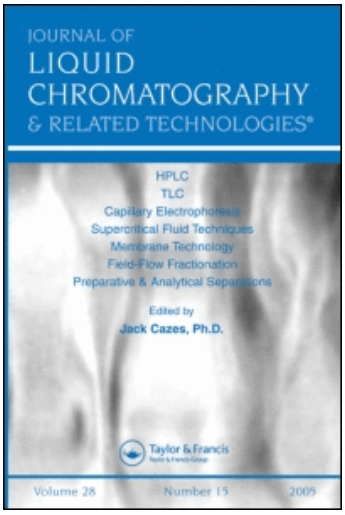

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Drug Assays for the Clinical Pharmacokinetics Laboratory

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To cite this Article Wilson, John M.(1987) 'Drug Assays for the Clinical Pharmacokinetics Laboratory', Journal of Liquid Chromatography & Related Technologies, 10: 2, 277 — 292
To link to this Article: DOI: 10.1080/01483918708066719
URL: http://dx.doi.org/10.1080/01483918708066719

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(2&3), 277-292 (1987)

DRUG ASSAYS FOR THE CLINICAL PHARMACOKINETICS LABORATORY

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ABSTRACT

The growth of high performance liquid chromatography (HPLC) has significantly aided the work of the clinical pharmacokinetics laboratory. Because of its precision, sensitivity, selectivity and flexibility many analytical problems can be solved with the purchase of one instrument. We illustrate how HPLC can function in Therapeutic Drug Monitoring (TDM), large scale pharmacokinetic studies and address clinical and pharmacological problems. We also provide a condensed summary of conditions for three assays of particular value to the Research Pharmacokinetics Laboratory; for antipyrine, indocyanine green (ICG) and propranolol.

INTRODUCTION

In the last ten to fifteen years, clinical laboratories have addressed the need for accurate and precise determinations of drug

concentrations in biological fluids including far ranging screening of fluids for substances of abuse. More recently, laboratories engaged in TDM and clinical toxicology, as well as research laboratories, have applied the analysis of drugs for the production of pharmacokinetic data. It will not be the intent here to define the function and role of the pharmacokinetics laboratory nor to detail how to establish same, for this has been adequately and persuasively presented by others (1-4). Instead it will be our purpose to specify how modern liquid chromatography can be used in pharmacokinetic applications, as well to present condensed methods for a number of important substances commonly used to obtain relevant biological information.

DISCUSSION

Some of the activities of a pharmacokinetics laboratory are listed in Table 1. This is not meant to be an all inclusive list, but merely to demonstrate the broad scope of activities such a laboratory might be required to provide. All of these activities involve analysis of drug byproducts in biological fluids to enable calculation of pharmacokinetic parameters (5). The most important of these are clearance, volume of distribution, half-life, absorption rate, bioavailability and the free fraction. In some instances one or two drug levels is sufficient for this purpose, but more often multiple levels are required. Quite often it is necessary for the assay to accurately measure blood or plasma levels orders of magnitude lower than the therapeutic range. Because of the need for sensitive and specific assays, as well as the broader require-

TABLE 1
Analytical Activities of the Pharmacokinetics Laboratory

1. Analysis of therapeutic drugs at steady state to determine efficacy (TDM)
2. Identification of drugs in blood or urine to assess potential toxicity (Clinical Toxicology)
3. Determination of unbound drug levels to assess the affect of disease state on biological disposition
4. Analysis of serial samples to allow calculation of pharmacokinetic parameters such as clearance, volume of distribution, and half-life and enable maintenance dose prediction
5. Multi-sampling after a single dose or over a dosing interval at steady state in a group of individuals to assess the population absorption elimination and distribution
6. Multi-sampling after intravenous and oral dosing to assess bioavailability
7. Measurement of drug in urine to assess the rate and extent of renal elimination
8. Measurement of metabolites in blood and urine to determine partial clearances through metabolism
9. Measurement of drug levels after different doses to assess linearity
10. Determination of levels for correlation to response, or to determine compliance

