BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **18**: 259–271 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.338

ORIGINAL RESEARCH

The Hummel–Dreyer method: impact in pharmacology†

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Received 24 September 2003; accepted 2 October 2003

ABSTRACT: Reviews devoted to drug–protein binding present mostly a variety of analytical methods applied to studying the unique process when the protein reversibly binds a low-molecular-weight substance—the drug. This report, however, reviews the great majority of papers presenting the application of one particular method introduced by Hummel and Dreyer in 1962: Measurement of protein-binding phenomena by gel filtration. The main criterion in reviewing published papers was the application of the Hummel–Dreyer method when the counterpart substances were the *drug* and the *human plasma protein*.

KEYWORDS: chromatography; capillary electrophoresis; drugs; drug–protein binding; interaction; reviews

INTRODUCTORY REMARKS

Primates, human beings included, stand on the youngest branch of the Tree of Life. Although philogenetically juvenile, the integrity and healthy functioning of their organism is under the very complex control of three systems: humoral, nervous and immunological.

From the point of view of humoral regulation, there are several organs/tissues which produce essential hormones. Deficiency of these endogenous substances can be compensated by introducing exogenous analogs—drugs.

Drugs at a therapeutically effective concentration should lead to diminished and even eliminated manifestations of the disease. Along with the assessment of signs and symptoms, there exists, however, an independent potential—the determination of the drug concentration in the target tissue. To measure the actual drug level, two modes of determination can be taken into account: non-invasive and invasive.

Optimally a series of non-invasive analytical methods, such as magnetic resonance spectroscopy (MRS), should be available. Yet, although very progressive, the existing MRS method is in general not sensitive enough to monitor the actual drug level in the tissue investigated. It may therefore be necessary to perform invasive organ tissue sampling. The most practical mode is sampling of blood, since the blood is in permanent contact with all organs within the body. The attributes indispensable for clinical pharmacologists to carry out therapy safely and rationally are: knowledge of the fate of distribution of drug molecules from the blood stream to the organs and back; and knowledge of the drug concentration in the blood.

Blood is not only a solvent of a whole series of essential endogenous substances but also a specific tissue exerting several physiologically relevant functions in the body. The 'macroscopic' components of colloidal tissue are represented by blood elements such as erythrocytes, leukocytes and platelets. Smaller by size are plasma proteins—serum albumin (HSA), α_1-acid glycoprotein (α_1-AGP), lipoproteins (L), immunoglobulins, etc. Permeation of these blood components to the extravascular space of the organism is considerably limited by the barrier represented by the vascular wall. Low-molecular-weight substances, including the molecules of a drug, do not create exclusively a true solution within the blood. A fraction of the drug molecules can be incorporated into blood elements and/or be reversibly bound to plasma proteins. Hence this drug fraction cannot diffuse into extravascular space to target the tissues. Precise information on the unbound, freely permeable, drug fraction is of utmost importance since it closely correlates with the pharmacological effects.

Insight into the principles of distribution of drug molecules in the organism, including the process of their reversible binding interaction particularly with two specific plasma proteins—HSA and α_1-AGP—is the subject of preclinical pharmacokinetic studies of any novel/prospective drug. Namely, protein binding of the drug can have important implications, especially when the drug molecules are highly/extensively bound to

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†Dedicated to Professor Emeritus Sébille Bernard François Louis, Laboratoire de Recherche sur les Polymères, UMR C7581 C.N.R.S. and Université Paris XII Val de Marne, France.

Abbreviations used: ED, equilibrium dialysis; FA, frontal analysis; GF, gel filtration; UF, ultrafiltration.

Published online 11 December 2003

plasma proteins. Several diseases are known to be accompanied by significant increases of the α_1-AGP level, which may result in decreased blood concentration of the free drug—dropping below the therapeutically effective value. On the other hand, the fraction of the unbound drug may reach a dangerous, even toxic level, as for example in the case of elevated concentrations of endogenous compounds such as fatty acids, which then compete for drug binding sites on the HSA macromolecule. Polytherapy may also involve competitive protein binding interactions.

STOICHIOMETRY OF REVERSIBLE DRUG–PROTEIN BINDING INTERACTION

Reversible binding interaction of a plasma protein, *P*, and a single drug, *D*, forming a drug–protein complex, *P–D*, can be described by the following equation:

$$P + D \underset{k_{diss.}}{\overset{k_{ass.}}{\rightleftharpoons}} P\text{–}D \qquad (1)$$

where $k_{ass.}$ is the second-order association rate constant, the unit of which is $l\,mol^{-1}\,s^{-1}$, and $k_{diss.}$ is the first-order dissociation rate constant, whose unit is s^{-1}. The equilibrium association constant $K_{ass.} = k_{ass.}/k_{diss.} = [P\text{–}D]/[P]\cdot[D]$ is hence measured in $l\,mol^{-1}$, while the equilibrium dissociation constant $K_{diss.} = k_{diss.}/k_{ass.} = [P]\cdot[D]/[P\text{–}D]$, equaling $1/K_{ass.}$, has the unity $mol\,l^{-1}$.

In drug–protein binding studies the fundamental interest is to determine the affinity, *K*, of the drug for the protein and the number of specific (saturable) binding sites, *n*, of the protein macromolecule for the drug. To calculate the values of *K* and *n* parameters a relationship similar to the Langmuir adsorption isotherm is used:

$$[B] = \frac{nK[F][P]}{1 + K[F]} \qquad (2)$$

where $[B]$ and $[F]$ represent the *bound* and *free* molar concentrations of the drug. However, since the protein macromolecule has usually more than one single class of binding sites for the drug, the following general equation has been exploited:

$$r = \sum_{i=1}^{N} \frac{n_i K_i [F]}{1 + K_i [F]} + \text{Const.} \times [F] \qquad (3)$$

where $r = [B]/[P]$ and the product Const. $\times$ $[F]$ represents a non-specific (unsaturable) binding term, which may but need not be manifested.

