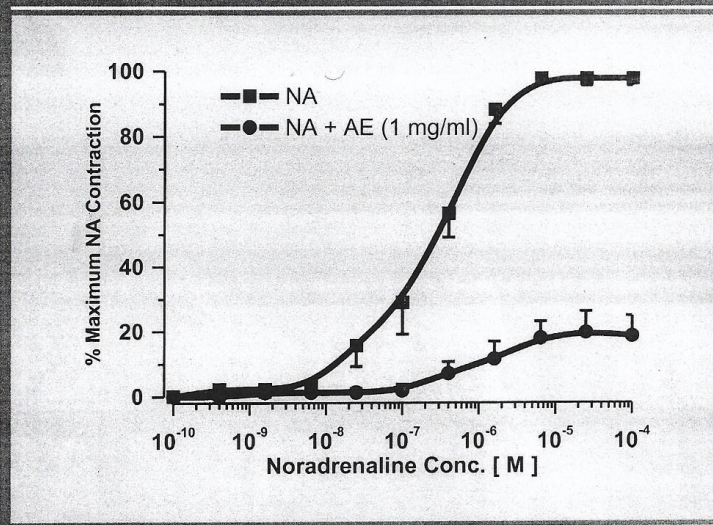


A HANDBOOK OF TECHNIQUES IN EXPERIMENTAL PHARMACOLOGY



EDITORS:

Raymond I. Ozolua | Enitome E. Bafor



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A Handbook of Techniques in Experimental Pharmacology is a bold attempt by over thirty academics and research scientists to stimulate and enhance biomedical research in Nigeria and other resource-poor countries. The book reduces time and energy often spent on internet searches for methods and models suitable for desired pharmacological investigations albeit without much scientific clarity. It condenses simple, relatively cheap, doable but reliable ways of investigating and interpreting the actions of drugs/substances. Although the emphasis of the book is on pharmacological and toxicological techniques, it is also enriched with chapters on experimental designs, ethics in research, and statistics, all of which are vital to quality biomedical investigations. The book contains helpful appendices such as formulae and uses of physiological salt solutions and buffers, and some biological parameters of common laboratory animals.

Postgraduate students, lecturers and researchers in pharmacology, pharmacy, medicine, physiology, biochemistry, medical laboratory science, science laboratory technology, and indeed biomedical scientists who are involved in investigating the actions of drugs and substances in biological systems will find the book a ready reference and a useful companion.


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*Patrick O. Erah, Shakirat I. Bello,
Kehinde A. Ganiyu & Winifred A. Ojieabu*

Introduction

Pharmacokinetics describes the movement (in Greek, *kinesis*) of a drug (in Greek, *pharmakon*) around the body. It is defined as “the study of the rates of absorption, distribution, metabolism and excretion (ADME) of a drug” (Ratain and Plunkett, 2003). Studies in pharmacokinetics are usually performed to examine the ADME of compounds which can be investigational drugs or approved drugs and can be carried out *in vitro* or *in vivo*. In clinical pharmacokinetics, the data gathered are vital in the design as well as the conduct of subsequent clinical trials. Furthermore, they are important for the evaluation of the efficacy and safety of new drugs and in post-marketing clinical trials. In general, the results of pre-clinical pharmacological and toxicological studies are compared with those from pharmacokinetic studies to obtain valuable information for the appropriate and safe conduct of clinical trials, and for the assessment of the mechanism of drug action. Pharmacokinetic studies are useful for the following (Archer, 2013):

- Determining the appropriate use of medicines based on patient characteristics, including disease and drug-metabolizing enzyme genotype,
- Forecasting the influence of pharmacokinetic drug interactions, and
- Providing information for therapeutic drug monitoring (TDM) available.

The traditional approach to pharmacokinetic studies relies on analysing drug concentrations in readily accessible body compartments such as blood, cerebrospinal fluid, faeces, saliva, skin blister fluid (microdialysates) and urine. Several other experimental approaches have been developed for direct assessment of drug kinetics. These are both *in vitro* and *in vivo* methods and include the more sophisticated methods such as those that follow the distribution of drugs between different organs and their elimination. Examples include two key imaging techniques namely magnetic resonance imaging (MRI) and position emission tomography (PET) (Probst *et al.*, 2016). In practice, differences often exist in the physical and chemical properties, pharmacological and toxicological effects of different compounds for the evaluation of ADME. Although the concentration of drugs in free plasma may not always correlate with those from other biological fluids, these measurements are often enough for TDM when combined with the measurements of laboratory parameters for kidney and liver functions. Therefore, it is important to select appropriate methods in

experimental pharmacokinetics. However, many scientists working in new drug discovery and development are faced with the great challenge of determining the most appropriate methods for the screening of compounds with optimal pharmacokinetic properties.

Sampling, Storage and Analysis of Drugs in Biological Samples

Before pharmacokinetic parameters can be determined, the pharmacokinetic specialist must first determine the route of administration of the drug, the dosage form, subjects to be selected, design of study, collection and storage of samples, analytical methods, etc.

Sampling

Pharmacokinetic data can be analysed in biological fluids and tissues. Biological fluids in which pharmacokinetic parameters can be determined include blood (plasma), urine, saliva and cerebrospinal fluid. Proper sample preparation reduces error in the analysis. The steps involved in bioanalytical methods are given in Figure 33.1.

