CHAPTER 15

In vitro *techniques for investigating drug metabolism*

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15.1 *Introduction*

The use of *in vitro* techniques to study drug metabolism allows comparison of the metabolism of a compound across species prior to administration to humans. In addition, the use of human *in vitro* systems provides more relevant information on the metabolites likely to be formed in clinical studies. Therefore, interspecies comparisons of the metabolite profile of a drug candidate may assist rational selection of the most appropriate species for safety assessment studies. Finally, potential clinical interactions, i.e. the ability of a drug candidate to affect the pharmacokinetics of other coadministered therapies (induction or inhibition of drug-metabolising enzymes) can also be investigated using human *in vitro* systems.

The liver is a major site for the biotransformation of xenobiotics and so many models of drug metabolism have primarily focussed on the liver and the enzymes contained within it. Investigation of drug metabolism has involved the use of liver

preparations which vary in their levels of cellular integrity. Systems range from the use of purified enzymes *in vitro* to whole liver perfusion *in situ*. For the purpose of this chapter, discussions will concentrate around the use of subcellular fractions (S9 and microsomes), isolated hepatocytes and liver slices.

The cytochromes P450 are a large family of enzymes involved in the metabolism of a wide range of structurally diverse xenobiotics. Cytochrome P450 enzymes are bound to the membranes of the endoplasmic reticulum, and concentrated sources used for the investigation of drug metabolism can be prepared from homogenised liver by differential centrifugation. The submitochondrial (S9) fraction of liver contains both cytosolic and membrane-bound enzymes but as a homogenate rather than an ordered cellular environment. The S9 fraction is prepared by sedimenting particulate matter, including cell nuclei and mitochondria, from homogenised liver at $9{,}000 \times g$ and is a crude tissue preparation; the impure nature of this preparation may lead to analytical difficulties. However, a more refined enzyme may be prepared from crude liver S9 by further centrifugation. Fragments of the endoplasmic reticulum reform into vesicles known as microsomes. The microsomal pellet is produced by centrifugation of the S9 fraction at $100{,}000 \times g$.

Microsomes are essentially an enriched source of the membrane-bound enzymes such as the cytochromes P450 and as such are probably the most commonly used system in the pharmaceutical industry to study *in vitro* cytochrome P450-mediated metabolism. Both S9 and microsomes retain enzyme activity following cold storage at $-80\,^{\circ}C$. Therefore, S9 and microsome preparations may be made and stored ready for use, an advantage for cross comparisons of metabolism between species and in the case of humans between different liver donors.

The parenchymal cells of the liver (hepatocytes) are a rich source of cytochromes P450. Hepatocytes can be isolated from liver tissue by enzymatic dissociation using collagenase perfusion. Once isolated, hepatocytes can be used to study drug metabolism and induction or inhibition of drug-metabolising enzymes by xenobiotics.

The major advantage of hepatocytes over microsomes for the study of drug metabolism is that, as a cellular system, hepatocytes contain many enzymes and enzyme cofactors not present in the microsomal fraction of the cells. For example, several of the enzymes responsible for conjugation of xenobiotics, such as the sulphotransferases, are located in the cytoplasm of the cell (see Table 15.1). Although some Phase II metabolic pathways can be studied using microsomes supplemented with appropriate cofactors (e.g. glucuronidation), they may be more easily investigated using hepatocytes.

Hepatocytes may be used as short-term suspension cultures (four hours) or may be placed into culture for investigation of drug-related enzyme induction or enzyme inhibition that requires longer periods of exposure to the test compound. One issue with hepatocytes that are placed into culture is that they undergo de-differentiation, i.e. the loss of specific cell characteristics, and this results in a rapid decrease in their content and activity of drug-metabolising enzymes, notably the cytochromes P450. As such, primary cultures of hepatocytes are not routinely used for the study

TABLE 15.1 *Cellular location of the major drug-metabolising enzymes*

Reaction	Enzyme	Cellular location	Substrates
Phase I reactions			
Hydrolysis	Esterases	Cytosol	Esters
		Mitochondria	
		Microsomes	
		Blood	
	Eposide hydrolase	Cytosol	Epoxides
		Microsomes	
	Peptidase	Microsomes	Peptides
		Blood	Glutathione conjugates
		Lysosomes	
Reduction	Mixed function oxidases	Microsomes	Azo
			Nitro
			N-oxides
			Arene oxides
			Alkyl halogenides
	Alcohol dehydrogenases	Cytosol	Alcohols
Oxidation	Mixed function oxidases	Microsomes	Alkanes
			Alkenes
			Arenes
			Amines
			Thiones
			Thioesters
	Monoamine oxidases	Mitochondria	Amines
	Alcohol dehydrogenases	Cytosol	Alcohols
	Aldehyde dehydrogenases	Cytosol	Aldehydes
Phase II reactions			
Glutathione conjugation	Glutathione transferases	Microsomes	Electrophiles
Glucuronide conjugation	Glucuronyl transferases	Microsomes	Phenols
			Thiols
			Amines
			Carboxylic acid
Sulphate conjugation	Sulfotransferases	Cytosol	Phenols
			Thiols
			Amines
Methylation	Methyl transferases	Cytosol	Phenols
		Microsomes	Amines
Acetylayion	Acetyl transferases	Cytosol	Amines
Amino acid conjugation	Transferases	Cytosol	Carboxylic acid
		Mitochondria	Aromatic hydroxylamine

of species differences in drug metabolism but are much more valuable in the study of drug-related enzyme induction or enzyme inhibition.

Liver slices represent a greater level of structural integrity compared to sub-cellular fractions or hepatocytes and may be prepared using instruments such as the Krumdieck tissue slicer. Tissue slices have the advantage that, compared to hepatocytes, they retain an intact three-dimensional structure, allowing cellular architecture and hence intercellular communication to be maintained. This cell–cell contact between different hepatic cell types is important in maintaining cell differentiation. For studies using human liver, tissue slices offer a major advantage over the preparation of hepatocytes, in that samples encapsulated by the Glisson's capsule (required for hepatocyte isolation) are not necessary for the preparation of slices and therefore use can be made of any fresh human liver samples. Liver slices, like hepatocytes, are capable of performing both Phase I and Phase II biotransformations.

The preparation, uses, advantages and disadvantages associated with the use of S9, microsomes, hepatoctyes and tissue slices will be discussed in the next section. This chapter has primarily focussed on hepatic *in vitro* preparations; however, it should be noted that not only the liver but a range of other tissues such as kidney, lung and intestine contain drug-metabolising enzymes and carry out xenobiotic metabolism *in vivo*. Therefore, *in vitro* metabolic preparations, especially S9, and microsomes could be prepared from any tissue to study extrahepatic drug metabolism.

