrifugation, but this may also remove the analytes from the sample. It is important to carefully consider the interaction of the analytes during the simple pretreatment clean-up strategy that is proposed.

Considering liquid matrices

The most often encountered liquid matrices requiring analysis are plasma, urine, whole blood and, to a lesser extent, serum. These are routinely generated from clinical, metabolic, toxicological, pharmacokinetic and bioavailability studies.

Plasma samples. Plasma is the liquid medium in blood in which blood cells are suspended. It accounts for ~55% of total volume, and it is important to recognise this when planning sample availability and backup for analysis. Plasma contains dissolved substances such as the products of digestion and waste products of metabolism including glucose, salts, amino acids, vitamins, hormones, lipids, proteins and urea, among other important substances that are required for either transport throughout the body or for elimination *via* the kidneys. The major solute of plasma is a heterogeneous group of proteins accounting for 7% of its weight.

It is recommended that at least 2.5 mL of blood is taken for an assay requiring 0.5 mL of plasma to allow availability for one repeat analysis in case of adversity during analyte determination. This will also allow for the precipitation of fibrinogen type substances that can occur during freeze and thaw cycles.

In most instances, serum can be processed for analysis in the same manner as plasma as it is essentially the same medium without clotting proteins, such as fibrinogen. However, it is important to consider the analytical goals and choose the medium that reflects the most accurate assessment required. An example would be amino acid determination. When blood clots, amino acids may be released from or metabolized by erythrocytes, leukocytes or platelets, which are then dispersed into the serum. Thus, serum has relatively higher and more variable concentrations of free amino acids than plasma. If a snapshot measurement of available free amino acids is the goal, then determination in plasma would be more representative.

Urine samples. The pH of the blood components is usually 7.3, but urine can vary from 4.5 to 8.2 and the analyte stability could be problematic. Urine also has a high variability of electrolytes, metabolites and urea, depending on diet and rate of urine production. Care must be taken as precipitates form very quickly because of hydrolysis of soluble conjugates or exogenous microbial action, which induces the production of ammonia and significantly raises the pH of the solution. Sampling urine during a period of time will show variability in endogenous background and pH. Possible N-glucuronide metabolites could be reconverted to parent compound, and analytical methods must consider this by diminishing this feature by adding buffers or antimicrobials.

Whole blood samples. Whole blood contains plasma and a suspension of white blood cells, red blood cells and platelets. The white blood cells deal with immunity and the breakdown of foreign bodies, and the red blood cells deliver oxygen to tissues and organs from the lung. The platelets aid clotting and provide protection at injured sites of the body.

If whole blood is sampled and left at room temperature, it will clot and form a brownish red waxy material that is difficult to handle. Operations with whole blood should be temperature controlled below 4 °C and anticoagulants added at the sample collection stage or contained in the sampling tubes; an example of this would be to use heparinized tubes that are defined in the sample collection protocol. The use of anticoagulant coated tubes is a convenient and consistent method of ensuring that all samples have similar amounts of anticoagulant. The choice of coating need not be heparin, but could be EDTA, citrate or fluoride. Apart from the primary role of preventing clotting of the blood, a secondary use may be to inhibit enzymatic action on the analytes of interest. Using fluoride can prevent the action of nonspecific esterases and would be useful if the compounds of interest are esters and require protection.

The most common anticoagulants used are sodium salts of heparin, EDTA, citrate and, to a lesser extent, fluoride. The choice of anticoagulant to be used in a study will consider its influence on the analytes, and their detection and determination. In the case of fluoride, it may be used to protect the analyte from enzymatic action. An example would be the inhibition of nonspecific esterases, which could hydrolyse the analyte ester or amide.

Consideration of solid matrices

The analysis of substances in tissue or faeces sometimes requires the understanding of targeted therapy concentrations of specific drugs or mass balance distribution of 'parent' and metabolites. The solid nature of these matrices poses a problem regarding effective extraction of the analytes. Tissues can vary in tensile strength, elasticity and hydration. They may also be subjected to biofluid or whole blood contamination, requiring a washing and drying step.

Tissue samples. Extraction of tissues, solid or semisolid biological samples (faeces) usually begins with a rupture of the sample fabric or macrostructure. Scalpels, scissors, mechanical blenders, grinders and ultrasonics supply shearing forces and can be used with or without solvents, and may involve freeze drying the matrix before grinding to a powder. Ultimately, the goal is to homogenize, liquidize or solubilize the matrix so that extraction fluid — usually buffers or solvents that are sometimes directly incorporated into the blending process — can be further separated and extracted to isolate and concentrate the determinants for analysis.

Some tissues, such as brain and liver, are easily broken down using mechanical means, while others that are elastic in nature, such as colonic tissue, require cutting and ultrasonics, or the use of proteolytic enzymes, such as bromelain, subtilisin carlsberg or proteinase K, to digest the protein structures.

The use of digests can be beneficial as cross-contamination issues are negligible. However, these substances may add interference or artefacts to the extract chromatogram, and metabolites may be reconverted to their parent drug because of hydrolysis.

Faecal samples. Faeces are waste products of the digestive systems in mammalian species. The composition is both diet and health dependent. In contrast to urine, it is microbiologically active and, depending on the size of the sample required, is best stored at –20 °C and freeze-dried. Samples are ground down and homogenized to powder, which is then extracted in a buffer or a buffer containing organic solvent solutions, and the liquid is separated using filtration or centrifugation. The solids may be washed a number of times and the solutions collected to maximize analyte extraction. Once the goal of solubilization of the analytes has been achieved, further separation and concentrative extraction procedures can take place using either liquid, liquid techniques with acid/base back extraction (if ionic) or solid phase extraction (SPE) technology.