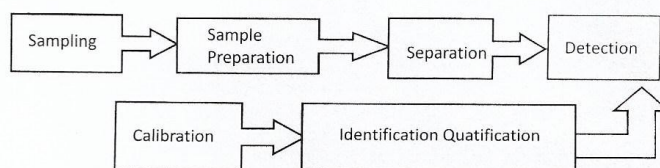


Figure 33.1: Steps in bioanalytical methods

In biological fluids, the preparation of any sample for the analysis of drugs consists of different unit operations that used to (i) release of the drug from a conjugate or biological matrix; (ii) remove endogenous compounds capable of interfering with the assay; and (iii) concentrate the analyte to improve its detection limits and handle the liquid. The various chemical and physical methods that can be utilised for this purpose include absorption, adsorption, chromatography, complex formation, crystallization, distillation, drying, extraction, filtration and ion exchange (Wu *et al.*, 2017). An example of a protocol for extracting weak acids is provided in Box 1 (Erah *et al.*, 1996; Erah, 1997).

Box 1: Example of protocol for the extraction of a wide variety of weak acids

- To 2 ml of plasma, urine or other homogenised matrix, 10 μ l of perchloric acid is added.
- The sample is thoroughly mixed and then centrifuged at 5000 g for 5 min.
- The supernatant is collected and neutralised with 10 μ l of sodium carbonate
- A quantity of 50 μ l of the sample is then injected into the HPLC column

Storage of samples

Prepared samples may be stored in liquid nitrogen (-180°C for long term storage) or in a deep freezer (-21°C for short term storage, often a few hours to days, depending on the type of analyte). The samples may not be stored in a refrigerator because the freezer in a refrigerator often has a temperature that ranges from 4°C to -5°C (when there is constant electric power supply) where many drugs and their metabolites can still degrade (Erah, 1997).

Assay Methods

Frequently used methods of analysis of drugs in biological samples include spectrophotometry, spectrofluorometric, high performance liquid chromatography (HPLC), mass spectrometry (MS) (Chen and Hsu, 2013), HPLC-MS (Dong, 2018), reversed-phase ultra-performance liquid chromatography (UPLC), and the tandem mass spectroscopy (UPLC-MS/MS) (Yan *et al.*, 2014), liquid chromatography and tandem mass spectroscopy (Erceg *et al.*, 2010), gas chromatography (GC), GC-MS, and immunoassay. Best approach is to look at already published work to determine suitable methods of assay for the compounds or metabolites to be determined or to develop and validate suitable assay methods. Every assay method must be validated before being used for evaluation. The basic validation parameters include specificity, linearity, range, precision, accuracy, detection and quantitation limits (Erah *et al.*, 1996), appropriate calibration curves and internal standards (a similar compound spiked into the sample and analysed along with the sample to correct any error in extraction of drug or metabolite into the final sample analysed). Any assay procedure adopted must be reproducible, precise, reliable, and efficient. As far back as 1992, an automated procedure for the sample preparation and chromatographic analysis of drugs and their metabolites in biological fluids using HPLC was developed (Carvalho, 2013).

Experimental Methods

Determination of Plasma Concentration Profile

There are different reasons why plasma concentration profile of a drug may be required. After initiating therapy, it may be necessary to evaluate the patient's response to the drug and then adjust the dosage regimen in case of ineffective therapy or appearance of undesirable effects. This may require the utilization of the drug plasma concentration profile of that individual. Other reasons include *in vivo* testing in which:

- ❖ the concentration of the drug or its metabolite(s) in biological fluid, such as blood, plasma, serum or tissues, is measured;
- ❖ the urinary excretion of the drug or its metabolite(s) is measured; and
- ❖ An appropriate pharmacological effect of the drug is measured.

Plasma drug concentration profile may also be required in well controlled clinical trials to establish both the safety and efficacy of the product. In establishing the plasma concentration profile, the drug may be administered to an animal or human. A simple example is the

administration of a single dose of paracetamol 1000 mg orally to a 35 years old man. Blood sample is collected just before the drug is administered (0 time) and then at specific time intervals. Knowing good time interval to use in the study is vital because if, for example, the investigator chooses to collect blood samples every hour and the maximum absorption phase occurs at 30 min, the maximum (peak) plasma drug concentration attained (C_{max}) and the corresponding time (t_{max}) will be wrongly estimated. Therefore, where the plasma drug concentration profile is unknown (as in the case of a new drug formulation), a preliminary investigation is usually desirable in determining the time intervals during which blood samples may be collected. An example is to collect blood samples at 10 min interval during the first hour and every 30 min subsequently for another 3 hours. When the plasma drug concentration is plotted against time, the investigator will have an idea of possible interval to use for the study.

In the hospital ward, where plasma profile of a drug is needed for individualizing a dosage regimen for a patient, an established clinical protocol is followed. For a new compound undergoing investigation, the number of subjects or animals needed to establish a profile is based on the requirements of the regulatory body. The first step is to prepare a study protocol with the appropriate study design for approval by the appropriate ethical committee for human (or animal) studies. Animals that can readily be used include dogs and monkeys. Collecting blood samples from small animals like rats and rabbits at the intervals required for a drug concentration profile may be a challenge.