15.2 *Preparation of liver subcellular fractions and hepatocytes*

15.2.1 Preparation of subcellular fractions

The most commonly used subcellular fractions in industrial drug metabolism studies are the S9 fraction and microsomal preparations. Both preparations are produced using differential centrifugation of tissue homogenates. In addition, microsomes can be prepared from S9 by calcium aggregation.

In order to preserve enzyme activity, buffers, tools and centrifuge rotors should be maintained cold for the duration of the preparation procedure, either by storage on ice or within a refrigerator.

Excised livers (or portions of liver from larger animals and man) are weighed and washed in a suitable ice cold buffer at pH 7.4. The initial buffer is removed and the liver is added to the buffer to give a 25 per cent homogenate. The tissue is scissor minced and then homogenised using a suitable 'soft' homogenisation technique such as the 'pestle and mortar' Potter Elvhejam homogeniser. More aggressive tissue disruption methods tend to reduce the enzyme activity of the final preparation.

The crude tissue homogenate is then centrifuged at $9{,}000 \times g$ for 20 minutes at 4 °C. This step pellets and therefore removes intact cells, cell debris, nuclei and

mitochondria from the crude cell homogenate. The supernatant represents the sub mitochondrial fraction (S9), which may then be quickly frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ prior to use.

The microsomal fraction may then be prepared from the S9 fraction by further centrifugation or by calcium precipitation. Centrifugation of the S9 at $100{,}000 \times g$ for one hour sediments the microsomal vesicles. After the supernatant is discarded, microsomes are resuspended in a volume of buffer (pH 7.4) equivalent to the original weight of tissue used. The centrifugation step may be repeated to further purify the preparation.

The preparation generally yields a protein concentration of a few milligrams of microsomal protein/millilitre tissue preparation. The purity and activity of the microsomal homogenate is dependent upon the preparation technique and will vary according to the aggressiveness of the homogenisation technique and subsequent temperature of the homogenate and degradation of the enzyme.

A number of additions to the preparation buffers have been suggested to increase the purity or enzyme activity of the final preparation. For example, the addition of EDTA in decreasing concentration to the preparation buffers (10 mM in homogenisation buffer, 1 mM in wash buffer and 0.1 mM in final buffer) is thought to stabilise flavin monooxygenase (FMO) activity (Sadeque *et al.*, 1992). The addition of potassium chloride (1.15 per cent w/v) to the homogenisation buffer further purifies the preparation by the removal of blood and cytoplasmic contaminants (Eriksson *et al.*, 1978). The addition of glycerol to the final storage buffer is thought to preserve enzyme activity on storage (Guengerich and Martin, 1998). Table 15.2 shows the composition of buffers used for the preparation of liver subcellular fractions.

An alternative method for the preparation of microsomes is to use calcium precipitation. This method is based on the calcium-dependent aggregation of the endoplasmic reticulum. Calcium chloride is added to the post-mitochondrial fraction to give a final concentration of 8 mM. The mixture is left to stand for five minutes with occasional mixing. Centrifugation at $27{,}000 \times g$ for 15 minutes will yield the microsomal pellet which may be resuspended as previously described.

The protein concentration of both S9 and microsomes should be determined prior to use in order to normalise incubation conditions between preparations (i.e. ensure the same amount of protein is added to each incubation). Protein measurement kits are commercially available and include the Lowry method (Lowry *et al.*, 1951) and the bicinchoninic acid method (Smith *et al.*, 1985).

TABLE 15.2 *Buffers used for the preparation of S9 and microsomes*

Homogenisation buffer	0.1 M phosphate buffer pH7.4 + 1.15% KCl (10 mM EDTA optional)
Wash buffer	0.1 M phosphate buffer pH 7.4 + 1.15% KCl (1 mM EDTA optional)
Storage buffer	0.1 M phosphate buffer pH 7.4 + 20% glycerol (0.1 mM EDTA optional)

The levels of active cytochrome P450 may be determined by the difference in spectrum (Omura and Sato, 1964). Determination of active cytochrome P450 gives an extra level of confidence in the activity of the preparation as well as greater consistency in incubation conditions if the incubations are normalised for cytochrome P450 concentration rather than microsomal protein concentration. In general, different spectra with S9 are not recommended as non-specific absorption and light scattering can occur due to the turbidity of this preparation.

Summary of the preparation of S9 and microsomal protein (by ultracentrifugation)

1 Carry out all steps at 4 °C.
2 Weigh the fresh or thawed liver sample.
3 Add four times the volume of homogenisation buffer to liver weight.
4 Scissor mince tissue then homogenise using a Potter Elvhejam or similar mechanical tissue homogeniser.
5 Centrifuge the homogenate at 9,000 × *g* for 20 minutes.
6 Combine the supernatants (S9 fraction) and snap freeze in liquid nitrogen. Store S9 fraction at −80 °C.
7 For the preparation of the microsomal fraction, centrifuge the S9 fraction at 100,000 × *g* for 1 hour.
8 Discard the supernatant and resuspend the pellet in buffer. Centrifuge the homogenate at 100,000 × *g* for 1 hour.
9 Discard the supernatant and resuspend the pellet in storage buffer (volume equivalent to the original tissue weight).
10 Snap freeze aliquots (1 or 2 ml) in liquid nitrogen and store at −80 °C

15.2.2 Preparation of hepatocytes

There are three methods routinely used for the preparation of hepatocytes. The methods involve removal of calcium from the tissue followed by treatment of the tissue with a solution containing collagenase. Calcium removal initiates the separation of cell–cell adhesion via calcium-dependent desmosomes. The collagenase treatment digests the architecture of the liver and allows the hepatocytes to be released. The first method is based on that of Berry and Friend (1969) and involves the perfusion of the liver *in situ*. The second technique was developed in 1976 by Fry and co-workers and involves digestion of liver slices, thus avoiding the need for perfusion. The benefit of this method is that an intact Glisson's capsule around the liver is not required, so use can be made of tissue that would otherwise be wasted. The third method is an adaptation of the *in situ* collagenase perfusion technique (Strom *et al.*, 1982; Oldham *et al.*, 1985). Small, end-of-lobe liver fragments surrounded

by an intact Glisson's capsule are perfused by cannulating the exposed vessels on the cut surface of the sample. This third method is the method routinely used in our laboratories because it can be used to isolate hepatocytes from several different species, including human, with minimal changes to the basic technique. An *in situ* perfusion technique would not be suitable to use with larger species such as the dog.