Assessing the chemical nature

Under controlled conditions and within a given environment, analytes can be assessed as being stable or unstable, either in themselves or in the bio matrix within which they have been sampled. Before an analytical determination takes place, immediate changes in environment and composition of the matrix may have to be performed to stabilize the analytes, possibly for storage and transport or for immediate analysis; for example, temperature control, antioxidant addition, addition of enzyme inhibitors and light exclusion.

Various strategies are employed to stabilize the analytes prior to storage and analysis, but the analyst must be aware of some of the possible pitfalls.

The molecular characteristics of the analyte should be identified, and a determination must be made of whether the analyte is nonpolar, polar or ionizable through pH control. The analyte's solubility and liquid chromatography properties should be assessed by looking at its structure and solubility in a variety of solvents.

Sample extraction. A key requirement to clean extraction prior to analysis is to exclude the bulk constituents of the matrix from the extract, as they have much less of an affinity or solubility for the extraction media or solvents than the analytes.

Examination of the extraction of spiked analytes from buffer and buffered matrix should be compared in terms of recovery. Lower extraction recoveries from the matrix could suggest that protein binding of analytes is present, or if SPE is the extracting media it may also suggest co-extraction of chemical species competing for and saturating adsorption sites on the bed, causing capacity issues that can result in analytes being poorly retained.

In some instances, an assumption that protein binding is taking place may be false especially when working with strongly polar moieties or compounds containing basic functional groups. If the extraction mode uses SPE, retention of molecules may be assisted by secondary interactions and the number of active sites affording retention will increase. The total number of retention sites on the column will be of sufficient capacity to completely retain the analytes from an otherwise compound-free buffer solution. However, the competitive interaction of many different compounds contained in a bio-matrix sample will take up many active retention sites and reduce the actual capacity of the column to retain the analyte of interest displaying an apparent observation found when real protein binding occurs. This greatly reduces the retention of analytes on the column as compared with retention of analytes in protein free surrogate (buffer) spiked samples.

Sample matrix effects. Analytes in *ex vivo* biological fluids can be subjected to a variety of reactions that are a function of the native chemistry of the molecule, and the inducements to interact and degrade with materials present in the matrix at the time of analysis. As discussed, some of these matrix substances have resulted during sampling, freeze/thaw and workup processes. Plasma and serum containing a high percentage of protein are particularly rich in enzymes that can convert analytes through hydrolysis and de-esterification. These enzymes may be inhibited through pH, temperature control or through the addition of inhibitory chemicals.

It is useful to study the stability of the analytes in a surrogate media, such as buffer, where most of the interactive tendencies of matrix are removed. However, some processes occur to a greater or lesser extent depending on the medium tested. Nifedipine, nitrendipine and nimodipine are dihydropyridine calcium antagonists that are prone to photo-oxidation degradation. Nifedipine has a half life of <1 h, depending on the transparency of the solvent when placed under normal daylight conditions. If nifedipine is contained in plasma or whole blood, the half life is far greater. The compound is stable when subjected to darkness or worked upon under yellow light. Light control during sampling, storage and analytical preparation reduces the problems associated with the analysis of these compounds and renders the analytical procedure simple and robust.

Case study

If oxidation of the analyte in the *ex vivo* sample is a problem, then care must be taken to ensure that the integrity of any metabolites is not adversely affected when protecting the 'parent' compound with antioxidants. The metabolites may be reduced back to the 'parent' compound. This has happened in our laboratory during the measurement of ascorbic acid and its metabolites from biological matrices.

The addition of substances, such as dithiothreitol (DTT), can convert the dehydroascorbic acid back to ascorbic acid and inaccurate higher concentrations of native ascorbic acid will be determined. To determine 'true' metabolized ascorbic acid accurately, the analytes were, immediately after the sampling procedure, protected in the matrix (plasma, urine and faeces) by the addition of metaphosphoric acid and stored in dry ice during transport and eventually at –80 °C.

Samples were carefully thawed, homogenized and centrifuged in light-protected environments kept below 4 °C. Duplicate samples of supernatant were taken and an aliquot of DTT solution added to one. Following incubation, both samples were transferred to auto-sampler injection vials, and HPLC analysis was performed with the dehydroascorbic acid measured as the difference between the DTT-treated sample and the nontreated sample.

Conclusion

The analysis of difficult samples can be rendered straightforward providing the physical and primary chemical nature of the matrix under examination is understood. In combination with chemistry knowledge of the functional activities of the drugs and metabolic pathways, a rationale for each of the key activities of sample collection, storage, isolation and concentration of the analytes can be formulated.

Emphasis on the exclusion of endogenous and exogenous substances during the extraction step, and protection of the determinants using physical, chemical and environmental friendly conditions during the analysis, will render the initially daunting analytical challenge attainable. Attention and care of the analytical column with recognition of co-extractable nonelutants will also render the chosen method robust.

Jim Hillier is Director of Scientific Operations for Tepnel Research Products and Services pharmaceutical outsourcing facility in Scotland. Jim has more than 30 years of experience in the practical application of analytical chemistry gained initially in the oil industry and later in contract research and development of analytical methods for the pharmaceutical industry. Jim's career has stretched from the development of techniques used to assign oil reservoirs to hydrocarbon generating source rocks, the synthesis of silica-based substances used in the isolation and purification of both DNA and RNA, and development of chromatographic methods used in toxicology and clinical studies. Jim is a graduate of the University of Strathclyde gaining a degree in pure and applied chemistry.

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