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A REVIEW ON HIGHLY SENSITIVE ANALYTICAL TECHNIQUES FOR TOXICOLOGICAL STUDIES IN BIOLOGICAL SAMPLES

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ABSTRACT

Highly sensitive analytical techniques have allowed a massive increase in the amount of pharmacokinetic data which is useful to estimate the toxicity of the particular drugs. Generally available analytical techniques may not provide sufficient information to identify the all these toxic substances present in biological fluids at low concentrations. So, modifications to generally available analytical techniques are required. They can provide high sensitivity and selectivity of the analytical method towards the particular type of toxic substances. They provide reliable, robust sensitive, specific, quantitative assay procedures for the determination of drugs and metabolites in body fluids. The sensitivity of these techniques is up to nano-grams and pico-grams. Originally, the most common extraction method was liquid–liquid extraction (LLE). Solid-phase extraction (SPE) has become an increasingly popular extraction method.

Keywords: Sensitive Analytical Techniques, Sample preparation, Choice of analytical methods and SPE-HPLC.

INTRODUCTION

Recent advances in analytical technology have allowed a massive increase in the amount of pharmacokinetic data available even though difficulties are often encountered in quantifying pharmacological effects, thus making clinical correlation difficult. The task of the analyst is to provide reliable, robust sensitive, specific, quantitative assay procedures for the determination of drugs and metabolites in body fluids, both during the early stages of a drug development programme, and later on in the life of the drug when attempts are being made to understand the differences in reaction of specific patients to the drug. Metabolites may occur in blood as well as in urine, and may they be pharmacologically active so that it does not sufficent to measure only the parent drug.

For toxicological screening during acute drug poisoning, laboratories must implement rapid and specific methods. Many laboratories use broad-spectrum screening to determine unknown agents in a patient's sample. Several studies have shown that toxicological screening frequently identifies unsuspected drugs in patients with drug overdoses[1]. In a majority of cases, all that is required to guide therapy is qualitative drug screening. Drug screening is an important part of a thorough toxicological workup. Urine, in particular, is an excellent specimen for this purpose because almost all drugs or their metabolites are excreted and concentrated in urine. Originally, the most common extraction method was liquid–liquid extraction (LLE). However, in recent years, particularly as drug testing has become more common in the workplace and in the athletic arena. Solid-phase extraction (SPE) has become an increasingly popular extraction method[2].

These resins, because they contained many classes of sorbent that were co-polymerized on a solid silica support, were able to be used for the separation of many different classes of compounds that had chemical and physical properties markedly different from one another. Since that time, SPE has been a widely used extraction technique in forensic

toxicology and the urine drug-testing market. The type of copolymer specifically designed for the urine drug-testing market is a cation-exchange/reversed-phase copolymer. This resin uses a combination of ion exchange and hydrophobic properties that allows for very clean extracts with high extraction efficiency. The literature has shown that copolymers used in SPE have been useful in extracting the full range of acidic, basic, and neutral drugs of abuse.

The advantages of SPE have become even more pronounced in recent years with the advent of semi-automated and automated SPE instruments. One study found that, in general, SPE was 12-fold less time consuming and fivefold less expensive than LLE[3]. Most semi-automated systems facilitate the aspiration of fluid through the columns by a vacuum system or vacuum box. These vacuum boxes often have some way to adjust and control the amount of vacuum.

THE NEED HIGHLY SENSITIVE ANALYTICAL TECHNIQUES

The activity of a drug is dependent upon its concentration in whole blood, plasma or serum. Since then clinical pharmacologists and clinicians have increasingly recognized the utility of measuring blood, serum or (more commonly) plasma levels of drugs - this being the best indicator available of the concentration at the site of action[4]. From the data thus obtained, various pharmacokinetic parameters can be calculated, such as half-life, area under the drug level - time curve (bioavailability), time taken to reach peak plasma level, clearance rate and apparent volume of distribution.

There are several reasons why the acquisition of such data is valuable[5]. Pharmacokinetic analyses provide a means for the comparison of different dosage forms of the same drug. A drug released too slowly may never reach a sufficiently high concentration at the site of action to be effective, or conversely, a rapid rate of release may give toxic side effects and then rapidly fall below the level necessary for effective therapy. Pharmacokinetic parameters are useful in the assessment of absorption and other processes enabling some rationale to be brought into the design of new drugs.