TABLE 2
Advantages of HPLC in Pharmacokinetic Analysis

1. The potential for the analysis of many different types of drugs of different molecular structures, different $_{p}$ka, differing molecular weights, sometimes within the same test framework.
2. The ease with which drug metabolites can be analyzed concurrently with the parent drug.
3. The ease of sample preparation in many cases allowing analysis with just a simple protein precipitation and with urine a dilution.
4. The high sensitivity of analysis for most organic compounds as well as the possibility of derivatization to enhance sensitivity.
5. The flexibility of solvent:solute systems allowing resolution to be obtained of even very small similar geometric and stereoisomers.
6. The availability of additional high sensitivity detection systems such as fluorescence, amperometry and mass spectrometry to extend sensitivity limits.
7. The diversity and preponderance of analytical columns of high effeciency in a highly competitive marketplace.
8. Liquid chromatography systems are easily adaptable for automation. In some cases the entire sample preparation can be automated.
9. Systems do not destroy the sample so that once analysis is complete samples can be recovered for additional studies.
10. LC systems in general tend to be highly specific and capable of producing very high precision.

ment for techniques of wide applicability and for many types of drugs, HPLC has become an indespensible analytical tool. Table 2 lists a number of reasons why HPLC is of value in pharmacokinetics.

Therapeutic drug monitoring in most instances can be satisfactorily achieved by monitoring trough serum levels after a drug has achieved steady state, that is, after a subject has adhered to a dosing regimen for at least four drug halflives. Table 3 lists a number of drugs, for which there is a consensus that therapeutic monitoring can be of value, and their therapeutic ranges, and references to HPLC assays that have been validated to possess acceptable accuracy and precision. It has been apparent over the last ten years that HPLC is a versatile and satisfactory technique when applied to the steady state analysis of therapeutic drugs.

In addition to total drug, insight into therapeutic management can sometimes be achieved by the analysis of unbound drug, since it is the unbound drug which in theory corresponds to the therapeutic effect. In a number of disease states, such as renal failure, hyperlipidemias, protein wasting diseases or trauma, protein binding can be altered and measuring the total drug may give misleading information.

Measurement of free levels is a more demanding analytical challenge. Determination of free fraction requires an additional stage, i.e. isolation of free drug from bound drug, and measurement of either naturally requires a more sensitive analysis. Separation may involve ultrafiltration, equillibrium dialysis,

TABLE 3

	Desired Range µg/ml	HPLC Ref.
Theophylline	10-20	6
Phenytoin	10-20	7
Phenobarbital	15-40	7
Ethosuximide	40-100	7
Valproic Acid	50-100	8
Carbamazepine	8-12	7
Primidone	5-12	7
Digoxin	0.8-2ng/ml	9
Gentamicin	Peak <10 Trough <2	10,13
Tobramycin	Peak <10 Trough <2	11,12
Amikacin	Peak <30 Trough <5	11,12
Chloramphenicol	Peak <20 Trough <12	14
Vancomycin	Peak <30 Trough <10	15
Procainamide	4-10	16,18
Tocainide	4-12	17
Lidocaine	2-5	18
Amiodarone	0.8-2.0	19
Cyclosporine	100-450 ng/ml*	20
Disopyramide	2-5	21
Amitriptyline	150-250 ng/ml**	22,23
Nortriptyline	50-100 ng/ml	22,23
Imipramine	150-300 ng/ml**	22-24
Desipramine	40-150 ng/ml	22-24
Quinidine	2-5	25

* Whole Blood

**Total Parent Drug Plus N-Desmethyl Metabolite

or ultracentrifugation. HPLC with UV detection is often times adequate for final analysis, though more sensitive detectors may be required to achieve sufficient sensitivity, as when measuring free levels for a very highly bound drug.

The pharmacokinetics laboratory may also be called upon to perform predictive analyses by analyzing therapeutic drugs at agreed upon times relative to a dosing regimen for the purpose of characterizing a subjects individual pharmacokinetic parameters, i.e. volume of distribution and clearance or elimination rate constant. Once these parameters are known they can be used to optimize dosing. This can be extremely beneficial when dealing with a drug for which the relationship between steady state plasma level and dose is quite variable, in a patient in whom therapeutic steady state levels need to be achieved rapidly and in patients receiving drugs with decidedly nonlinear pharmacokinetic parameters.