The method described below is used for the isolation of hepatocytes from end-of-lobe liver samples (wedge biopsies). Table 15.3 details the composition of buffers and

TABLE 15.3 *Buffers and solutions for the isolation of hepatocytes*

Isolation buffer:	10 × EBSS	100 ml
Perfusion buffer	(without calcium and magnesuim)	30 ml
	7.5% sodium bicarbonate solution	870 ml
	purified water	pH adjusted to 7.4
Isolation buffer:	Perfusion buffer	490 ml
Chelating buffer	25 mM EGTA (in 0.1 M NaOH)	10 ml pH adjusted to 7.4
Isolation buffer:	Perfusion buffer	150 ml
Collagenase buffer	1 M $CaCl_2$	300 μl
	Trypsin inhibitor	~10 mg
	Collagenase H	12 units (rat) 24 units (dog, human, pig)
Dispersal buffer	Sodium chloride	4.15 g
	Potassium chloride	0.26 g
	HEPES	1.19 g
	Purified water	500 ml pH adjusted to 7.4
	BSA	5.0 g
Culture medium	William's medium E	500 ml
(for suspensions)	200 mM L-glutamine	10 ml
Culture medium	William's medium E	500 ml
(for monolayers)	200 mM L-glutamine	5 ml
	Penicillin (10,000 U/ml)/Streptomycin	5 ml
	(10,000 mg/ml)	0.5 ml
	Insulin (250 U/ml)	0.5 ml
	δ-aminolevulinic acid (1 mM)	0.5 ml
	Transferrin (5 mg/ml)	0.5 ml
	Hydrocortisone (3.6 mg/ml)	0.5 ml
	Zinc sulphate (5 μM)	
Culture medium	Chee's essential medium	500 ml
(for monolayers)	200 mM L-glutamine	5 ml
	Penicillin (10,000 U/ml)/	5 ml
	Streptomycin (10,000 mg/ml)	

solutions required for the isolation of hepatocytes. Figures 15.1 and 15.2 show the apparatus used for the isolation procedure and the perfusion of whole lobes of rat liver.

All buffers and solutions should be maintained at 37 °C with the exception of the dispersal buffer which should be kept at 4 °C. All buffers used during the perfusion should be maintained at pH 7.4 by continuous gassing with carbogen (95 per cent oxygen, 5 per cent carbon dioxide).

Exposed vessels on the cut surface of the liver tissue (or vessels entering the individual lobes for rat liver) are cannulated using 20 gauge (rat) or 16 gauge cannulae (human, dog, pig). The tissue is perfused with chelating buffer for approximately 5 minutes at a flow rate of approximately 6 ml/min/cannula (rat) or 12 ml/min/cannula (human, dog, pig). The chelating buffer is then washed out by perfusion buffer for approximately 5 minutes. The tissue is then perfused with collagenase buffer, which should be recirculated after 3–4 minutes. The collagenase perfusion should continue until the cells beneath the Glisson's capsule become spongy to the touch. This should take approximately 20–25 minutes for rat liver and anything up to 1 hour for the isolation of human hepatocytes. The time required for human hepatocyte isolation varies depending upon the age, status and fibrotic nature of the liver sample used.