There are many factors such as kidney damage, drug interactions, eating habits, enzyme deficiency and liver damage[6] that affect the behavior of a drug within a particular patient and knowledge of pharmacokinetics can help to tailor a dosage regimen to a specific individual (therapeutic monitoring).

This correlation between pharmacological or toxicological effect and drug concentration in biological fluid[7], with the aim of achieving safer and more effective therapy[8] has been the main stimulus to achieving reliable analytical methods. Recent advances in analytical technology have allowed a massive increase in the amount of pharmacokinetic data available even though difficulties are often encountered in quantifying pharmacological effects, thus making clinical correlation difficult.

The task of the analyst is to provide reliable, robust, sensitive and specific quantitative assay procedures for the determination of drugs and metabolites in body fluids, both during the early stages of a drug development programme, and later on in the life of the drug when attempts are being made to understand the differences in reaction of specific patients to the drug. Metabolites may occur in blood as well as in urine, and may they be pharmacologically active so that it does not suffice to measure only the parent drug.

ADVANTAGES OF SENSITIVE ANALYTICAL TECHNIQUES

1. Sensitivity: The analytical sensitivity of an assay is that assay's ability to detect a low concentration of a given substance in a biological sample, whether that substance is blood glucose or HIV-1 pro-viral DNA. This type of sensitivity is expressed as a concentration (for example, in mg/dL or in gene copies/50 million cells) [9]. If lower the detectable concentration, greater the analytical sensitivity. Synonyms for analytical sensitivity include "limit of detection" and "minimal detectable concentration." Analytical sensitivity may also be expressed in terms of an assay's ability to detect a change in concentration.
2. Specificity: Analytical specificity is the ability of an assay to exclusively identify a target substance or organism rather than similar but different substances (insulin rather than pro-insulin; HIV-1 rather than HIV-2) in a sample or specimen.[10]
3. Ability to identify the very low concentrations (i.e. nano-grmas, pico-grams, etc).
4. Separating acidic, neutral, and basic drugs in a single run.[11]
5. Quantification of structurally unrelated drugs in a single analytical run, resulting in substantial reductions in analytical time, turnaround time, and cost.

METHODS AVAILABLE FOR THE ANALYSIS OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

The interpretation of drug concentration data is valid only if the analytical procedure used measures the drug or metabolite that it is intended to measure, and if it does so with acceptable precision and accuracy. Improvements in instrumentation have led to a wide array of methods allowing differing degrees of sensitivity and specificity to be available. Quantification is based on the fundamental relationship that the signal produced by the measuring instrument is dependent on the concentration (or amount) of the compound being measured. Since some loss usually occurs during sample preparation, the calibration curve has to be based on values for pure compound 'spiked' into the original medium, eg. control plasma.

1. U. V. AND VISIBLE SPECTROPHOTOMETRY
2. LUMINESCENCE SPECTROSCOPY
3. RADIODERIVATIZATION
4. ELECTROCHEMICAL TECHNIQUE
5. LIGAND METHODS
6. THIN LAYER CHROMATOGRAPHY
7. GAS LIQUID CHROMATOGRAPHY
8. HIGH PRESSURE (PERFORMANCE) LIQUID CHROMATOGRAPHY

GENERAL POINTS CONCERNING CHOICE OF METHOD

The final choice of an analytical technique to provide the end step in a drug assay programme is frequently not easy since there is no absolute method of choice. The procedure employed should, however, meet the specificity and sensitivity requirements for the individual study being undertaken. Factors such as the amount of time and effort expended also play a part when analyzing biological samples, due to the fact that it is usually necessary to carry out preliminary manipulation of the samples prior to the end step. RIA offers advantages since sample preparation is often unnecessary and very small sample volumes can be used. However, the development of a RIA is a relatively difficult, time consuming and sometimes uncertain process since it requires: (a) Synthesis of a hapten of the drug which can be coupled to a protein to produce the immunogen, (b) Production of a potent antiserum, (c) Synthesis of highly labeled drug and (d) Testing the system for cross-reactivity due to metabolites.