An example of detailed procedure is the determination of artemeter-lumefantrine in patients by Nyunt *et al.* (2016). First, it is ensured that each subject or animal is not currently on the drug to be evaluated or allergic to it; at least a drug-free week is allowed prior to evaluation. Then the subject is allowed to fast overnight and thereafter, blood sample is obtained from the subject just before a usual dose of the drug is administered under medical supervision. Blood samples (2-5 ml) are collected at predetermined intervals into appropriate vials with heparin (to prevent clot). The blood is centrifuged at 5000 g for about 5 min using a microcentrifuge to obtain plasma. This plasma may be kept frozen (preferably at -20°C to -180°C) until analysed. Drug concentrations in the plasma are measured using suitable analytical approach (such as high performance liquid chromatography, HPLC) that minimizes interference from endogenous materials. Further details of sample preparation and instrumentation are discussed above under *Sampling and analysis of drugs in biological samples*. A graph of the plasma concentrations of the drug against time for that subject is plotted. Where more than a subject is involved, the mean of the plasma drug concentrations at each time may be plotted against time. As appropriate, the area under plasma drug concentration curve often called area under the curve (AUC), the t_{max} and the C_{max} for each subject or the appropriate average values may be determined from the profile(s) as follows:

(a) Trapezoidal method

Consider the following values obtained after the oral administration of 300 mg of phenytoin tablet:

Time (h)	1	2	5	10	15	20	30	40	50
C_p (mg/L)	0.65	2.0	3.55	4.05	3.6	3.2	2.0	1.2	0.75

The above data is entered into Microsoft Excel sheet. Values of the logarithm (log) of the plasma concentration (C_p), i.e., $\log_{10} C_p$, are determined and plotted against the time values.

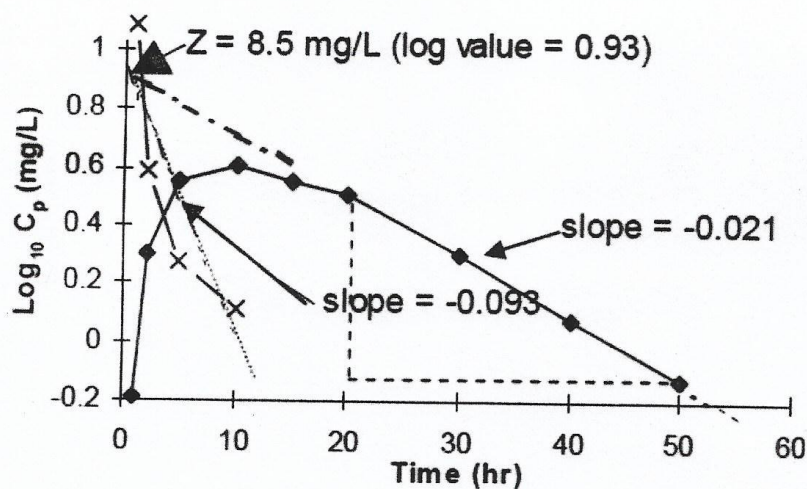


Figure 33.2: Plasma concentration profile of 300 mg phenytoin following oral administration.

The elimination rate constant for phenytoin ($-k_e$) may be determined from the slope of the log-linear portion using the relationship: $-k_e = 2.303 \times \text{slope}$. Half-life ($t_{1/2}$) may be determined using the relationship: $t_{1/2} = 0.693/k_e = 0.693 / (2.303 \times \text{slope})$. The $C_{\max}$ and the corresponding $t_{\max}$ can be estimated directly from the graph. Absorption rate constant (k_{ab}) may be calculated by the method of residuals as follows:

- ✓ The log-linear line is extended to zero time (intercept Z) as shown in Figure 33.2.
- ✓ $\log_{10} C_p$ values on this extended line ($\log_{10} C_p^*$) are obtained at each of the time points until the attainment of the decline phase of the concentration-time plot (in this case

the time points are 1, 2, 5 and 10 h).

- ✓ The real $\log_{10} C_p$ values are subtracted from these projected values (Figure 33.2).
- ✓ These new values ($\log_{10} C_p - \log_{10} C_p^e$) [Table 1] are plotted and a straight line is drawn through the points (the zero-time intercept of this line will also be Z). The slope of this new line (-0.093 shown in Figure 33.2) is used to calculate k_{ab} (slope = $k_{ab}/2.303$).
- ✓ Half-life for absorption ($t_{1/2(ab)} = 0.693/k_{ab}$) is calculated.
- ✓ AUC from zero to infinite time ($AUC_{0-\infty}$) after the oral administration of the drug is calculated using any of the two following methods:

(i) $AUC_{0-\infty} = Z/k_e - Z/k_{ab}$ (Z is 8.5 mg/l in Figure 33.2).

(ii) *Trapezoid approximation method:*

- ✓ The log-linear portion of the plot is extended downwards to meet the time axis (infinity time). Note the last C_p value ($C_{pn} = 0.75$ mg/l) and the corresponding time ($t_n = 50$ h)

$$\checkmark AUC_{0-n} = \int_0^n C_p dt = \sum (t_1 - t_0)/2 \times (C_0 + C_{p1}) + (t_2 - t_1)/2 \times (C_{p2} + C_{p1}) + \dots$$

$$(t_n - t_{n-1})/2 \times (C_{pn} + C_{pn-1})$$

- ✓ AUC from time zero to the last time C_p (AUC_{0-n}) can be measured as follows:

$$\checkmark AUC_{0-\infty} = \sum_{\alpha} C_p dt = AUC_{0-n} + (t_{\infty} - t_n)/2 \times (C_{p\infty} + C_{pn})$$

- ✓ $C_{p\infty}$ is zero but t_{∞} is unknown and $AUC_{n-\infty} = C_{pn}/k_e$ hence,

$$AUC_{0-\infty} = AUC_{0-n} + C_{pn}/k_e \text{ where } C_{pn} \text{ is the last measured } C_p$$

Table 33.1: Example of Microsoft Excel approach of pharmacokinetics parameters calculation