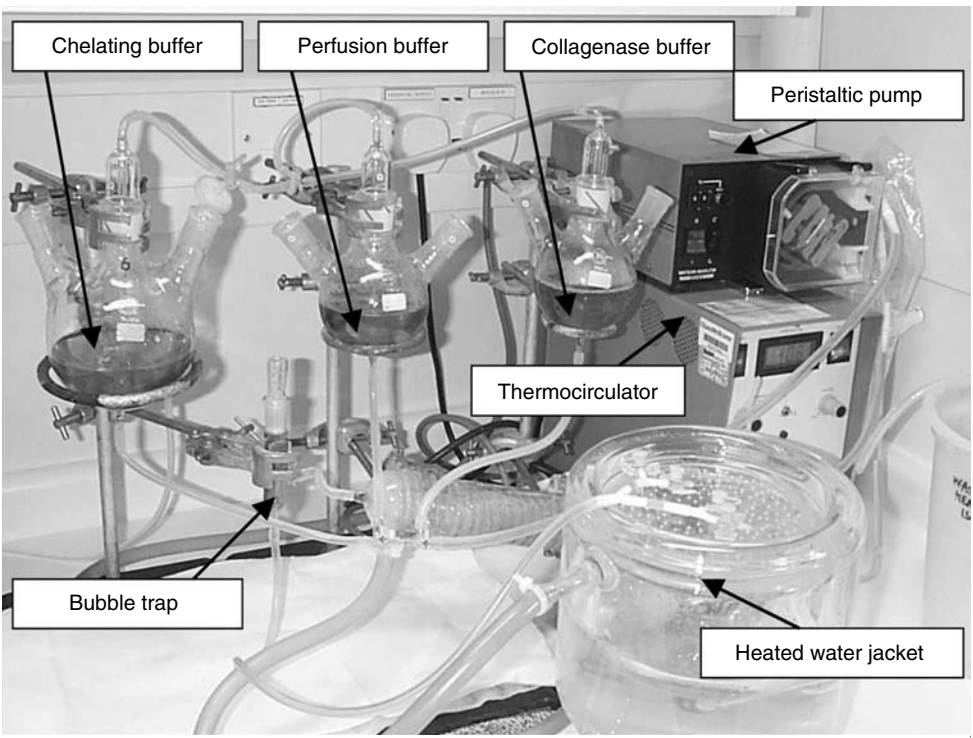

FIGURE 15.1 *Apparatus used for the isolation of hepatocytes.*

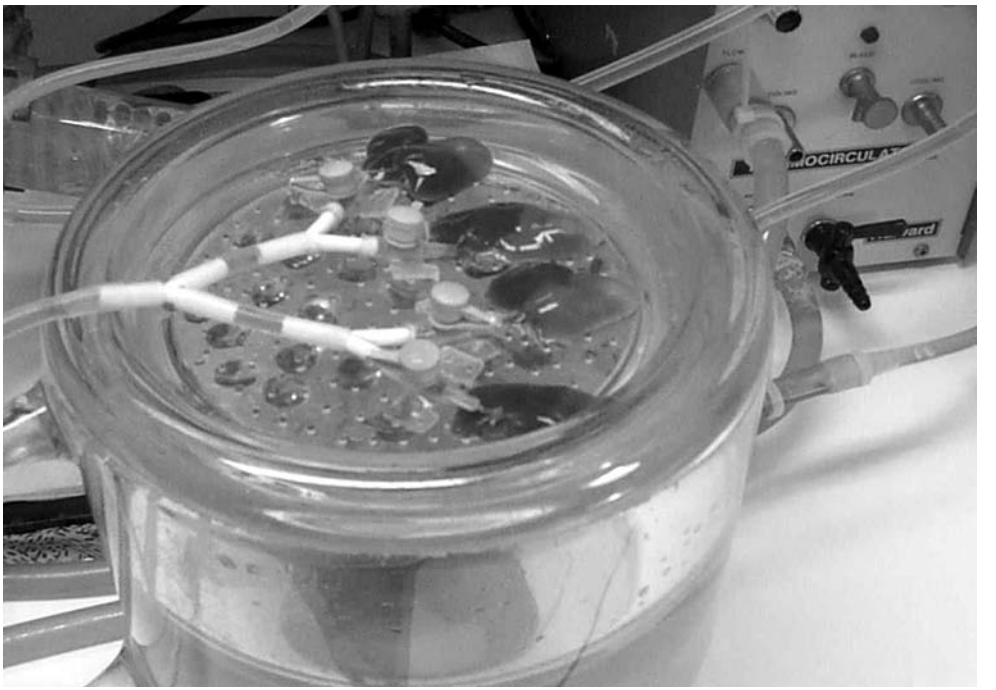

FIGURE 15.2 *Isolation of hepatocytes: perfusion of rat liver lobes.*

Once the extracellular matrix has dissociated sufficiently the perfusion is stopped, avoiding possible rupture of the Glisson's capsule.

The tissue is placed into a shallow dish containing dispersal buffer and using forceps, the Glisson's capsule is carefully peeled away releasing isolated cells into the dispersal buffer. The forceps can be used to gently 'comb' the cells to help release them from the tissue.

The cell suspension is filtered through nylon bolting cloth (64 μm pore size) pre-wetted with dispersal buffer containing DNAse I (5 mg/100 ml) to remove large clumps of tissue and cells. DNAse removes DNA that has been released from damaged cells which causes cells to clump together. All subsequent manipulations of the cell suspension are carried out at 4 °C. The low temperature minimises the activity of any cytotoxic enzymes that may have been released from damaged cells and to preserve drug-metabolising enzyme activity. Viable cells are sedimented by centrifugation ($50 \times g$ for 5 min) at 4 °C. The cells are washed twice more as previously described but without DNAse I in the final wash.

The final cell pellet is resuspended in a suitable incubation buffer such as William's medium E (plus appropriate supplements) and the viability of the preparation assessed using an appropriate cell viability test, of which the simplest is the trypan blue exclusion test. Trypan blue is a large molecular weight dye which

is excluded from viable cells with an intact cell membrane. However, non-viable cells with a damaged membrane will take up the dye resulting in a blue-stained nucleus. After treatment with trypan blue, the total number of cells isolated in combination with the number of viable and non-viable cells can easily be determined using light microscopy and a haemocytometer. Generally, for small animal species, a viability of >90 per cent is required before use.

The remaining cell suspension is now ready to be diluted appropriately for use.

Summary of hepatocyte isolation (two-step collagenase digestion)

1 Perfuse tissue with chelating buffer.
2 Perfuse tissue with perfusion buffer.
3 Perfuse tissue with collagenase buffer until extracellular matrix has dissociated.
4 Place tissue into dispersal buffer.
5 Break open Glisson's capsule and tease out cells into the buffer.
6 Filter cells, with DNAse I and centrifuge ($50 \times g$, 5 minutes).
7 Filter cells, with DNAse I and centrifuge ($50 \times g$, 5 minutes).
8 Filter cells, without DNAse I and centrifuge ($50 \times g$, 5 minutes).
9 Re-suspend cell pellet in medium.
10 Determine cell viability and total cell number using trypan blue exclusion.
11 Dilute remaining cells.
12 Use in suspension or plate cells out as monolayers.

15.2.3 Preparation of liver slices

There are a number of different techniques for producing liver slices, and a similar choice of incubation systems. A number of commercial tissue slicers are available, such as the Brendel-Vitron and Krumdieck machines, which allow thin slices of liver (thickness *ca*. 250 μm) to be reproducibly cut from cores of tissue. These cores may be prepared freehand or by using a mechanised borer. The cores are then placed in the machine, and slices cut using a microtome, which allows the thickness of the prepared slices to be controlled. The core, and the slices prepared from it, is kept submerged in ice-cold physiological buffer. Following this, prepared slices are harvested and used in the incubation system, the choice of which may depend on the aim of the experiment. If slices need to be prepared aseptically this may be possible as some mechanised slicers are autoclavable.

Summary of liver slice preparation

1 Obtain fresh liver.
2 Cut cylindrical cores from the tissue.

3 Place cores into the tissue slicer and prepare slices in ice-cold physiological buffer.
4 Transfer slices into incubation system of choice.

15.3 *Use of subcellular fractions, hepatocytes and liver slices to study drug metabolism*

15.3.1 Subcellular fractions

There are a number of uses for subcellular fractions within the pharmaceutical industry. However, the major uses within drug metabolism are to screen potential drug candidates for metabolic stability and as 'metabolite factories' to produce potential metabolites for identification with more ease than by extraction and analysis of drug-related material from biological fluids following *in vivo* drug administration.

Qualitative experiments investigating the metabolic profile of potential drug candidates (Adams *et al*., 1981; Acheampong *et al*., 1996) have been used to confirm the presence of metabolites in animals and man and therefore validate long-term and expensive toxicology testing.

Incubations are prepared by addition of the compound, enzyme preparation, buffer and appropriate enzyme cofactors. The addition of organic solvents to enzyme incubations should be kept to a minimum as organic solvents may affect enzyme activity (Chauret *et al*., 1998). Choice of the appropriate enzyme cofactor is also an important consideration. For example, the preparation of microsomes produces an endoplasmic reticulum-rich fraction containing membrane-bound cytochromes P450. All soluble enzyme cofactors are lost during the process. Therefore, oxidations by cytochromes P450 will not proceed without the addition of the reduced form of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) or an NADP(H)-regenerating system to the microsomal incubations.

Following incubation, and simple 'cleanup' techniques such as the addition of organic solvents to precipitate proteins, the metabolites obtained may be analysed using high-performance liquid chromatography (HPLC), in conjunction with mass spectrometry (LC–MS) or nuclear magnetic resonance (LC–NMR). In this way, detailed information of the metabolic profile of a compound may be determined. Species-specific metabolism or the production of pharmacologically active or toxic metabolites may be quickly screened and investigated.