Although it is sometimes possible to develop specific analysis for drugs in biological samples using non-chromatographic methods, generally some form of chromatography is necessary. Until recently GLC has been the technique most commonly used since it combines highly sensitive and possibly selective on-line detectors with high separating efficiency. Nowadays more and more assays employ HPLC since it offers several advantages over GLC. For example, there is no requirement for volatility (only solubility), and as a result highly polar substances including zwitterions can be chromatographed and underivatized. Another advantage is that compounds which are thermally unstable do not present a problem with HPLC. Furthermore, the range of options in HPLC is greater than for GLC since with the latter the major variables are the liquid stationary phase and temperature, whereas with HPLC one can use a wide range of mobile phases along with various column types. Sample preparation for HPLC can also be simpler in many cases[12], since aqueous eluents are frequently used for drug and metabolite analysis, occasionally allowing direct injection of biological fluid.

Over a relatively short period HPLC has thus become the major tool used for the measurement of drugs and metabolites in biological fluids. The availability of some highly selective, sensitive detectors has contributed to this, yet it is the lack of a suitably wide range of these that is the greatest drawback. The development of reliable, highly sensitive and specific detectors such as coupling MS to HPLC, complemented by novel derivatization reactions to enable more compounds to evoke a response to the specific detectors available, will probably result in the continuing growth in the application of HPLC to trace analysis.

SAMPLE PREPARATION APPROACHES

An analytical method for the quantitative analysis of a drug or metabolite in a biological fluid usually requires some form of isolation procedure. Often the compound of interest is present at very low concentrations and the matrix contains numerous endogenous compounds, some present at much higher levels than the compound to be measured. In human studies, plasma and urine are the most common biological matrices although serum, whole blood, saliva, faeces and tissue are also used. When analyzing plasma samples a common means of getting a protein free solution is to precipitate the proteins so they can be removed by centrifugation. Deproteinization can be undertaken in several ways, such as the addition of an acidic reagent eg. trichloroacetic acid, addition of an organic solvent miscible with water (eg. Acetonitrile), heating the

biological fluid or use of reagents of high ionic strength. A related technique (particularly useful with tissue samples) is digestion of the biological material by proteolytic enzymes. When analyzing urine many drugs or metabolites may be present as conjugates, particularly as glucuronides or sulphates, and these are seldom amenable to solvent extraction. Although there are some examples where conjugates have been chromatographed directly by HPLC, it is more common to hydrolyze them, either enzymatically or using acid, to release the actual drug or metabolite.

RECENT TECHNIQUES

UNIFORM SOLID-PHASE EXTRACTION PROCEDURE FOR TOXICOLOGICAL DRUG SCREENING IN SERUM AND URINE BY HPLC WITH PHOTODIODE-ARRAY DETECTION[13]

MATERIALS AND METHODS: Serum and urine samples, a liquid chromatogram equipped with a built-in DAD and an automated sampler. On-line printing of ultraviolet spectra, wavelength ratios, and peak purity data was generated by printer interfaced with the instrument. The analytical column consisted of a 10 x 2.1 mm (i.d.) pre-column and a 150 x 4.6 mm (i.d.) main column packed with Hypersil 5 mm (particle size) octadecylsilane (C_{18}). Column temperature was maintained at 45 °C.

REAGENTS AND MATERIALS: Potassium dihydrogen phosphate, Ttriethylamine buffer solution, Phosphoric acid (all SupraPure grades), Acetonitrile, methanol (both HPLC grade), Sodium octyl sulfate, ammonium acetate, and b-glucuronidase type H-2 (from *Helix pomatia*). All drugs of pharmaceutical quality in tablets, capsules, or injection form and stock solutions are prepare at 10 g/L in 800 mL/L acetonitrile and store at 4°C. For solvent extraction, Toxi-TubeTM A was used. These tubes are designed for use with 5-mL urine samples; contain sodium carbonate and bicarbonate to give a pH of 9.0 in a mixture of dichloromethane and dichloroethane.

CHROMATOGRAPHIC CONDITIONS: The analytical flow rate was 1.0 mL/min., gradient elution generated by the proportional mixing of two solvents: Solvent A contained 50 mL/L acetonitrile and solvent B contained 500 mL/L acetonitrile, both in 50 mmol/L phosphate buffer, pH 3.0, containing 375 mg/L sodium octyl sulfate and 3 mL/L triethylamine was used. Solvent gradient conditions changed linearly from 15% B to 90% B in 20 min, stayed at 90% B for 5 min, and returned to 15% B in 3 min. Total run time was 30 min, the analysis time being 28 min. To ensure stabilized analytical conditions, we waited 6 min between runs.