Dose prediction has been shown to be satisfactory based on the measurement of a single plasma level drawn after an initial test dose of drugs such as theophylline, chloramphenicol, lithium, amitriptyline, nortriptyline, imipramine and desipramine (26-28). Other drugs may require two or three levels and nonlinear curve fitting for satisfactory predictive performance. For example, aminoglycosides have been successfully dosed based on the measurement of a trough and peak plasma level drawn around a single dose (30). Additionally accuracy can be achieved by drawing an additional level subsequent to the peak to allow determination of the plasma halflife (29).

Beyond the scope of therapeutic drug monitoring, the pharmacokinetics laboratory may need to estimate these parameters and others more precisely for the purpose of evaluating the potential value of a particular drug in relation to others. Measurement of clearance, bioavailability and volume of distribution at steady state are commonly desirable parameters to know. Their estimation is achieved through area under the curve measurements involving multisampling over a single dose or dosing interval (31, 32). Sampling should be undertaken until drug is no longer measurable so that as much of the total area from time zero to infinity (AUC) can be characterized by analysis. The remainder can be calculated by extrapolation from the last measurable quantity and time point. Two factors make HPLC a very effective technique in this regard. Injection volume can be increased considerably to decrease minimum detection limits to achieve measurement of low levels with undiminished precision and because HPLC lends itself easily to automation the many samples collected over the dosing interval can be analyzed in replicate to achieve greater statistical validity.

Estimation of other kinetic parameters such as absorption, distribution and elimination rate constants, compartmental volumes and interdepartmental rate constants require additional nonlinear curve fitting, the validity of which is aided by the high precision which can be achieved with HPLC. Automation can again help to deal with the large numbers of samples generated in these types of studies.

Pharmacokinetic applications are not limited to therapeutic monitoring and large scale studies. Pharmacokinetics is a powerful tool for evaluating pharmacologic issues. Clearance is an important clinical parameter which can be assessed by appropriate analysis. Renal clearance can be measured by monitoring urinary excretion relative to the serum concentration as in equation 1.

$$Clr = \frac{\text{Amt Drug in Urine}}{(\text{Hrs of urine collection}) \times \text{drug concentration in serum}} \quad (1)$$

Hepatic clearance is more difficult to assess directly, but since most drugs are metabolized in the liver, metabolic clearance can be obtained by subtracting the renal clearance from the total body clearance. Hepatic function can be assessed in a more general sense by administering a test drug such as antipyrine (See Appendix). Antipyrine has two important properties. It is unbound and eliminated entirely by liver metabolism. This means that total body clearance will be equal to the intrinsic clearance of the liver. Antipyrine can then be used as a gross measurement of hepatic function.

Hepatic clearance can be influenced by a number of factors, blood flow to the liver, plasma protein binding and the intrinsic metabolizing capability of the liver. Blood flow can also be measured by administering a test drug, in this case, indocyanine green (ICG). ICG is used because it has a very high extraction ratio (>0.9) by the liver. It is taken up so rapidly by the liver cells that measurement of its disappearance from plasma is an accurate indicator of liver blood flow. ICG is preferably measured by HPLC (See Appendix) because upon standing it will degrade to form products which will interfere with spectrophotometric assays.

Both intrinsic hepatic clearance and blood flow can be assessed by the administration of a drug such as propranolol (33). Oral propranolol is completely absorbed but extensively removed from the liver on the first pass via the portal vein. With oral administration intrinsic hepatic clearance is equal to its oral clearance.

$$Cl_{int} = \frac{\text{Oral Dose}}{\text{AUC oral}} \tag{2}$$

When also given intravenously, blood flow can be calculated using both the areas under the I.V. and oral curves.

$$\text{Blood Flow} = \frac{D_{oral}\, D_{iv}}{D_{oral}\, AUC_{iv} - D_{iv}\, AUC_{oral}} \tag{3}$$

Therefore administration of propranolol by two different routes can indicate wheter changes in kinetic parameters are a result of changes in blood flow or intrinsic hepatic clearance (See Appendix).