Quantitative metabolism may be used in the initial stages of research to screen and then rank potential drug candidates according to their metabolic stability. The advent of combinatorial chemistry has enabled chemists to rapidly produce large numbers of novel synthetic compounds. Metabolic stability (or instability) is

a crucial factor in the development of any new medicine, so high-throughput methodologies employing subcellular fractions have now been developed to enable the metabolic stability of large numbers of compounds to be investigated in the minimum amount of time (Eddershaw and Dickins, 1999). Compounds with inappropriate metabolic stability can therefore be removed from research and development at an early stage.

15.3.2 Hepatocytes

Hepatocytes provide the pharmaceutical industry with a whole cell system to study the metabolism of drug candidates. Hepatocytes can be cultured in suspension in the presence of a test compound for up to 4 hours. The culture medium routinely used in our laboratories is William's medium E containing 4 mM L-glutamine. The medium does not contain phenol red as this indicator dye is metabolised by several Phase II drug-metabolising enzymes and therefore could compete for metabolism by these enzymes and prevent Phase II metabolism of the test compound (Driscoll *et al.*, 1982).

15.3.3 Liver slices

As a whole cell preparation, liver slices offer an alternative to hepatocytes and perfused liver in the study of metabolism.

Liver slices have a variety of uses in the study of drug metabolism. The ease and speed of their preparation facilitates the retention of metabolic activity and they are therefore useful as predictive tools when studying the biotransformations of drugs *in vitro*. They can be used as 'metabolite factories' to produce sufficient quantities of metabolite for characterisation and identification. Because the amount of liver tissue per slice will never be identical, however, there can be greater individual variability between slices compared with hepatocyte incubations when used in quantitative studies, for instance in drug interaction studies.

The choice of incubation system may depend on the aim of the experiment. A dynamic organ culture system may be used, where the slice is supported on a wire mesh in a vial-containing medium and placed on rollers in an oven maintained at 37 °C so that the slice moves in and out of the liquid phase (Smith *et al.*, 1986). Alternatively, slices may be placed in a conical flask, or in 12- or 24-well plates, and incubated with agitation in a temperature-controlled incubator. Whichever system is used, a pre-incubation of the slice in fresh culture medium (generally up to 2 hours duration) is carried out to allow sloughing of cells from the cut surfaces of the slice and ensure homeostasis. For short-term incubations, such as simple assessment of xenobiotic biotransformation in a manner similar to the use of hepatocyte suspensions, the slices may be incubated in a tissue culture medium such as

Williams' medium E (supplemented with glutamine) containing the test compound. Generally for a short-term culture, sufficient oxygen may be introduced to the system by pre-gassing the medium with carbogen. Alternatively, vials with a hole drilled in the lid to facilitate oxygen transfer may be placed in the incubator. For longer-term incubations, such as studying the biotransformation of slowly metabolised xenobiotics or the potential for induction of drug-metabolising enzymes (Lake *et al*., 1997), the medium is usually supplemented with a number of additives such as foetal calf serum, hormones and antibiotics. In addition, for longer-term cultures, the medium may need to be changed regularly and adequate oxygenation maintained by placing the culture plates in a tissue culture incubator.

At the end of incubation both the medium and the slice are usually analysed, as metabolites may be retained within the slice.

15.4 In vitro–in vivo *correlations*

All the systems discussed so far can be used to measure the metabolic clearance of a compound *in vitro*. The data obtained from any of these systems can then be scaled using various models to provide an estimate of the clearance of that particular drug *in vivo*.

Subcellular fractions, especially microsomes (Houston, 1994; Houston and Carlile, 1997; Iwatsubo *et al*., 1997) have been used to estimate rates of metabolic clearance of a particular drug *in vitro* in an attempt to estimate rates of metabolic clearance of a drug *in vivo*. This may be achieved either by quantifying rates of metabolite production, or by calculation of the rate of disappearance of substrate from the incubation by metabolism. Disappearance plots have the advantage that knowledge of the metabolic profile of a compound is not required before investigation commences, thus enabling the study of drug candidates whose metabolic profile is unknown. However, the disadvantage of this approach is that substrate may be removed from the incubation matrix by methods other than metabolism such as non-specific irreversible binding to cellular proteins.

The first step in quantitative prediction of the metabolic clearance of a drug candidate *in vivo* is achieved by determination of the intrinsic clearance of a drug. Intrinsic clearance is the ability of an enzyme system to metabolise a drug in the absence of any interfering constraints such as plasma protein binding or blood flow. Intrinsic clearance is quite simply the ratio of Michaelis Menton parameters K_m and V_{max}, as shown below.

$$\text{Rate of metabolism} = \text{intrinsic clearance} \times \text{substrate concentration } [S]$$

$$\text{Intrinsic clearance} = \frac{\text{rate of metabolism}}{\text{substrate concentration}}$$

Michaelis Menton states that

$$\frac{V_{max} \cdot [S]}{K_m + [S]}$$

When [*S*] is 10 per cent or less than K_m the equation reduces to

$$\frac{V_{max} \cdot [S]}{K_m}$$

Therefore intrinsic clearance $= V_{max}/K_m$

Once intrinsic clearance has been calculated as a rate (i.e. nmoles of parent turned over or metabolite produced/min/mg of microsomal protein), then the intrinsic clearance is scaled to predict clearance that would occur in the liver *in vivo*. For example, the literature average for the amount of microsomal protein is approximately 45 mg microsomal protein/g liver.

The effects of plasma protein binding and blood flow on the clearance process are then modelled using one of a number of liver models. The simplest model is the venous equilibration model shown below; however, more complex models including the parallel tube and dispersion models may also be used (Wilkinson, 1987; Saville *et al*., 1992). Essentially the models vary in the estimation of the concentration drop of parent compound across the liver. This will be most important for highly metabolised compounds where the concentration drop across the liver will be greatest. Under these circumstances the venous equilibrium model will give the lowest prediction of clearance, the parallel tube model the highest prediction and the dispersion model prediction will be in between the two.

15.4.1 Venous equilibrium model

$$\text{Clearance } \textit{in vivo} = \frac{Q \cdot Fu \cdot \text{intrinsic clearance}}{Q + Fu \cdot \text{intrinsic clearance}}$$

where Q = hepatic blood flow; Fu = unbound fraction of drug in the blood. The final result is an estimation of hepatic metabolic clearance which may then be used to predict the plasma pharmacokinetics of a drug in animals or man *in vivo*.

This technique, although at present used with caution, could in the future serve to estimate the metabolic clearance of compound in animals and man and reduce the need for widespread and intensive testing of the pharmacokinetics of compounds in animals. In theory, only compounds with appropriate kinetics would be investigated further leading to a reduction of research cost and effort.