Time (h)	C_p (mg/l)	$\log_{10} C_p$	Projected $\log_{10} C_p$ ($\log_{10} C_p^e$)	$C_p^e - C_p$	$t_x - t_{x-1}$	$(C_p + C_{p-1})/2$	$\sum_{\alpha} (t_x - t_{x-1}) \times (C_p + C_{p-1})/2$
0	0	0	0.929346	0.929346	0		
1	0.65	-0.18709	0.908226	1.095313	1	0.325	0.325
2	2	0.30103	0.887106	0.586076	1	1.325	1.325
5	3.55	0.550228	0.823746	0.273518	3	2.775	8.325
10	4.05	0.607455	0.718146	0.110691	5	3.8	19
15	3.6	0.556303			5	3.825	19.125
20	3.2	0.50515			5	3.4	17
30	2	0.30103			10	2.6	26
40	1.2	0.079181			10	1.6	16
50	0.75	-0.12494			10	0.975	9.75

Sum (AUC_{0-50}) = 116.85 h.mg/l

(b) Pharmacokinetics software

Instead of the above relatively manual approach in determining the pharmacokinetic parameters, pharmacokinetic software such as WinNonLin (Certara, 2018) and GraphPad Prism (GraphPad, 2018) can be helpful. A list of some other useful software is available at <https://www.pharmpk.com/soft.html> (accessed 9th June, 2018). These software can provide all the needed pharmacokinetics parameters and the graphs.

Determination of Bioavailability and Bioequivalence

Bioavailability is a measurement of the rate and extent (amount) of therapeutically active drug which reaches the general circulation. That is, the rate at which a drug appears in the systemic circulation and the relative amount of administered dose [or the fraction (F) of drug absorbed] which reaches the systemic circulation. Although it can be determined following a single or multiple doses of a drug, more information concerning the rate of absorption is obtained after a single dose than after multiple dosing. However, multiple dosing provides a more closely representation of the usual clinical situation, and plasma concentrations are often higher than those after a single dose. Steady state plasma concentration is usually obtained after multiple dosing at a fixed-dosing interval for four or five elimination half-lives.

“Two pharmaceutical products are bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives, and their bioavailability values, in terms of rate (C_{max} and t_{max}) and extent of absorption (area under the curve), after administration of the same molar dose under the same conditions, are similar to such a degree that their effects can be expected to be essentially the same” (WHO, 2016). The United States Food and Drugs Administration (FDA) defined bioequivalence as the “absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study” (FDA, 2017). Bioequivalent products are expected to be therapeutically equivalent. Thus, FDA considers “two products to be bioequivalent if the 90% confidence intervals of the relative (ratios) mean of the log-transformed exposure measure (C_{max} , $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$) of the test (e.g. generic formulation) to reference (e.g. innovator brand formulation) is within 80% to 125% and the t_{max} for the products are also similar”. For some regulatory bodies, the fraction of dose absorbed (F) should also be similar for both test and reference products. Therefore, bioequivalence may often be determined in terms of fraction of dose absorbed (F), t_{max} , C_{max} and $AUC_{(0-\infty)}$. Although the extent of absorption of some pharmaceutical equivalents or pharmaceutical alternatives may be equivalent and their rates of absorption are different, they may be considered bioequivalent if such differences in the rate of absorption is intentional and reflected in the labelling and not considered essential to the attainment of effective body drug concentrations on chronic use, or are considered medically insignificant for the particular drug product studied.

For both the National Agency for Food and Drug Administration and Control (NAFDAC) of Nigeria and FDA, establishing bioequivalence in the *in vitro* and/or *in vivo* testing of specified drug products is a condition for marketing of the products.

The problems in determining bioequivalence include lack of an adequate study design, inability to accurately measure the drug analytes (including metabolites and enantiomers), and lack of systemic absorption. Bioequivalence studies for which objective blood concentrations cannot be obtained require either a pharmacodynamic study, clinical trial or an *in vitro* study that has been correlated with human *in vivo* bioavailability data (WHO, 2016).

First, the analytical methods of the drugs in the formulation are sourced from the literature or manufacturer's information. Efforts are then made to reproduce the methods and ensure they are working correctly. Next, a protocol for the investigation is prepared using the guidelines of the appropriate Ethical Review Board (Committee).

Institutional Review Board for animal studies may be based in a research institute or University. A cross-over study design and random allocation of subjects into the control and test groups using 2 x 2 table are often considered in bioequivalence studies. The *in vitro* characteristics of the drug molecule including the physicochemical properties of the product (appearance, taste – if possible, colour, shape, consistency, pH, viscosity, etc) as well as the disintegration and/or dissolution profiles are determined depending on whether the drug is in liquid or solid dosage form. This is followed by the recruitment of the appropriate number of subjects (non-pregnant healthy volunteers or animals) for the *in vivo* studies described below under bioavailability or bioequivalence. Most regulatory bodies require 13-15 subjects but NAFDAC may allow at least 5 subjects. It is important to ensure that no subject has been on the drug for at least a week prior to the investigation.

Bioavailability: After an overnight fast, blood sample is obtained from the subject just before the drug is administered. A single dose of the test formulation is administered orally (for oral bioavailability) or via the route being investigated and blood samples (2-5 ml) are collected at appropriate intervals. The same dose of the test drug is then administered intravenously to the same subject after a washout period (a period over which the drug would have been completely cleared from the blood) and blood samples are collected at the same time intervals. The plasma drug concentration profile and AUC are determined as earlier described and the bioavailability (F) calculated as AUC_{oral}/AUC_{iv} where AUC_{oral} represents the AUC of the plasma concentration profile obtained following the oral route and AUC_{iv} represents the AUC of the plasma concentration profile obtained following the intravenous route.