ANALYTICAL METHODS

Equilibrium dialysis (ED) is invariably the leading reference analytical method to study reversible drug–protein binding interaction. The two compartments of a dialyzer, separated by a membrane impermeable for *P* and *P–D*, simulate exactly the intra- and extravascular spaces. The unfavorably slow process of spontaneous diffusion at ED of the free, uncomplexed drug can be speeded up by applying commercial ultrafiltration (UF) devices, which have become popular for monitoring clinical blood plasma/serum samples. Along with ED and UF tools, several further separation principles/techniques have been introduced for the qualitative and/or quantitative analysis of the reversibly interacting drug-protein system (Wood and Cooper, 1970; Sebille, 1990; Sebille *et al*., 1990; Oravcová *et al*., 1996b; Hage and Tweed, 1997; Hage, 2001).

The aim of this review is to outline advantages and to underline limitations of a liquid chromatographic separation method—the method of Hummel and Dreyer. Of the over one thousand published papers citing the use of the Hummel–Dreyer method, only those articles are reviewed where the subject of the investigation was the reversible binding interaction between a drug and a plasma protein of humans (see columns 1 and 2 in Tables 1–4).

HUMMEL–DREYER METHOD

In 1962 Hummel and Dreyer published a preliminary note entitled 'Measurement of protein-binding phenomena by gel filtration'. Their method, devised to detect reversible binding interaction between the macromolecules of a protein and the molecules of a low-molecular-weight substance, may be introduced as follows:

A solution of the low-molecular-weight substance with the molar concentration $[D]$ flows continuously through a column packed with a gel filtration (GF) medium exclusive for the macromolecules of *P*. On injecting a sample of *P*, dissolved in a solution of the low-molecular-weight substance with a concentration $[D]$ identical to that percolating through the GF column, two 'limiting' situations may occur:

(1) If the macromolecules of the protein do not bind the molecules of the low-molecular-weight substance, invariably one single peak corresponding to the *P* macromolecules should be detected at the column interstitial void volume.
(2) In case of the occurrence of reversible binding phenomenon, a trough must be observed after a peak detected at the excluded volume of the column (see also Fig. 1).

Thus, in other words, the appearance of the trough in the elution profile provides proof of the binding of *D* by *P* macromolecules.

Cross-linked dextran gel Sephadex® G-25, coarse (novel code 'G-25-300'), was chosen by Hummel and

Table 1. Application of the Hummel–Dreyer method in protein-binding studies with a single drug

Single drug[a]	Plasma protein	Method	Column packing material	Reference
Ceftriaxone	HSA[b]	HPLC	Sep-Protein	Mohler *et al.* (1990)
Chlorpromazine	α_1-AGP	GF	Sephadex G-25-150	Friedman *et al.* (1985)
Daunorubicin[c]	HSA	GF	Sephadex G-200	Demant and Friche (1998)
Diazepam	HSA	HPLC	LiChrosorb Diol	Thuaud *et al.* (1983)
Doxorubicin[d]	HSA	GF	Sephadex G-200	Demant and Friche (1998)
Furosemide	HSA	HPLC	Glycophase G	Sebille *et al.* (1978)
Phenytoin	HSA	HPLC	LiChrosorb Diol	Melten *et al.* (1985)

[a] See the Appendix.
[b] Under similar experimental conditions, Mohler *et al.* (1990) assayed the binding interactions of ceftriaxone and the injected sample of diluted human serum.
[c] Under similar experimental conditions, Demant and Friche (1998) assayed the binding interactions of 4-demethoxydaunorubicin [58957-92-9] with HSA (see also the Appendix).
[d] Under similar experimental conditions, Demant and Friche (1998) assayed the binding interactions of 4′-deoxy-4′-iododoxorubicin and 13-dihydro-4′-deoxy-4′-iododoxorubicin with HSA (see also the Appendix).

Table 2. Application of the Hummel–Dreyer method in protein-binding studies with a drug racemate

Drug racemate[a]	Plasma protein	Method	Column packing material	Reference
Amlodipine, *rac*-	HDL; LDL	HPLC	LiChrosorb Diol	Oravcová and Sojková (1995)
Carvedilol-(*RS*)	α_1-AGP; HSA	HPLC	LiChrosorb Diol	Oravcová *et al.* (1996a)
Isradipine-(±)-(*RS*)	HDL; LDL	HPLC	LiChrosorb Diol	Oravcová and Sojková (1995)
Isradipine-(±)-(*RS*)	α_1-AGP; HSA	HPLC	LiChrosorb Diol	Oravcová *et al.* (1995a)
Phenobarbital, *rac*-	HSA	HPLC	LiChrosorb Diol	Melten *et al.* (1985)
Propranolol, *rac*-	α_1-AGP	HPLC	LiChrosorb Diol	Šoltés *et al*. (1985)
Propranolol, *rac*-	α_1-AGP; HSA; HDL; LDL	HPLC	LiChrosorb Diol	Bree *et al.* (1986)
Warfarin, *rac*-	HSA	HPLC	GFF-S5-80	Pinkerton and Koeplinger (1990)
Warfarin, *rac*-	HSA	HPLC	Waters I-125	Sun *et al.* (1984)
Warfarin, *rac*-	HSA	HPLC	Glycophase G	Sebille *et al.* (1978)

[a] See the Appendix.

Table 3. Application of the Hummel–Dreyer method in protein-binding studies with a drug enantiomer

Drug enantiomer[a]	Plasma protein	Method	Column packing material	Reference
Carvedilol-(*R*)	α_1-AGP; HSA	HPLC	LiChrosorb Diol	Oravcová *et al.* (1996a)
Carvedilol-(*S*)	α_1-AGP; HSA	HPLC	LiChrosorb Diol	Oravcová *et al.* (1996a)
Isradipine-(+)-(*S*)	α_1-AGP; HSA	HPLC	LiChrosorb Diol	Oravcová *et al.* (1995a,b)
Isradipine-(−)-(*R*)	α_1-AGP; HSA	HPLC	LiChrosorb Diol	Oravcová *et al.* (1995a,b)
Pirprofen-(+)	HSA	HPLC	LiChrosorb Diol	Oravcová *et al*. (1991a)
Pirprofen-(−)	HSA	HPLC	LiChrosorb Diol	Oravcová *et al*. (1991a)
Propafenone-(*R*)	α_1-AGP	HPLC	LiChrosorb Diol	Oravcova *et al.* (1991b); Šoltés *et al*. (1994a)
Propafenone-(*S*)	α_1-AGP	HPLC	LiChrosorb Diol	Oravcova *et al.* (1991b); Šoltés *et al*. (1994a)
Propranolol-(+)	α_1-AGP	HPLC	LiChrosorb Diol	Oravcova *et al.* (1989)
Propranolol-(−)	α_1-AGP	HPLC	LiChrosorb Diol	Oravcova *et al.* (1989)

[a] See the Appendix.