15.4.2 Hepatocytes

Hepatocytes, like subcellular fractions, may also be used for both quantitative and qualitative studies and studies to examine drug interactions as previously outlined. The use of whole cells provides more metabolic pathways for metabolism of a test compound and as hepatocyte number in the whole liver is well established, the scaling from *in vitro* data generated in hepatocytes gives a better estimation of *in vivo* clearance.

15.4.3 Liver slices

Liver slices have also been used as a tool for the prediction of the intrinsic clearance of drugs, in a similar manner to hepatocytes (Worboys *et al.*, 1996). This application demonstrates one of the limitations of tissue slices, namely that the contribution of cells in the centre of the slice may be unrepresentative of the overall clearance due to the need for drug to diffuse to the centre. Also, although slices are cut very thinly (about 200–300 μm) and are maintained in conditions to maximise the supply of oxygen to the slice, the centre cells may become necrosed with time and thus not contribute to the turnover of drug. Finally, metabolites, or remaining parent drug, may be retained within the slice leading to over- or underestimation of clearance if only the medium, and not the contents of the slice itself, is studied at the end of the incubation period.

15.5 *Advantages and disadvantages of the* in vitro *systems used to study drug metabolism*

The cellular locations of the main enzymes responsible for xenobiotic metabolism have already been shown in Table 15.1. This table highlights the differences in the distribution of enzyme types between different *in vitro* liver preparations.

15.5.1 Subcellular fractions

The advantages and disadvantages of using subcellular fractions to study drug metabolism are summarised in Table 15.4. S9 is a crude cell preparation and contains a range of both cytosolic- and membrane-bound enzymes. Therefore, this fraction yields a wide range of biotransformation products. Whilst S9 is a crude preparation the microsomal preparation is a highly purified enzyme preparation that contains only those enzymes bound to the endoplasmic reticulum *in vivo*.

There is also a difference in the availability of cofactors for certain enzyme reactions between preparations. S9, as a crude preparation, should also contain the

TABLE 15.4 *The advantages and disadvantages of subcellular fractions for the study of drug metabolism*

	Advantages	Disadvantages
S9	Ease of preparation	Crude preparation
	Contains soluble and membrane-bound enzymes	High protein concentration
	Ease of storage	
	Contains cofactors	
	Prepared from frozen tissue	
Microsomes	Ease of storage	Requires ultracentrifuge
	Prepared from frozen tissue	No cofactors
	Membrane-bound enzymes	

necessary cofactors for enzyme activity. However, experience has shown that the addition of suitable cofactors (e.g. NADPH) may be important in retaining suitable cytochrome P450 activity.

Although microsomes contain cytochromes P450, the preparation will not carry out redox reactions *per se* due to the lack of cofactors available to aid in the reaction cycle. NADPH or an NADP(H)-regenerating system must be added to incubation mixtures for biotransformations to proceed. In addition, although microsomes contain membrane-bound glucuronyl transferases, the cofactor uridine diphosphate glucuronic acid (UDPGA) and also detergents need to be added for microsomes to carry out Phase II glucuronidation reactions. The detergents are required to release the enzyme from the centre of the microsomal vesicle where access to the substrate is impaired.

S9 and microsomes have the advantage that they may be prepared in large quantities from both fresh and frozen tissue. Once prepared, the tissue preparations may be stored at $-80\,^{\circ}C$ without a significant drop in enzyme activity. This means that large numbers of experiments may be carried out using the same preparation facilitating comparison between experiments.

Disadvantages of using subcellular fractions include interactions due to large concentrations of non-specific protein within the matrix. Protein may bind and essentially remove the drug from the incubation medium leading to an overestimate of the stability of the drug. Concentrations of non-specific protein are much higher in the S9 fraction than in the microsomal fraction. Large amounts of protein may interfere with drug metabolism directly or hinder analysis of the experimental incubates.

Metabolism may also be impaired in subcellular fractions due to the build-up of Phase I metabolites which will effectively inhibit the turnover of parent material (product inhibition). This will lead to an underestimation of metabolic turnover.

In contrast, the intimate association of the drug with the enzyme under *in vitro* conditions with subcellular fractions may drive a reaction that may not occur *in vivo*. For example, a drug with a low volume of distribution may not become available within the cell for interaction with the enzyme *in vivo* and therefore the metabolic

instability of a compound may be overestimated. In addition, the activity of drug transporters in the cell membrane may serve to regulate the intracellular levels of drug *in vivo*. This may lead to inaccurate estimations of metabolic clearance using subcellular preparations due to incorrect assumptions of the amount of drug interacting with the enzyme *in vivo*.

Finally, the activity of subcellular fractions is highly dependent upon assay conditions, for example, the activity of cytochromes P450 is dependant on buffer concentration and pH. Each cytochrome P450 has an optimum buffer and buffer concentration (Wrighton and Gillespie, 1998).

15.5.2 Hepatocytes

Advantages and disadvantages of using hepatocytes to study drug metabolism are shown in Table 15.5.

Hepatocytes may be used in short-term suspension incubations similar to those for subcellular fractions or may be kept in long-term culture. This section relates to short-term suspensions; hepatocyte culture will be discussed later.

Hepatocytes are structurally intact liver cells and therefore represent a step up in tissue integrity with respect to subcellular fractions. The presence of an intact cell membrane means that access of the drug to the drug-metabolising enzymes is controlled in a similar way to that *in vivo*. For example, access into or out of the cell may be controlled by the physical presence of the cell membrane and/or by drug transporters (both influx and efflux) present in the cell membrane.

Hepatocytes not only contain a full complement of enzyme activity but also the cofactors necessary for the completion of metabolic pathways. Hepatocytes therefore have advantages over the use of subcellular fractions with respect to Phase II and Phase III metabolism. In addition, because of the full complement of enzyme systems, metabolism can proceed normally from Phase I to Phase II metabolism and therefore build-up of Phase I metabolites (and therefore product inhibition) should not occur to the same extent as in microsomes.

Due to the presence of an intact cell membrane, enzyme activity in hepatocytes is much less dependent on the buffer or medium composition compared to subcellular fractions and, after centrifugation to lyse the cells, hepatocyte suspensions are much cleaner and therefore easier to analyse than subcellular fractions.

Table 15.5 *The advantages and disadvantages of using hepatocytes for the study of drug metabolism*

	Advantages	**Disadvantages**
Hepatocytes	Full enzyme compliment Culture methods available	Need fresh tissue Storage limited No cell–cell contact

Disadvantages of hepatocyte preparations include the need for fresh tissue for cell isolation, although a number of authors (Coundouris *et al*., 1993; Zalenski *et al*., 1993; Swales *et al*., 1996) claim to have preserved the enzyme activity of isolated cells through cryopreservation, thus reducing the need for re-preparation of cells.