Bioequivalence: Samples of the drug formulation to be tested (test drug) and standard formulation, usually the *innovator brand or an* already registered drug from a reputable manufacturer (reference drug) are obtained. Using a cross over design, the subjects are randomly assigned to the days they will visit the investigation unit to receive either the test or the control drugs. After an overnight fast, blood sample is obtained from each subject just before the drug is administered. A single dose (or multiple doses) of one of the two drugs is administered and blood samples (2-5 ml) are collected at appropriate intervals. After a washout period, the same dose(s) of the second drug is then administered to the same subject and blood samples are collected at the same time intervals. The plasma drug concentration profiles, AUC_{0-12} , $AUC_{0-\infty}$, t_{max} and C_{max} for each of the drugs for each subject are determined as described above, and compared. AUC_{0-12} is the AUC from zero time to the time the last blood sample was collected while $AUC_{0-\infty}$ is the AUC from zero time to the time the drug is completely cleared from the plasma. If the 90% confidence intervals of the relative mean of the C_{max} , AUC_{0-12} and $AUC_{0-\infty}$ of the test drug to the reference drug is within 80% to 125% and the t_{max} for the products are also similar, the two products are bioequivalent.

Prediction of Human Clearance and Volume of Distribution

Allometric scaling of pharmacokinetic data has been used to correlate pharmacokinetic parameters in pre-clinical species with those of humans. This allows the predictions of clearance and volume of distribution in humans (VD). An example of how this predictive approach for Vd using “average fraction unbound in tissues” method is provided below while other predictive methods can be found elsewhere (Nagar and Korzekwa, 2017).

Procedure for Prediction Using the Method of Average Fraction Unbound in Tissues

Experimentally determined values for Vd (in units of litres/kg) and plasma protein binding obtained from pre-clinical studies for different animal species is used, along with standard values for extracellular fluid volumes, plasma volumes, and other parameters to calculate the fraction unbound in tissues (f_u) in the animal species. The f_u for each pre-clinical species is given by:

$$\frac{V_r f_u}{[Vd_{ss} - V_p - f_u V_e] - \left[(1 - f_u) \frac{R_e}{i} V_p \right]}$$

Where, f_u = unbound fraction in plasma (or serum); Vd_{ss} = steady state volume of distribution (in litres/kg); V_p = plasma volume (in litres/kg), V_e = extracellular fluid volume (in litres/kg); V_r = “remainder of the fluid” volume (in litres/kg); R_e/i = ratio of binding proteins in extracellular fluid (except plasma) to binding proteins in plasma.

After calculating f_u for each of the pre-clinical species, an average of all values for the

compound is obtained. This average $f_{u, \text{avg}}$ is assumed to be equal to $f_{u, \text{human}}$ and is used, along with the value of the fraction unbound in human serum/plasma (f_u) determined experimentally, to predict human $V_{d, \text{human}}$ (in units of litres/kg) using appropriate human values for V_p , R_{ef} in the following equation:

$$V_{d(\text{human prediction})} = V_p + [f_{u(\text{human})} \cdot V_c] + \{[1 - f_{u(\text{human})}] \cdot R_{ef} \cdot V_p\} + V_i \cdot f_{u(\text{human})} / f_{u(\text{average})}$$

Regional Pharmacokinetics

Different regional approaches exist for the evaluation of drug pharmacokinetics. These include intestinal permeability, artificial perfused organs, *in vivo* regional blood sampling for drugs, tissues slices and cell cultures, post-mortem tissue biopsies, etc.

Estimating Intestinal Permeability

In vivo drug absorption can be predicted using *in vitro* and *in situ* approaches. The three most commonly applied methods used to predict the fraction of the drug absorbed are: (1) rat *in situ* single-pass intestinal perfusion; (2) rat everted intestinal rings; and (3) the *in vitro* human colon adenocarcinoma (Caco-2) cell line (Wang *et al.*, 2015; Yang *et al.*, 2017). These assessment methods are principally applicable to drugs absorbed through passive diffusion (Yang *et al.*, 2017).

Caco-2 cells model is excellent for passive transcellular pathway or comparing models with parallel artificial membrane permeation (Erah, 1997). Drugs that are completely absorbed usually have a high permeability coefficient ($P_{app} > 1 \times 10^{-6}$ cm/s) in Caco-2 cells. However, the limitations in the use of Caco-2 cells are: (1) they cannot be used in the screening of formulations that are sensitive to pharmaceutical excipients; (2) the cells have low tolerability to organic solvents; (3) they are assayed in microwells, which do not represent the whole system; and (4) they do not accurately predict the side or adverse effects, which is a common experience with drugs in the context of the human body or target organ system.

Procedure for Caco-2 monolayers: The Caco-2 cells monolayers are grown in culture T-flasks (75 cm² or 175 cm²) in a CO₂-incubator at 37°C with 5% CO₂ and 90% relative humidity using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1 mM of non-essential amino acids, 100 U/ml of penicillin, 0.1 g/ml of streptomycin, 10 mM of sodium bicarbonate, and 10% foetal bovine serum (FBS) as the culture medium. When the confluency of the cells is 70%–80%, they are split at a ratio of 1:3–1:5. For the transport experiments, the cells are harvested with trypsin-EDTA and seeded on polycarbonate filters (0.4 µm pore size, 1.13 cm² growth area for 12-well plate or 0.33 cm² growth area for 24-well plate) into a Transwell cell culture chambers using a density of approximately 75,000