Table 4. Application of the Hummel–Dreyer method in protein-binding studies with a drug *plus* a competitor

Drug *plus* a competitor[a]	Plasma protein	Method	Column packing material	Reference
Furosemide *plus* Warfarin, *rac*-	HSA	HPLC	Glycophase G	Sebille *et al.* (1978)
Warfarin, *rac*- *plus* Ibuprofen, *rac*-	HSA	HPLC	GFF-S5-80	Pinkerton and Koeplinger (1990)
Warfarin, *rac*- *plus* Fatty Acids	HSA	HPLC	LiChrosorb Diol	Sebille *et al.* (1979)
Warfarin, *rac*- *plus* Sodium Dodecyl Sulphate	HSA	HPLC	LiChrosorb Diol	Sebille *et al.* (1979)
Warfarin, *rac*- *plus* Furosemide	HSA	HPLC	Glycophase G	Sebille *et al.* (1978)

[a] See the Appendix.

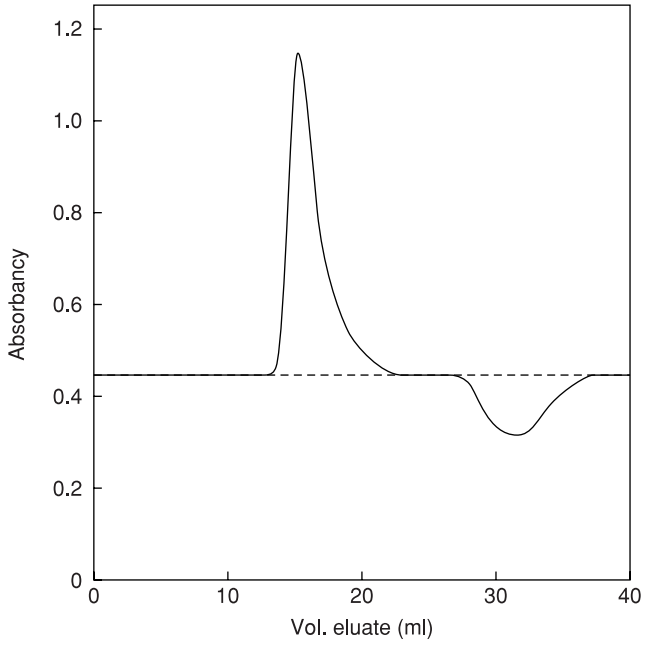

Figure 1. Elution profile of the 285 mμ absorbancy accompanying the passage of pancreatic RNAase through a column of Sephadex G-25 gel which was equilibrated with 2′-cytidylic acid (Hummel and Dreyer, 1962; with permission).

Dreyer (1962) at their pioneer GF study of the reversible binding interaction between a low-molecular-weight substance—2′-cytidylic acid (cytidine 2′-monophosphate; 323.2 Da)—and a protein, pancreatic ribonuclease A [pancreatic RNAase; ~13,700 Da (Laemmli, 1970)]. The fractionation range of the G-25-300 type of Sephadex® for globular proteins, i.e. 1000–5000 Da, assures that both the pancreatic RNAase as well as its unimolecular (1:1) complex with 2′-cytidylic acid are excluded from the sorbent pores and hence both co-migrating high-molecular-weight sample components are co-eluting in one single peak (cf. Fig. 1). Moreover, as evident from Fig. 1, the GF column used (100 cm in length) allowed a relatively large sample load (2 mg of the protein injected), maintaining a plateau clearly recognizable between the detected peak and trough. As reported (Hummel and Dreyer, 1962), even weak reversible binding interactions may be studied by this method provided that the concentration [*D*] of the substance run in the mobile phase is sufficiently high.

The rationale of the Hummel–Dreyer method is that the rate of the stabilization of the composition of the *P–D* complex, passing through the chromatographic column, is much higher than the speed of the separation process itself (Pinkerton and Koeplinger, 1990). Since this is an intrinsic feature, the Hummel–Dreyer method is generally declared to be an equilibrium liquid chromatographic separation method for studying reversible ligand–macrobiomolecule interactions (Šoltés *et al.*, 1994b; Šoltés and Sebille, 1994, 1997). It is important to emphasize explicitly that: the state of the equilibrium [cf. equation (1)] is governed by the concentration [*D*] set in the mobile phase.

GF VS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DESIGN OF THE HUMMEL–DREYER METHOD

The end of the 1960s and the beginning of the 1970s were characterized by an explosion in both the number of producers of individual modules applicable for building up a high-pressure/performance liquid chromatographic (HPLC) apparatus and the number of suppliers of complete, integrated HPLC machines. Surprisingly, however, it took almost a decade for the first application of the Hummel–Dreyer method to the HPLC assay arrangement to appear in the year 1978 (Sebille *et al.*, 1978). The authors studied reversible binding interaction of HSA vs furosemide and *rac*-warfarin (see also Tables 1, 2 and 4). They compared the innovated Hummel–Dreyer method with another established HPLC arrangement, that of the frontal analysis (FA). The year 1978 is thus classifiable as a milestone in the re-establishment of the Hummel–Dreyer method. In the 1980s and later on, the HPLC arrangement has become dominating, although a minor use of the GF design (cf. also Table 1) can also be recorded (Friedman *et al.*, 1985; Demant and Friche, 1998).

Column packing materials

The soft gel material of Sephadex®, suited for GF, would naturally collapse at the high pressure applied at the HPLC arrangement of the Hummel–Dreyer method. The primary selection of Glycophase G™ (Sebille *et al.*, 1978), a rigid HPLC column packing material, was very soon replaced by Sebille *et al.* (1979) by LiChrosorb® Diol, a 5 or 10 μm silica gel sorbent (porosity of 100 Å) covered by a diol phase. Although some similar column packing materials, e.g. Sep-Protein, a 20 μm spherical silica gel sorbent (porosity of 300 Å) lined with a hydrophobic polymer (Mohler *et al.*, 1990), were successfully used, LiChrosorb® Diol is to date one of the most exploited HPLC materials for running the Hummel–Dreyer method (see Tables 2 and 3).