15.5.3 Liver slices

Table 15.6 highlights some advantages and disadvantages of liver slices in the study of drug metabolism. Their main advantage lies in the ease of preparation and the maintenance of cell–cell contact, and thus they offer a supplement or alternative to other whole cell systems for particular applications. The cryopreservation of liver slices has been investigated with a view to the long-term storage of human tissue which retains its drug-metabolising capacity, and results in this area, particularly using the vitrification technique, have been encouraging (de Kanter *et al*., 1998). Further details on the use of tissue slices may be found in a number of articles (e.g. Parrish *et al*., 1995; Bach *et al*., 1996; Ekins, 1996; Thohan *et al*., 2001).

15.6 *The study of drug interactions using* in vitro *systems*

Although this subject is dealt with in more detail in Chapter 16, it is also pertinent to mention in this chapter as *in vitro* drug interaction studies are generally carried out using either liver subcellular fractions or isolated hepatocytes.

15.6.1 Drug–drug interactions: subcellular fractions and hepatocytes

A number of clinically important drug interactions (reviewed by Landrum-Michalets, 1998) have highlighted the need to investigate the enzymology of drug metabolism reactions and determine whether potential drug interactions may occur in the clinic.

Table 15.6 *The advantages and disadvantages of using liver slices for the study of drug metabolism*

	Advantages	**Disadvantages**
Liver slices	Quick, easy to prepare Cell–cell contact No use of proteolytic enzymes All cell types present Cryopreservation potential	Need fresh tissue Necrosis of slice centre Long-term culture difficult Quantitative studies variable

Subcellular fractions and suspension cultures of isolated hepatocytes have been used for this purpose. Inhibition of the metabolism of drug A in the presence of drug B can be determined. Competitive inhibition of or by the drug candidate molecule can be established and any potentially dangerous interactions be further investigated. By investigating competitive inhibition using a competitor whose metabolism is attributable to a single cytochrome P450, such studies can also elucidate the cytochromes P450 involved in the metabolism of a potential drug candidate.

15.6.2 Drug interactions: monolayer culture of hepatocytes

One major disadvantage of primary culture of hepatocytes is the rapid loss of enzyme expression which occurs as the cultured cells undergo de-differentiation. In particular, there is a loss of cytochrome P450 expression in monolayer culture of hepatocytes.

Monolayer cultures of hepatocytes are therefore used most often to study enzyme induction and inhibition. Induction and inhibition of the levels of drug-metabolising enzymes present within a cell play a key role in some adverse drug reactions. Certain drug entities may induce or inhibit production of drug-metabolising enzymes, often via an effect on gene expression.

By placing hepatocytes into primary culture and providing them with substrata comprised of extracellular matrix proteins such as collagen, together with supplemented tissue culture media, hepatocytes can be maintained for several days or weeks. Because primary cultures can be maintained for these prolonged periods, changes in gene expression can be studied.

Freshly isolated hepatocytes can be plated out to form primary cultures. Hepatocytes placed into culture on collagen-coated plastic adopt a flattened morphology (see Figure 15.3). Hepatocytes do not proliferate in culture and so must be seeded at a density such that the cells form confluent monolayers once they have attached.

Monolayer cultures of hepatocytes are used to investigate the induction of cytochromes P450 by potential drug candidates. Despite the initial loss of cytochrome P450 activity in monolayer cultures of hepatocytes, the levels of individual cytochromes P450 can be increased again by treatment with certain chemical-inducing agents. Table 15.7 shows the most commonly used inducing agents together with the cytochrome(s) P450 that they induce.

To study the induction potential of a potential drug candidate, hepatocyte monolayers are prepared and left for 24 hours before treatment with known inducing agents or the potential drug candidates to be investigated. The cells are dosed daily for three days. Induction of the cytochromes P450 can be determined in several ways.

Increases in the catalytic activity of the cytochromes P450 in treated cells can be established by incubating the control and treated cells with a probe substrate

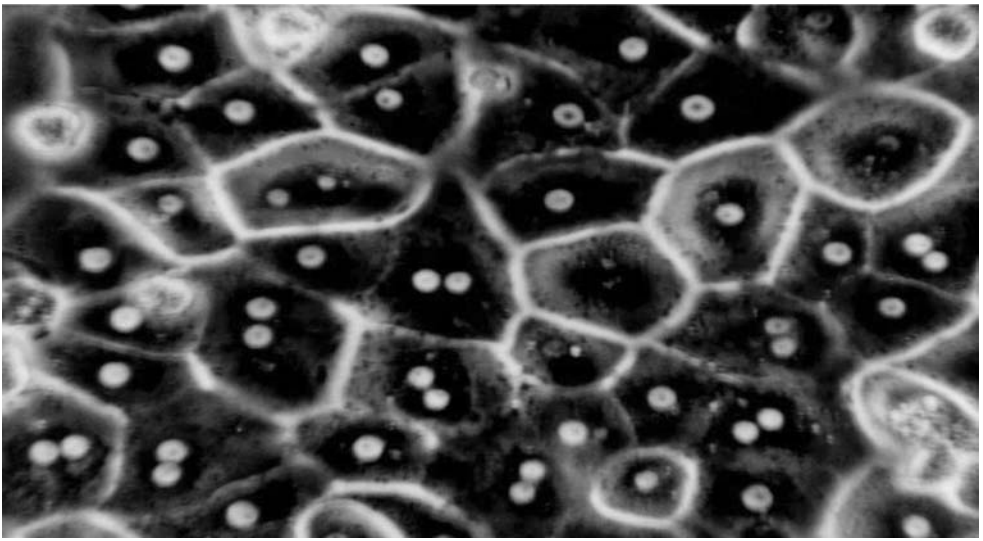

FIGURE 15.3 *Rat hepatocytes in monolayer culture. Cells were plated at* 1×10^5 *cells per* cm^2 *on collagen-coated dishes.*

specific for the cytochrome P450 of interest. Table 15.7 also shows probe substrates commonly used for assessing the activity of the different cytochromes P450.