cells/cm². The culture medium is revitalized every 24–48 h and allowed to grow for 18–21 days before being used for the transport studies to allow maximum expression of transporters (e.g., P-glycoprotein). For proper integrity of the cells, the transepithelial electrical resistance (TEER) and the permeability of mannitol (low cellular permeability) through the cells are measured. Only Caco-2 monolayers with TEER values between 400 and 800 Ω.cm² (24 wells) and permeability of ³H-mannitol lower than 0.5×10⁻⁶ cm/s are used. In each experiment, a reference compounds with high cellular permeability, such as mannitol or propranolol, is included. The apparent permeability coefficient (P_{app} , expressed in cm/s) is calculated as:

$$P_{app} = V_R A \times C_0 \times \frac{dC}{dt}$$

where, V_R is the volume of the drug in the receiver chamber (ml), A is the surface area (cm²) of the filter, C_0 is the initial concentration of drug (μg/ml) in the donor chamber, and dC/dt is the initial slope of the cumulative concentration (μg/ml) in the receiver chamber with time (t). If the P_{app} values in the apical-to-basolateral direction are not equal to those in the basolateral-to-apical direction, or if the P_{app} values decrease with increasing test compound concentration, further incubations may be done to determine the involvement of specific transporter or efflux systems (Yang *et al.*, 2017).

Human Cryopreserved Hepatocyte Methods

Epithelial cells cultures from the barriers in the skin, intestine, blood/brain, and blood/lung, are used widely to evaluate drug absorption. Primary hepatocytes isolated from the liver are invaluable in the *in vitro* assessment of drug-drug interactions, hepatotoxicity, metabolism and transporter for phases I and II drug metabolizing enzyme activities. Cryopreserved primary hepatocytes (Life, 2017), HepaRG cell lines (Marion *et al.*, 2010), 3-D liver tissue models, and bioprinted (Murphy and Atala, 2014) liver “strips” are effective for the evaluation of drug metabolism.

An aliquot of the drug solution (100–300 μM) in dimethylsulfoxide is added to a vial containing buffer of hepatocyte suspension. The hepatocytes (2 million cells/ml) are pre-incubated for 5 min at 37°C along with the vial containing a mixture of the drug and buffer (1–3 μM). To reduce the P450-inhibitory effects of dimethylsulfoxide, the final mixture is diluted to 1% (v/v), to mimic the incubation conditions used in a typical drug discovery programme and to counter issues of solubility. Then, aliquots are removed at time intervals (5, 10, 20, 40, 60, and 90 min), quenched in ice-cold methanol and the samples are then frozen for an hour at -20°C. They are finally centrifuged at 3500 rpm for 15 min at 4°C and the supernatants are collected and analysed (McGinnity *et al.*, 2004; Shibany *et al.*, 2016).

Artificially Perfused Regions

Artificially perfused regions (*in situ* or isolated) from animal can be perfused via cannulated afferent and efferent blood vessels, with an artificial nutrient. Like Caco-2 cells, the rat *in situ* single-pass intestinal perfusion and the rat everted intestinal rings are two of the most widely used methods primarily applicable to drugs that are absorbed through passive diffusion. Also, drug elimination has been evaluated in isolated perfused livers of a number of species. An example is the evaluation of the uptake and elution of alphaxolone in the rat lung (Upton *et al.*, 1990).

A rat is sacrificed and the abdominal wall opened. About 6 cm is excised from the small intestine, clipped at both ends and cannulated to allow drug injection while inside a thermostatically-controlled chamber containing physiological salt solution (PSS) of the following composition (mM): NaCl: 118, KCl: 4.7, CaCl₂: 2.5, MgSO₄: 1.2, glucose: 2, NaHCO₃: 14.9, KH₂PO₄: 1.2. It is ensured that the preparation is filled with the PSS and continuously bubbled with a 95% O₂ and 5% CO₂ gas mixture at a pH of 7.4 and temperature of 37°C using a peristaltic pump to prevent drying. After an equilibration period of 60min, aliquot of the PSS is withdrawn into a sample vial, and the drug solution in PSS (0.1 ml) is injected into the preparation through the cannula. Samples of the PSS are then withdrawn from the chamber at time intervals for analysis of the drug permeating through the intestine into the chamber. Each time PSS is withdrawn from the chamber, an equivalent volume of PSS is used to replace it. The process is repeated four times or more and the concentration of the drug in the chamber determined. A plot of the drug concentration against time is made and the pharmacokinetic parameters estimated.

Post-Mortem Tissue Biopsies

This method can be used to determine the concentrations of drugs in specific regions of the body (Bhatia and Ingber, 2014). However, a large number of animals are required to evaluate the time-course of drug concentrations as many animals are required at each time-point to minimize the effects of inter-animal variability. In this method, it is difficult to prove that the manner of sacrificing the animals does not alter the regional drug concentrations even though some authors believe that rapid killing of the animals minimizes this problem (Upton *et al.*, 1990).

At least 5 animals (e.g. rats) are selected for each of the predetermined time that the concentration of the drug in the tissue is to be measured. For example, if drug concentration is to be obtained at 0, 1, 2, 4, 6, 8 and 10 h, 35 rats are used. At baseline, the drug is administered to all the animals and 5 of them are rapidly sacrificed. Thereafter, 5 rats are sacrificed at 1, 2, 4, 6, 8 and 10 h. Once an animal is sacrificed, biopsy sample of the post-mortem tissues is obtained and preserved at -20°C until required for analysis of the drug content. The biopsy samples are assayed for the drug using standard sample preparation and sample analysis techniques.

Tissue Slices and Cell Culture

Slices of tissues, cell cultures or suspensions can be used to study the uptake of drugs from an incubation medium, most commonly by measuring the decrease in drug concentration in the medium instead of the increase in drug concentration in the slice. However, with this approach, it is difficult to determine whether the drug uptake from the medium is due to drug metabolism or distribution. This is because the uptake of drugs into fat *in vivo* is complex (Bickel, 1984).