The 'paradox' of a longer retention of the basic than the acidic drugs involved in working with LiChrosorb® Diol, which is due to reversible adsorption of bases by residual silanol groups of the sorbent, sometimes calls for variation of the HPLC column length. When running basic drugs it is generally shortened and with acidic ones it is prolonged.

Along with column packing materials suitable for 'desalting' proteins under size-exclusion conditions, a

restricted access HPLC column was introduced by Pinkerton and Koeplinger (1990), which is also applicable for reversible drug–protein binding studies (Table 2). The highly efficient internal surface reversed-phase column packing material (GFF-S5-80), which is exclusive for serum proteins, allows partitioning of small molecules within the pores capped by a hydrophobic tripeptide. This type of material, packed in a short column (5 cm), can be used to advantage for studies when, along with a drug run in the HPLC mobile phase, a competitor (another drug added into the eluent) is attracted against HSA binding sites (Table 4; Pinkerton and Koeplinger, 1990).

DETECTION

Spectrophotometers belong to the most popular HPLC detectors. Their linearity in a wide solute/drug concentration range has been especially appreciated. At a high drug level run in the mobile phase, however, it is sometimes necessary to select a proper wavelength setting—different from the drug's absorption maximum.

If a detection method can discriminate the competitor from the drug, competitive binding studies can be conducted easily (Sebille *et al.*, 1978, 1979). However, when such discrimination cannot be made, then the species must be separated (Pinkerton and Koeplinger, 1990).

PLASMA PROTEINS

The vast majority of pre-clinical drug–protein binding studies have been performed employing plasma proteins derived from healthy volunteers. Human serum albumin and α_1-acid glycoprotein are the two most important drug-binding proteins in blood plasma. The involvement of lipoproteins, immunoglobulins and other blood constituents, which may also act as drug-complexing macrobiomolecules, has been less frequently investigated during the pre-clinical pharmacokinetic stages.

All plasma proteins are inherently chiral. Non-polar, dipole and coulombic forces, as well as hydrogen bonding and steric effects—all these weak interactions/forces cooperatively participate in the reversible fixation of the given drug molecule closely to the particular region(s) on the protein.

The two principal binding sites on human serum albumin—the warfarin-azapropazone and indole-benzodiazepine—have been well characterized. The binding of drugs to human α_1-acid glycoprotein is, however, thought to occur at one single hydrophobic pocket/cleft within the protein part of the α_1-AGP macromolecule (Noctor, 1993).

HSA

Human serum albumin is a highly water soluble globular protein (66,462 Da; www.pdb.ufmg.br/pdb/cgi/explore.cgi?job=chains&pdbId=1AO6&page=20&pid=262651041627279) that has a strong negative charge (–17). The HSA polypeptide consists of a sequence of 585 L-aminoacidic residues interconnected intramolecularly by 17 disulfidic bonds (Fig. 2). From the point of view of the higher-order structure of the HSA macromolecule, the polypeptide presents both the α-helix (45–75%) and β-sheet (<18%; [Honoré, 1990]). The mean plasma concentration of albumin in healthy adults, 43 g L^{-1} (~0.65 mmol L^{-1}), represents approximately two-thirds of the total plasma protein level. Although hyperalbuminemia is a relatively rare pathological condition, hypoalbuminemia occurs frequently in severe hepatic and renal disorders.

One of the main physiological functions of albumin is the transport of a whole series of reversibly bound endogenic substances, such as fatty acids, bilirubin, L-tryptophan, etc. (Carter and Ho, 1994). Of the compounds with exogenic nature, HSA readily interacts with acidic drugs ($pK_a < 7$) with the affinity K of this interaction being usually in the interval of 10^4–10^6 L mol^{-1}. Basic drugs tend to associate with HSA generally in a low-affinity but high-capacity manner. For instance, approximately 50% of propranolol found in plasma was loosely bound to albumin (Noctor, 1993).

It has been clearly established that the presence of fatty acids in albumin increases its non-specific binding, thus an essentially fatty acid free HSA sample—the brand A 1887 (Sigma, St Louis, MO, USA)—was employed in several studies (Sebille *et al.*, 1978; Thuaud *et al.*, 1983; Bree *et al.*, 1986; Oravcová *et al.*, 1991a, 1995a,b, 1996a).

α_1-AGP

The macromolecule of human α_1-acid glycoprotein consists of a single polypeptidic chain with a sequence of 183 L-amino acid residues interconnected with two intrachain disulphide bonds (Fournier *et al.*, 2000). Five separate heteropolysaccharidic structural units are covalently bound to the polypeptide (Fig. 3). These units consist of neutral hexose (14%), *N*-acetylglucosamine (14%), sialic acid (11%) and fructose (1%). The protein part of the α_1-AGP macromolecule is formed of a higher-order structure of the α-helix type (21%), β-form (21%), and a section of eight β-turns (β-loops). The isoelectric point of α_1-AGP is 2.7. Consequently, at physiological pH, the glycoprotein macromolecule (44.0–44.1 kDa) is negatively charged (Israili and Dayton, 2001).

The plasma concentration of α_1-AGP varies within the interval 0.4–1.0 g L^{-1} (~9–23 μmol L^{-1}). As a result