SAMPLE PREPARATION: To 3 mL of urine or serum in a plastic tube, we added an equal volume of sample diluent (0.4 mol/L phosphate buffer, pH 6.0) and vortex-mixed the samples for 1 min. SPE columns were inserted into the reduced-pressure manifold and conditioned by washing once with 3 mL of methanol followed by 3 mL of conditioning solution (0.2 mol/L phosphate buffer, pH 6.0). We made sure the columns did not dry out before the specimens were applied. We poured the samples into each column reservoir, from which they were drawn slowly through the column at a flow rate of 1 mL/min. The columns were washed with 3 mL of wash buffer (50 mL/L methanol in 0.2 mol/L phosphate buffer, pH 6.0) and the fluid was drained by passing air through the columns for 10 s. Concentrated drugs were eluted with 750 mL of a mixture of 100% methanol and 10% ammonia at a slower flow rate of 0.5 mL/min. For urine analysis in cases of acute poisoning, we injected 25 mL of the extract directly into the chromatograph. To enhance the sensitivity of drug detection at lower concentrations, we evaporated the SPE extract under nitrogen gas after adding a few drops of 1 mol/L HCl to reduce the volatility of some drugs (e.g., sympathomimetic amines), the dried residue was then reconstituted in 150 mL of solvent B for HPLC injection. In case of insufficient sample volume, we adjusted the reconstitution volume according to initial loading volume of the specimen, to give a fixed concentration factor of 20-fold. That is, a 1-mL serum sample can be concentrated to a 50-mL extract for sensitive serum analysis.

DETECTION AND IDENTIFICATION: The retention and spectral data of 300 toxicologically relevant compounds (therapeutic and illicit drugs, their metabolites, and endogenous substances) determined by gradient HPLC procedure have been recorded. The basic library for pure compounds was initially established by analyses of injections of diluted drug solution (1:200 dilution of drug stock solution in solvent B). During each run, the absorbance was recorded at 210 nm, with 8-nm bandwidth; the reference wavelength was 550 nm, with 40-nm bandwidth. Ultraviolet spectra over the range 210–350 nm were automatically extracted if the peak signal was >3 m*A* (threshold setting) and the peak covered >0.1 min (peak width setting). The built-in microprocessor output processed peak signals immediately to the on-line printer, plotting overlaid ultraviolet curves for apex and down slope, and reporting both the *A*210 nm/*A*230 nm ratio and the factor of peak purity (ideal 5 1.000). To construct a

spectral library, use an Excel-5TM electronic workbook on a 486-PC, digitalizing ultraviolet spectra for pure products, for drug metabolites, and for matrix peaks in serum or urine processed under the same analytical conditions. Arranged according to retention time, each entry was assigned a view area that also documented relevant information. Each library entry includes identification parameters, metabolite profiles, chemical structure and properties, pharmaco- and toxicokinetic data, clinical presentation of overdose, and a brief note on the disposition of the drug in humans. In addition, we have included three indexing lists for quick searches according to (*a*) tentative identification according to retention times, absorption maxima, and *A*210 nm/*A*230 nm ratios. (*b*) computer search by name to view the relevant information by use of the Excel-5 View Manager function. (*c*) cross-reference to the bench log books for detailed inspection. The log books compile actual printouts of chromatogram and ultraviolet spectra of parent drugs and metabolites in real cases of drug overdose/drug abuse. The manual but systematic search routine allows quick identification in urgent cases.

ESTIMATION OF DETECTION LIMITS FOR QUALITATIVE IDENTIFICATION: Quantitative control materials for therapeutic drug monitoring and drugs of abuse detection were obtained from commercial sources. Serum or urine controls were processed in three separate runs in the same manner as the routine samples. SPE extracts were injected into the HPLC without further enrichment. For drugs that were positively identified, the mean absorption signals of the peaks were related to its assigned concentration. To assess linearity of detection, assay the serum controls at all three target concentrations. The estimated detection limit for qualitative identification is thus a function of extraction efficiency, chromatographic peak symmetry, and photometric absorptivity of individual drugs.

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