For tissue slices, animals are sacrificed rapidly and the small tissue slices obtained are weighed rapidly. The slices are then placed in a 0.9% sodium chloride solution to remove the blood or content, blotted on a filter paper, and then weighed for wet weight. The drug is added to PSS in a chamber under the conditions described in “artificially perfused regions” and tissue slices are then dropped into the PSS. Reduction in drug concentration in the medium is measured over time intervals bearing in mind the new concentration of drug in the medium each time the PSS is sampled for drug assay and the volume removed replaced with equal volume of the PSS (Fry and Bridges, 1977; Zhang *et al.*, 2017).

Tissue Homogenates

In vitro, this method can be used to evaluate the rates of metabolism and tissue binding of drugs. Homogenization usually causes the destruction of the structure of the tissues leading to small groups of cells or damaged cells. As a result, drugs easily pass through the homogenate. The binding of drugs like antipyrine, imipramine, phenobarbital, phenylbutazone, salicylic acid, and thiopental in rats has been evaluated from diluted tissue homogenates in multi-chamber dialysis apparatuses (Upton *et al.*, 1990).

After oral dosing, the experimental animals are quickly sacrificed and the tissue samples cut out are weighed rapidly. The samples are then placed in a 0.9% sodium chloride solution to remove the blood or content, blotted on a filter paper, and weighed again for wet weight. The slices of tissues are individually homogenized in normal saline (1:4; weight/volume). Drug is added to the homogenate and the time-course of the drug concentration in the homogenate is determined using similar approach to that of post-mortem tissue biopsies (Upton *et al.*, 1990; Zhang *et al.*, 2017).

Regional Blood Sampling In Vivo

The afferent and efferent blood of a region can be useful in gathering regional pharmacokinetic information. Since arterial blood is often assumed to be homogeneous, the concentration of drug entering most organs of the body is a representation of the concentration of drug in blood sampled from any arterial vessel. Thus, the concentrations of lignocaine in the aorta and femoral artery of humans are similar after a bolus injection of the drug (Hatherell *et al.*, 2011). Blood samples from the artery and the appropriate peripheral vein can easily be used to evaluate peripheral regions, such as the arms or legs. This method

has also been used to characterize the uptake of drugs into the lungs using central venous and arterial blood sampling. It has also been used to evaluate the uptake of drugs into the lungs. For example, thiopental has been studied using the human forearm through the radial artery and cubital vein catheters. However, the visceral organs require more elaborate cannulation methods (Upton *et al.*, 1990).

The subjects to be used (humans or animals), none of who has been on the drug to be investigated for at least a week or 8 half-lives of the drug, are recruited. After an overnight fast, the appropriate dose of the drug is administered after collecting blood sample from the regional artery which may be cannulated, if possible. Then, blood samples are collected at predetermined intervals and the procedure earlier described for the determination of pharmacokinetic parameters in blood samples followed.

Surgical Transplantation

This method generally requires the use of large animals such as dogs or sheep. The studies can be facilitated by surgically transplanting an organ to another location in the animal. Infusion of drug into a peripheral vein of the lower body corresponds to a portal infusion of the same drug, while infusion into a peripheral vein of the upper body leads to systemic administration. This preparation has been used to investigate the elimination of quinidine and morphine in dog liver (Upton *et al.*, 1990).

As an example, the kidney of a sheep is surgically transposed to a pouch under the skin of the neck after an overnight fast. The renal artery and vein are then anastomosed to the carotid artery and jugular vein. As the animals remain healthy, the transposed kidney continued to make urine. A blood vessel in the neck is cannulated and both urine and the blood leaving the kidney are collected. The drug is then administered and its concentration in the urine and blood are monitored as earlier described (Upton *et al.*, 1990).

External Counting

This is a relatively old method in which concentrations of radio-labelled drugs is assessed in specific regions of the body using external counting methods. Example is the investigation of the uptake of ^{14}C -aliphatic amine and ^{14}C -chlorpromazine by the lungs. Nuclear magnetic resonance has been used for the non-invasive evaluation of the amount of halothane in the brain of rabbits (Thomas *et al.*, 1993).

The drug to be used for the investigation such as erythromycin is radio-labelled using ^{14}C . It is then administered to the subjects after an overnight fast and an external gamma counter is used to monitor the drug in some organs such as lungs (Thomas *et al.*, 1993; Upton *et al.*, 1990).

Non-Animal-Based Methods

Animals which include rats, rabbit, monkeys, dogs are frequently employed in pharmacokinetics studies in an attempt to simulate what might happen when a medication is administered by a human. The obvious genetic and physiological differences between humans and other animal that result from these studies may not reflect potential human responses. Volume of blood, profile of the metabolic enzymes, permeability across barriers, gastric environment, clearance, and other factors in animal models differ substantially from those of humans (Giorgi *et al.*, 2012; Grime and Paine, 2013). Some examples on a number of sophisticated methods by which pharmacokinetic parameters can be measured are provided below.

Microdosing

This is a pre-clinical safety evaluation technique for pharmaceuticals which involves highly controlled trials of new drugs in humans. It allows gathering of accurate information on the movement of the drug during absorption and metabolism in the body. The use of ultralow doses in this technique, ensures that the trials conducted are safe (Tewari and Mukherjee, 2010).

Each group of subjects recruited for the study initially receive small dose of the drug for investigation and plasma levels are monitored as previously described. Increasing higher doses are similarly investigated and once the right dose is found, it is used for the determination of the required pharmacokinetic parameters.