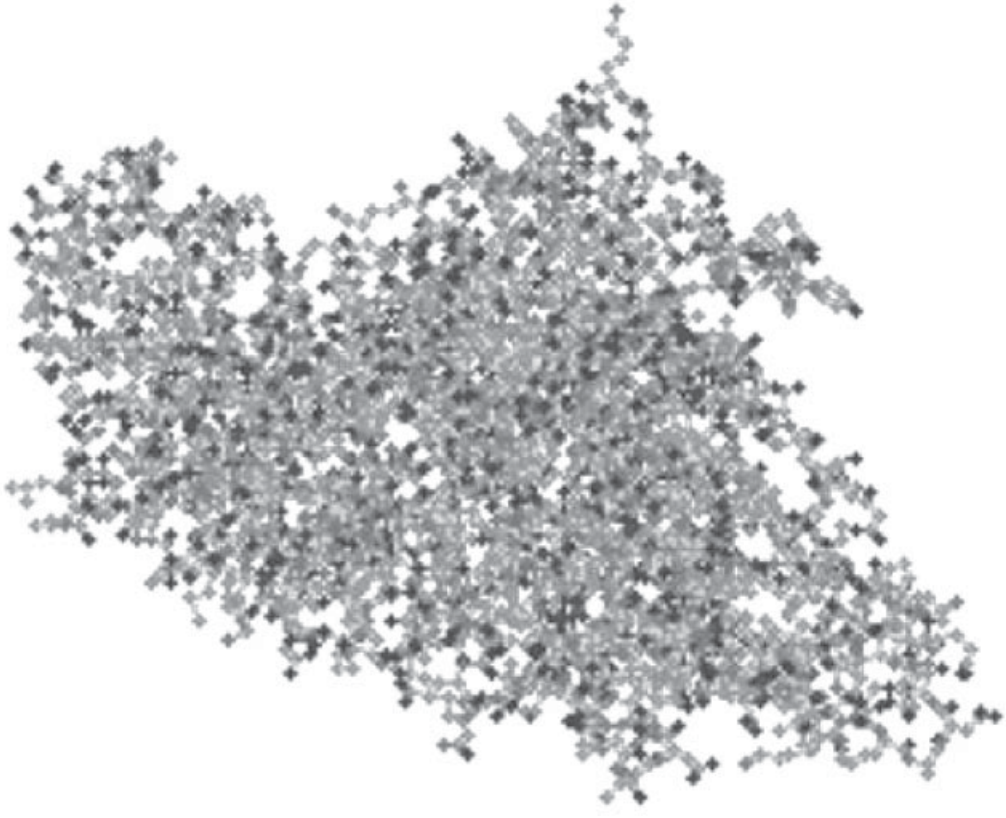

Figure 2. Human serum albumin; created by Carter DC, Chang B, Ho JX, Keeling K and Krishnasami Z www.isat.jmu.edu/users/klevicca/VISM/Spermine/SA/Main.html.

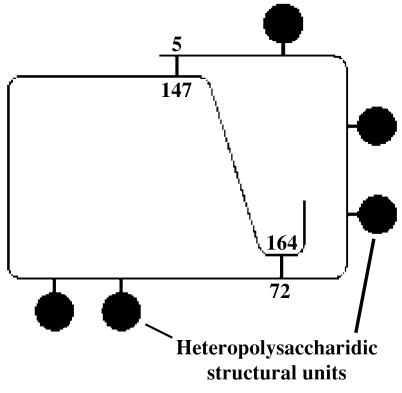

Figure 3. Alpha$_1$-acid glycoprotein www.raell.demon.co.uk/chem/CHIbook/a-agp.jpg.

of certain diseases, however, levels up to 3.0 g L^{-1} have been reported. Of the xenobiotics, α_1-AGP interacts preferentially with basic drugs (pK_a > 7) where the affinity K of this interaction reaches the value within the interval 10^4–10^7 L mol^{-1} (Kremer *et al*., 1988; Israili and Dayton, 2001). Although, due to its high content of sialic acid residues, α_1-AGP is an acidic protein, the binding of basic drugs appears to be driven by hydrophobic rather than electrostatic forces. Namely, removal of the sialic acid residues from the α_1-AGP macromolecule does not significantly reduce its affinity against basic drugs (Noctor, 1993). Human α_1-AGP can also bind acidic drugs such as phenobarbital (Fournier *et al*., 2000).

The macromolecule of human α_1-acid glycoprotein exhibits only low enantioselectivity. Thus in drug binding to α_1-AGP, clinically important effects due to stereospecificity are much less frequently observed than in binding to HSA.

Lipoproteins

By their structure, lipoproteins are complexes, associates assuming a spherical particulate shape. The hydrophobic core, consisting of esters of cholesterol and triacylglycerols, is covered by a hydrophilic layer, which is composed of phospholipids, cholesterol and the apolipoprotein (Fig. 4). From the point of view of specific density (D), lipoproteins have been grouped into high- (HDL), low- (LDL) and very low-density lipoproteins (VLDL).

The primary physiological function of lipoproteins is the transport of lipids. The cross-interaction of lipoproteins with low-molecular-weight drugs may have the nature of an association/complexation of drug molecules with the apolipoprotein and/or of a 'solubilization' of the drug molecules in the lipoprotein hydrophobic core. Most plausibly, however, the 'interaction' process itself

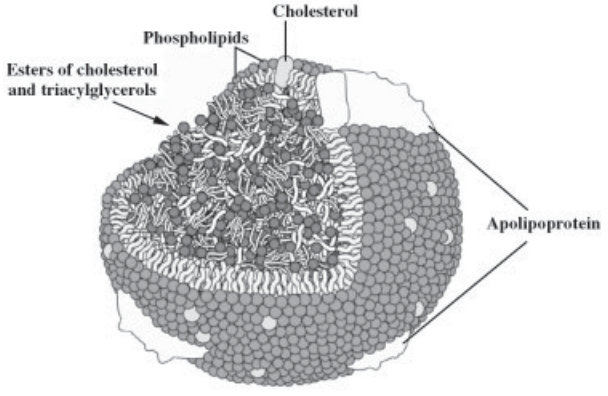

Figure 4. Lipoprotein http://cwx.prenhall.com/horton/medialib/media_portfolio/text_images/FG16_05.JPG.

would be essentially the dissolution of the drug in the lipoprotein particle rather than a specific attachment of drug molecules to a binding site.

The importance of lipoproteins in the 'binding' of a wider range of drugs may be enhanced in disease states such as hyperlipoproteinemia. However, although determinations of binding parameters of a particular drug to a group of lipoproteins were attempted (Table 2), the observations/deductions may be misleading since lipoproteins by themselves are associates—their composition may be significantly destructed/decomposed during the (zonal) HPLC run.

DRUGS

Most drugs are synthetic organic compounds possessing ionizable basic or acidic functional groups. Dissolved in water, the drug molecules are thus positively or negatively charged. Although charged, due to the organic character of their molecules, the drugs are poorly soluble in aqueous solution(s) at neutral physiological conditions (pH 7.4). To modulate the bioavailability of *the active principle*, the drug preparations are often converted to salts, e.g. basic substances to hydrochlorides, acidic drugs to Na-salt preparations.

Enantiomers of a racemic drug could, and often do, have different biological activities as a consequence of their selective, stereochemical interaction with macrobiomolecules in the living organism. This is why drug–protein binding studies have become increasingly concerned with pure, single drug enantiomer(s) (Table 3).