Changes in the amount of cytochrome P450 protein can be determined by gel electrophoresis and Western blotting. Following treatment with the inducer or

TABLE 15.7 *Cytochromes P450: commonly used inducing agents and probe substrates*

Cytochrome P450	Inducing agent(s)	Probe substrate(s)
1A	β-naphthoflavone	7-ethoxyresorufin
	3-methylcholanthrene	Phenacetin
2A	Barbiturates (phenobarbitone)	Coumarin
2B	Phenobarbitone	7-pentoxyresorufin
		7-methoxyresorufin
2C	Rifampicin	Diclofenac (2C9)
		Tolbutamide (2C9)
		S-mephenytoin (2C19)
2D	None known	Bufuralol
		Debrisoquine
2E	Ethanol	Chlorzoxazone
	Isoniazid	*p*-nitrophenol
3A	Rifampicin	Testosterone (6β-hydroxylation)
	Dexamethasone	
	Phenobarbitone	Midazolam

potential drug candidate, the cells are harvested and the microsomal fraction prepared from the cells. Microsomal proteins are then separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose. The nitrocellulose is then incubated with anti-cytochrome P450 antibodies, specific for each individual form. Once these antibodies have bound the cytochromes P450 on the membrane, the membrane is then incubated with secondary antibodies containing a linked enzyme such as alkaline phosphatase. Alkaline phosphatase is used to drive a colourimetric reaction which results in the formation of a coloured band where the cytochrome P450 band was transferred from the gel. The intensity of this coloured band is proportional to the amount of cytochrome P450 originally present in the sample. A similar method is used by cytochrome P450 ELISA kits although there is no need for gel separation as the antibody-binding steps are performed in 96-well plates. The basic principles of these techniques are depicted in Figure 15.4.

Basic protocol for induction studies using hepatocyte monolayers

1 Isolate hepatocytes and seed cells onto collagen-coated plasticware.
2 Seed cells in suitable tissue culture medium containing 10 per cent foetal calf serum (for example, William's medium E or Chee's medium).
3 Allow cells to adhere for 2 hours and replace medium with serum free medium.
4 After 24 hours, add inducing agents or test chemicals to culture medium.
5 Dose daily for three days.
6 Wash cell in PBS and incubate cells with a specific probe substrate. Analyse for metabolism of substrate and compare treated to untreated cells.
7 Harvest cells into PBS and prepare the microsomal fraction.
8 Separate the cytochrome P450 protein from other proteins using SDS-PAGE.
9 Analyse by Western blotting.

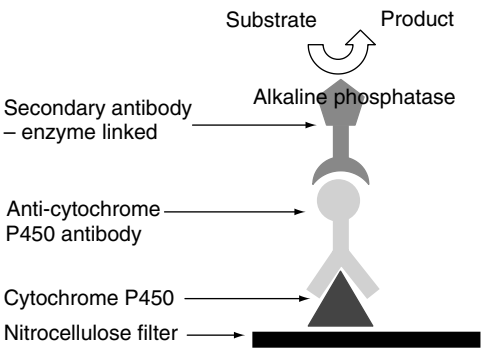

FIGURE 15.4 *Principles of blotting using enzyme-linked secondary antibodies.*

15.6.3 Drug interactions: liver slices

Liver slices can also be used to study the induction of cytochromes P450. They can be incubated in culture to examine the induction potential of xenobiotics on the cytochrome P450 enzymes of animal species (Lake *et al*., 1993); however, this work has been extended to use liver slices to assess induction in human tissue (Lake *et al*., 1996, 1997). Liver slices may also be used to assess the inhibitory potential of xenobiotics on drug-metabolising enzymes, particularly as slices retain both Phase I and Phase II metabolism *in vitro*.

15.6.4 Other *in vitro* models

So far this chapter has concentrated on the use of liver S9, microsomes, hepatocytes and liver slices and their use in studying drug metabolism. There are other *in vitro* models that can be used; however, these are not widely used by the pharmaceutical industry at present although may be more widely used in the future.

Due to the problems observed with hepatocytes when placed in culture, i.e. cell de-differentiation and subsequent reduction in the levels of cytochromes P450. A number of techniques have been developed to prolong the activity of hepatocytes in culture; these techniques include the use of liver spheroids, collagen sandwiches and hollow fibre bioreactors.

Hepatic cell lines have also been investigated for the study of drug metabolism. These techniques are briefly described below.

Liver spheroids

Liver spheroids are clusters of hepatocytes which are formed by culturing freshly isolated cells on dishes coated with poly-(2-hydroxyethylmethacrylate) (p-HEMA), a positively charged coating to which the hepatocytes are unable to attach. Gentle shaking on an orbital shaker encourages the hepatocytes to aggregate to form spheroids. Liver spheroids offer the advantage that, like liver slices, the cells are ultimately maintained in a three-dimensional structure allowing cell–cell communication to re-establish (Juillerat *et al*., 1997). The spheroids are capable of metabolism for prolonged periods of time and the cytochromes P450 can be induced by chemical-inducing agents (Amman and Maier, 1997). Within the spheroids, functional bile canaliculi are formed (Hamilton, 1998).

Collagen sandwich cultures

Culturing hepatocytes in collagen sandwiches has been shown to extend the viability of cultured hepatocytes in terms of cell membrane integrity and P450

enzyme stability (Lecluyse *et al*., 2000). Cells are cultured onto collagen-coated flasks in the normal way; however, after a period of time (*ca*. one day) the hepatocytes are immobilised in a further layer of collagen.

Hollow fibre bioreactors

Hollow fibre technology has been used clinically to provide extra-corporeal liver assist devices to maintain patients waiting for liver transplants or until spontaneous recovery due to hepatic regeneration occurs. Bioreactors have been seeded with human or pig hepatocytes and the cells perform the synthetic, metabolic, detoxification and excretion functions of the liver for patients with liver failure. Clinically, the devices are used for relatively short time periods (8 hours). However, as the system provides a dynamic culture environment for the culture of hepatocytes, it is under investigation for its suitability for use in the study of drug metabolism within the pharmaceutical industry. The bioreactor could be seeded with hepatocytes from a variety of species and if the system could be maintained for prolonged periods of time, in effect providing an artificial liver to work with, then it may be possible to study all aspects of liver function and in particular drug metabolism, induction of drug-metabolising enzymes and chronic hepatotoxicity.

Hepatic cell lines

There are several hepatic cell lines available for use in studying drug metabolism. Most have been derived from hepatomas or adenocarcinomas. Human hepatic cell lines, mainly HepG2 and Hep3B, are of particular interest to the pharmaceutical industry. One problem with using these cells, however, is that their complement of drug-metabolising enzymes does not reflect those present in freshly isolated human hepatocytes. HepG2, the more widely used human hepatic cell line, is suitable for investigating several of the Phase II pathways of metabolism and metabolism by cytochrome P4501A. However, there appears to be little catalytic activity of the other forms of cytochrome P450 in these cells. This may be due to a loss of expression of these forms as the cell line has been repeatedly passaged (undergone cell division) over the years.

15.7 *References*

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