Microphysiological Systems

These are revolutionary systems also called “human-on-a-chip” or “physiome-on-a-chip” systems. Each is an integration of engineering and advanced biology using flexible systems that contain small reservoirs of cells joined by fluid-containing channels on small silicone or polymer chips. Larger platforms with dishes of cells joined with tubes that can configure different organs such as kidney, skin and skeletal muscle (Figure 33.3) are available (Edington *et al.*, 2018). These systems permit the linkage of different organs and their flexibility allows researchers to study metabolism and distribution of drugs with many different organs (Ghaemmaghami *et al.*, 2012; NCATS, 2017).

The drug is administered orally or intravenously to the subjects and after an overnight fast. Different organs such as kidney, skin and skeletal muscle of the subject are configured into the system as appropriate and samples containing the drug (eg, diclofenac) are collected from each compartment. The samples are then quantified for parent drug and its primary metabolite(s). Individual points represent measurements from different platforms. The data collected are analysed with pharmacokinetic models.

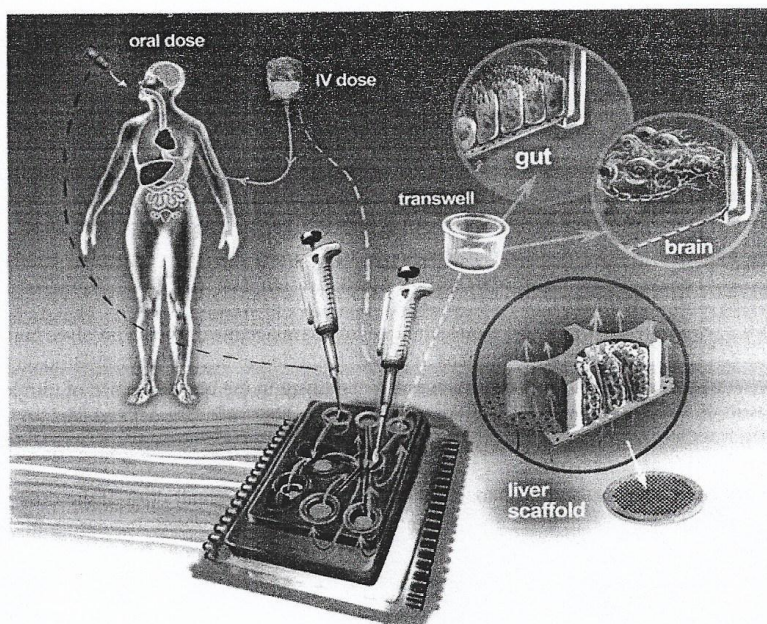


Figure 33.3: A microphysiological platform.

Computational Methods

The advances in information and communication technologies have enabled the development of a number of *in silico* tools to help predict the pharmacokinetic parameters and properties of drugs. *In silico* is the identification of the drug target molecule using bioinformatics tools. The two types of *in silico* drug design are the ligand based drug design and the structure-based drug design. Unlike the ligand-based drug design that relies on the knowledge of other molecules which bind to the biological target of interest, structure-based drug design relies on the knowledge of three dimensional structure of the biological target obtained using methods such as x-ray crystallography, NMR spectroscopy and homology modelling. Different databases allow the comparison of new drugs with existing drug profiles (Law *et al.*, 2014). Computers programme can analyse vast amounts of data and generate predictive algorithms and models based on the chemical structure of drugs (Hsiao *et al.*, 2013). Other than speed, a major advantage of computational approaches is their ability to take individual variations into account which is not possible with animals.

First step is to determine the biochemical basis of the disease process selected. Knowing the exact step(s) in the pathway that altered in the diseased state and regulation of the pathway as well as the three dimensional structures of the molecules involved in the process are critical. The second step is to select a target (enzyme, receptor or nucleic acid) at which to disrupt the biochemical process. The protein BLAST (Basic Local Alignment Search Tool, NCBI, 2018) is then performed for all the genes/proteins with respect to *Homo sapiens*. The least matching molecule in human is selected and another BLAST is performed. As the query sequence matched best, the required target molecule is selected and its structure obtained from The Research Collaboration for Structural Bioinformatics (RCSB) Protein Data Bank (PDB). The crystal structure of the target protein is then obtained from PDB database. This is followed by the selection of ligands/drugs (lead identification) during which high throughput screening of natural product and synthetic compound libraries are done to screen out lead compound. Here a 3D structure of the compound and target is docked. Part of the scoring functions is the determination of the pharmacokinetic properties as well as quantification of the energy of protein/ligand interactions such as hydrogen bond, electrostatics, Van der Waals, hydrophobic p/p etc. Criteria to be fulfilled include efficacy, potency, chemical stability, pharmacodynamic and physiochemical properties, Lipinski's "rule of five", properties, metabolic stability, toxicological aspects, ease of chemical synthesis, chemical optimization potential, and patentability (Kumar, 2013).

Another aspect of *in silico* is to determine the active site of the target protein and visualize it. Lead optimization using quantitative structure activity relationship (QSAR) is done to refine the 3D structure of the lead compounds (Kumar, 2013).

Conclusion

Both animal and non-animal based experimental techniques are available for the evaluation of pharmacokinetic parameters and properties. Despite the limitations of many of these techniques, they have been successfully used in assessing the location of drugs in tissues, movement across biological barriers, concentration of drugs in tissues, and other pharmacokinetic data. The advances in computer technology have enabled revolutionary computational approaches which speed up the process and allow data comparison which would otherwise not have been possible.

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