CONCENTRATION OF THE DRUG RUN IN THE MOBILE PHASE

In healthy human blood plasma proteins exist at approximately 37°C, pH 7.4. On processing the Hummel–Dreyer method these conditions can be conveniently met by thermostating the chromatographic column and working with buffered solutions. Phosphate buffer of physiological concentration (0.067 mol L^{-1}; pH 7.4) has been most often employed to dissolve the given drug. For a quick saturation of the chromatographic column a concentrated drug solution is first applied as the eluent. This drug concentrate—stock drug solution—is further diluted with the blank phosphate buffer to obtain the desired working drug solution(s)—the eluent(s). The task to be fulfilled on preparing the appropriate eluents so as to perform the drug–protein binding study correctly, is to set the drug concentrations with care, usually in a drug concentration range that is as wide as possible. Moreover, solutions containing a drug sensitive to light, oxidation, etc. must be adequately protected. In studies of the influence of a competitor (Table 4), the eluent, along with the drug, contains naturally a proper concentration of the competitor as well.

Similarly, the samples assayed are prepared by appropriately mixing the phosphate-buffered stock protein solution with the drug concentrate and the blank phosphate buffer. It should, however, be mentioned that the sensitivity of the phosphate buffered drug–protein solution to contamination by ubiquitous microorganisms (molds) is very high.

CONCENTRATION OF THE PLASMA PROTEIN INJECTED

Experimental conditions on performing the Hummel–Dreyer method are rather different from those occurring *in vivo* in patients. From the point of view of the albumin concentration, the HSA level in samples assayed varies usually around 2 g L^{-1}, i.e. about 5% of the physiological level. Moreover, on injecting a volume aliquot into the chromatographic column, the albumin concentration is immediately decreased due to the spread of the sample in the eluent stream. Thus the instantaneous [*P*] and [*P–D*] concentrations passing through the chromatographic column are varying (Parsons, 1980). However, as reported, for example, for the HSA–warfarin binding interaction, the obtained results were identical for the injected albumin concentration range 1–10 g L^{-1} (Sebille *et al*., 1980). Yet, this observation does not warrant that use of the Hummel-Dreyer method will result in identical data independently of the protein concentration assayed.

Contrary to HSA, on studying the drug interaction with human α_1-AGP, the difference between the *in vivo* protein level and the concentration in the samples assayed is reported to be virtually nil. The dependence of the results obtained on the injected α_1-AGP concentration has however not been investigated so far.

CHANGES IN CONFORMATION OF THE PROTEIN

The molar concentration of HSA in blood plasma is approximately 650 μmol L^{-1}. Such a high protein concentration, although injected onto the HPLC column (Melten *et al.*, 1985), is immediately diluted in the column head. The diluted albumin can and does demonstrate a considerable conformational mobility. It has been observed that dilution of the acceptor (receptor preparation or albumin) increases the affinity K and/or the number of binding sites n (Pedersen and Lindup, 1994) against a given substrate—a drug.

Many solutes that bind to albumin have been demonstrated to induce reversible conformational changes in the protein. That is also why a well-defined HSA sample is recommendable to easier compare/harmonize the results obtained by applying the Hummel–Dreyer method. On studying the human α_1-AGP binding interaction with drugs, the selection of the protein sample itself can be also critical. It is namely known that commercial α_1-AGP samples are slightly non-homogenous since heterogeneity is a reality in both the protein and the carbohydrate portions of the α_1-AGP macromolecules in the organisms (Israili and Dayton, 2001).

CAPILLARY ELECTROPHORETIC DESIGN OF THE HUMMEL–DREYER METHOD

When the sample assayed is injected onto the HPLC column head, the equilibrium between the interacting components is immediately reestablished in accordance with the (free) drug concentration set in the mobile phase. Both the protein and the drug–protein complex co-elute in the column interstitial void volume while the low-molecular-weight drug migrates according to its pore volume penetration.

In capillary electrophoresis (CE) the separation is based on the differences in electrophoretic mobilities of the sample species. Since the protein macromolecule is much larger and carries much more charge than the molecule of the drug, it is reasonable to assume that by binding of the drug to the protein neither its charge nor its molecular mass will be significantly altered. Consequently, both the protein and the drug–protein complex will have the same electrophoretic mobility. Thus methods similar to those developed by Hummel and Dreyer can be used with capillary electrophoresis. These assumptions, although theoretically valid, were however accompanied by numerous drawbacks observed on arranging the Hummel–Dreyer method in the CE design (Kraak *et al.*, 1992).

As reported by the same research group, application of the FA arrangement in CE presents the method of choice. However, although the CE–FA design is still very often used to characterize reversible drug–protein binding interaction, it is inevitable that in CE both the protein and the *P–D* complex formed are under a voltage current and thus the macromolecules may change their shapes, by which new binding sites are generated or the existing sites are modified/degenerated.

GF/HPLC ASSAY RUNS

Equilibrium in the chromatographic column can be simply tested on injecting a small volume aliquot drawn from the reservoir of the eluent. The detection of the unperturbed baseline within the whole interval of retention times is proof that equilibrium conditions have been reached. Analogously, any sample whose composition will be identical with the one run in the mobile phase should be 'invisible' when injected into the apparatus (Šoltés *et al.*, 1996).

Simultaneously, injection of a probe of the blank phosphate buffer should result in the detection of a maximal baseline negative deflection (trough) at the retention time corresponding to the drug. Thus this sample 'mimics' the situation when 100% drug would be bound to protein.

On injecting a real sample, containing the protein assayed on its affinity to reversibly bind the drug, in the case of a *P–D* complex formation a negatively oriented peak (trough) must be detected at the retention time of the drug. The trough area/height is a measure of $[B]$. In other words, detection of 50% of the maximally observable trough area/height means that in the investigated sample $[B] = {}^1/_2[F]$. Hence, on injecting a novel sample with the total drug level equaling $1.5 \times [F]$, no trough will appear in the chromatogram.