We have already indicated that measurement of protein binding, the third factor in assessing changes in clearance can also be facilitated using HPLC.

Metabolism of drugs often occurs via multiple routes and these can be differentiated by calculating partial metabolic clearances. This is facilitated by measuring metabolite plasma levels as well as that of parent drug. The partial clearance or formation clearance (Cl_f) to a specified metabolite is given by

$$Cl_f = \frac{Cl_m\, (AUC_m)_p}{(AUC_p)_p} \tag{4}$$

where $(AUC_m)_p$ and $(AUC_p)_p$ are the areas under the curve for metabolite and parent drug after administration of the parent drug, and Cl_m is the clearance of the metabolite, obtained by administering the metabolite. Often with HPLC, metabolites can be measured in the same assay with the parent drug. Antipyrine, because it undergoes a number of oxidative transformations, can be used an index of various metabolic activities (34). Measurement of antipyrine and metabolites in urine can be accomplished by HPLC (36).

Some drugs exist in multiple forms and HPLC offers the opportunity to assess their pharmacokinetics and pharmacodynamics independently. Doxepin is administered as cis:trans geometric isomers in proportion of 15:85(cis:trans). Many drugs have chiral centers, some more than one, and are administered as racemic or other mixtures. Instead of giving one drug, two are actually being given, which may have different, even antagonistic, pharmacologic effects and which may also have different clearances and volumes of distribution (35). HPLC offers the potential of separating and individually quantitating the isomers to allow a more complete assessment of the drug's pharmacokinetic profile. A polarimeter or circular dichroism detector placed in line with a UV detector holds the potential, not only for quantitation, but also providing information as to absolute configuration.

CONCLUSION

The focus of this report has been the place of HPLC in the pharmacokinetics laboratory. It should not be concluded that

other instrumentation does not also have valuable and important contributions to make. A laboratory with a firm foundation must have a diverse mix of instruments to deal effectively with the varied challenges provided by modern pharmaceutical sciences. HPLC complements and overlaps the utility of other instrumentation providing analytical approaches to the solution of practical problems. It has significantly advanced the practice of pharmacokinetics.

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APPENDIX

Condensed HPLC Assay For Antipyrine and Metabolites

Mobile Phase	61.3:35:3.15:0.16 [Hexane:Dichloromethane: Methanol:Ammonia (25%)]
Detection System	UV 254nm
Column Type	5μm Silica 125x6mm (Nucleosil 50-5)
Flow Rate	1.6ml/min 25°C
Sample Prep	Urine extraction Dichloromethane:Isopropanol-Ammonium Sulfate (pH 4.0)
Internal Standard	N-Acetylbenzylhydrazide
Retention Times	Antipyrine 1.5 min, 4-Hydroxy-Antipyrine 2.5 min, Norantipyrine 3 min., IS 6.5 min, 3-Hydroxymethylantipyrine 9 min.
Reference	36

Condensed HPLC Assay for ICG

Mobile Phase	47:3:50 [Acetonitrile:Methanol:0.05M (KH_2PO_4 - Na_2HPO_4 Buffer (pH6)]
Detection System	UV (225nm)
Column Type	10µm C18 250x4.6mm (HIBAR II)
Flow Rate	2ml/min.
Sample Prep	Protein Precipitation 1.6:1 in Acetonitrile
Internal Standard	Diazepam
Retention Time	~ 4 min. (IS), ~ 6 min. (STD)
Reference	37

Condensed HPLC Assay For Propranolol

Mobile Phase	25:75 [Acetonitrile:KH_2PO_4-H_3PO_4 Buffer (pH 2.7)]
Detection System	Fluorescence Exc - 285nm Em-350nm
Column Type	10nm C18 250x4.6mm (Partisil 10-ODS)
Flow Rate	1.5ml/min at 50°C
Sample Prep	Hexane Extraction (pH 9.6) Hexane Wash (pH 2.7)
Internal Standard	Pronethalol
Retention Time	4.6 min (IS) 6.3 min (STD)
Reference	38