DETERMINATION OF ONE PAIR OF [*F*], [*B*] VALUES

Although two runs—an injection of the blank phosphate buffer and that of the real sample—are generally fully sufficient to estimate the percentage of drug binding, a more exact procedure called 'internal calibration' has been proposed and established (Hummel and Dreyer, 1962). During this calibration several real samples with total drug level equaling, for example, $0.5 \times [F]$, $1.0 \times [F]$, $1.5 \times [F]$ and $2.0 \times [F]$ are randomly analyzed. If the unbound drug concentration in the sample is less than $[F]$ set in the mobile phase, then a trough will appear in the chromatogram. If the unbound drug concentration in the sample is higher than that run in the eluent, then a peak will be detected

at the drug retention time. The detector responses plotted against the drug deficit or the drug excess in the samples analyzed allow that drug excess value to be found at which no peak will be detected at the retention time of the drug. This value is thus the unknown $[B]$, which is in equilibrium with the $[F]$ drug level set in the mobile phase.

The main purpose of using the internal calibration technique is to find the concentration of the drug which just compensates for the drug deficit in the sample analyzed or to determine that drug excess which eliminates the appearance of the second peak on the chromatographic record. Since the dependence of the trough—peak areas/heights on the amounts of the drug added to the injected samples is usually linear, the concentration of the bound drug which abolishes the detector response can be precisely interpolated.

DETERMINATION OF A SET OF $[F]_i$, $[B]_i$ DATA

Determination of one pair of the $[F]$, $[B]$ values allows calculation of the percentage of bound drug: $\{100 \times [B]/([F] + [B])\}$. Usually however, the task of the reversible drug–protein interaction study is to determine a set of $[F]_i$, $[B]_i$ data to construct the binding isotherm—the plot $[B] = f([F])$—and/or to calculate the binding parameters, n and K values.

It is comprehensible that, while the determination of one single $[F]$, $[B]$ pair needs, after equilibration of the chromatographic column, no more than four or five analyses, on determining the $[F]_i$, $[B]_i$ data set the measurements require numerous analytical runs and several settings of the equilibrium. Although automatization of the whole series of the measurements has been attempted (Pinkerton and Koeplinger, 1990), even working 24 h per day, the determination of a collection of $[F]_i$, $[B]_i$ data takes a few days.

GRAPHICAL AND MATHEMATICAL ANALYSIS OF BINDING DATA

The $[F]_i$, $[B]_i$ or better the $[F]_i$, r_i data determined have usually been plotted into a semilogarithmic r vs $\log[F]$ graph. When the reversible drug–protein binding interaction is characterized exclusively by specific (saturable), one or more, classes of binding sites then the r vs $\log[F]$ graph turns to an S-shape curve with the maximum of the ordinate value (r_{max}) reaching the unknown, n or $\Sigma\, n_i$, value.

The particular case of the occurrence of only one specific (saturable) class of binding sites can be checked simultaneously by constructing a Scatchard plot in the transformed scale $r/[F]$ vs r. In this plot the intercept on the abscissa should equal n. Thus the two graphical presentations of the binding data, i.e. r vs $\log[F]$ and $r/[F]$ vs r, can be used to estimate if the interaction can be characterized simply by one single population of specific (saturable) binding sites on the protein.

However, the Scatchard plot—a common graphical presentation of binding data—is often nonlinear. Despite several articles (Kermode, 1989; Šoltés *et al*., 1989; Zierler, 1989) calling attention to incorrect treatment of Scatchard plots, erroneous interpretations of nonlinear Scatchard plots remain frequent. In the case of nonlinearity of the $r/[F]$ vs r plot along with no tendency of the r vs $\log[F]$ graph to turn into an S-shape curve, the correct conclusion must be that the drug–protein interaction cannot be characterized simply by one specific (saturable) class of binding sites. The risk of uncritical choice of the equation

$$r = \frac{n_1 K_1[F]}{1 + K_1[F]} + \frac{n_2 K_2[F]}{1 + K_2[F]} \tag{4}$$

characterizing the reversible drug–protein binding interaction by the existence of two simultaneously manifested specific (saturable) classes of binding sites has recently been analyzed in detail (Šoltés and Mach, 2002).

A simple way of mathematically treating the binding data, which graphically are represented by a curvilinear Scatchard plot, is to suppose that along with a specific (saturable) class of binding sites the reversible drug–protein binding interaction is by its nature characterizable by a non-saturable binding term (Taira and Terada, 1985). This situation can thus be simply described by using a summation of Langmuir- and Freundlich-type isotherms:

$$r = \frac{nK[F]}{1 + K[F]} + \text{Const.} \times [F] \tag{5}$$

As demonstrated several times, this type of $r = f([F])$ dependence in particular has one unambiguous solution, i.e. one true objective set of parameters n, K, and Const. (Šoltés *et al*., 1994a; Šoltés and Mach, 2002).

Although equation (5) seems to be a rational compromise, another description of the reversible binding interaction was introduced by Tobler and Engel (1983). Their linear programing approach, the affinity spectra, allows estimation not only of the most probable value of N [equation (3)], but also the n_i, K_i guesses—the inputs necessary to solve equation (3) by a nonlinear iterative procedure. The advantage of using the Tobler–Engel program has so far been exploited by evaluating data on the reversible interaction of human α_1-AGP with the enantiomers of propafenone (Šoltés *et al*., 1994a; Šoltés and Mach, 2002).

HUMMEL–DREYER METHOD STRENGTH

Analogously to ED, the Hummel–Dreyer method allows the reversible drug–protein binding interaction to be studied in a homogenous liquid phase. Since the column packing material used is inert, the equilibrium association/dissociations processes proceed in the mobile phase. Moreover, the Hummel–Dreyer experimental approach can be classified as unique since it enables the free drug concentration to be controlled as the true independent variable. Thus on investigating the reversible drug–protein binding interaction isotherm by this method, the $[F]_i$ values can easily be set equidistantly through the $[F]$-scale of the $[B] = f([F])$ isotherm.

HUMMEL–DREYER METHOD WEAKNESS

Despite the fact that the number of drug moles bound by 1 mole of the protein, r, depends exclusively on the drug concentration set in the eluent $[F]$, the gradual dilution of the drug–protein complex, occurring inevitably during the chromatographic process, can influence the interaction equilibrium. As a consequence of this fact, the determined binding parameters have in some casses to be classified as apparent values.

CONCLUDING REMARKS

The Hummel–Dreyer method has entered the fifth decade of its existence. In the light of the relatively low citation rate concerning the use of this method in pharmacology, it may appear that the researchers involved do not consider it to be the method of choice. The main weakness of the approach is the fact that following injection, the sample containing the *P–D* complex becomes gradually diluted on passing the chromatographic column. The potential alteration of the protein conformation involves modification of binding sites, which may even result in only apparent $[B]_i$, r_i values. Treatment of such apparent data by exploiting equation (4) invariably decreases the measure of validity of the resulting parameters of binding interaction.

An important aim of the given overview is to appeal on relevant researchers to establish a standard experimental protocol for studying the given interaction with regard to both methodology and analysis of binding data (Šoltés and Mach, 2002).

Acknowledgement

The grants VEGA no. 1047, 2049, and 2053 from the grant agency for Sciences, Bratislava, Slovak Republic are gratefully acknowledged.

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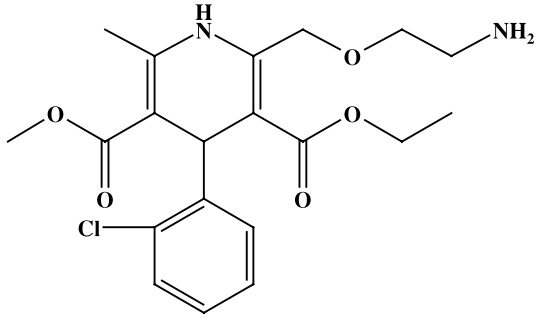
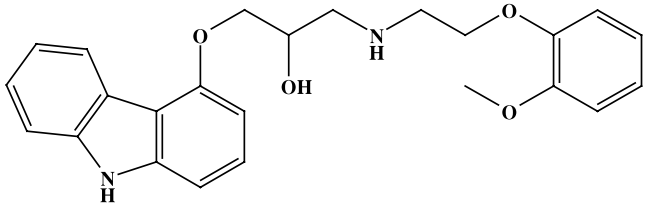
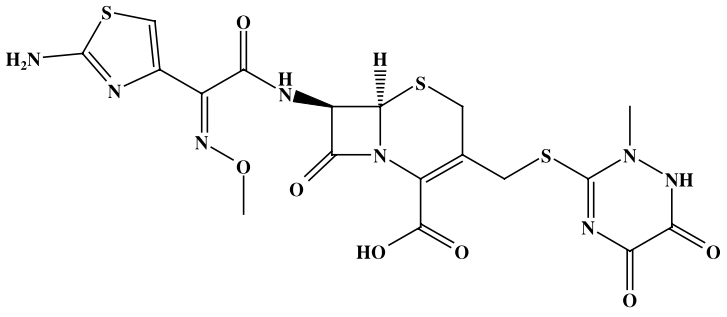
APPENDIX



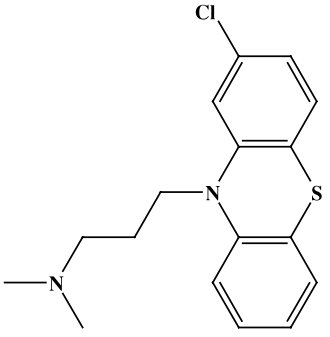
Scheme I. Amlodipine [88150-42-9]; long-acting calcium channel blocker.



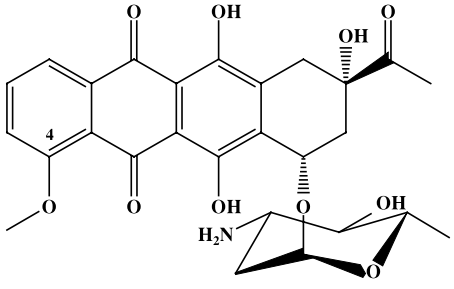
Scheme II. Carvedilol [72956-09-3]; non-selective β-adrenergic blocking agent with α_1-blocking activity.



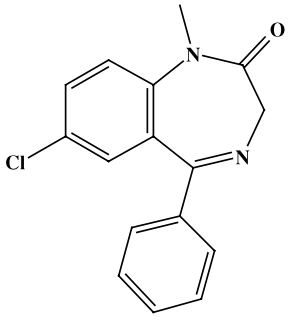
Scheme III. Ceftriaxone [74578-69-1]; semisynthetic, broad-spectrum cephalosporin antibiotic.



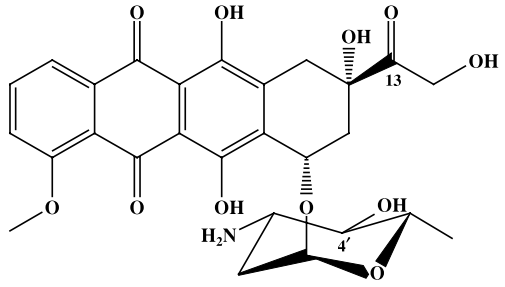
Scheme IV. Chlorpromazine [50-53-3]; its principal pharmacological actions are psychotropic.



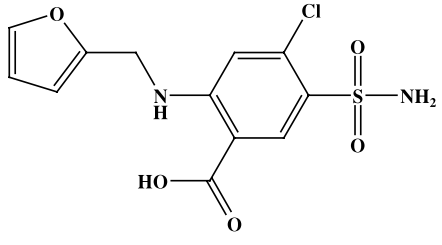
Scheme V. Daunorubicin [20830-81-3]; antineoplastic.



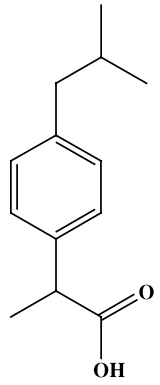
Scheme VI. Diazepam [439-14-5]; benzodiazepine anxiolytic and sedative-hypnotic.



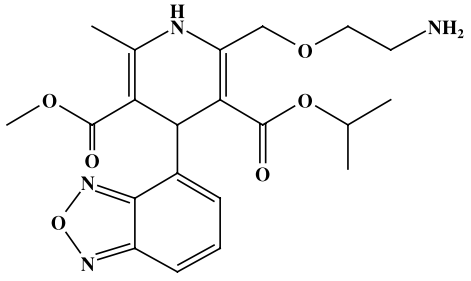
Scheme VII. Doxorubicin [23214-92-8]; anthracycline antibiotic and antineoplastic.



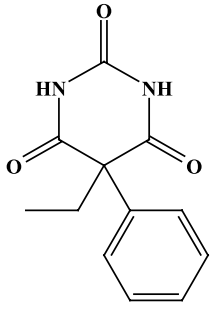
Scheme VIII. Furosemide [54-31-9]; diuretic.



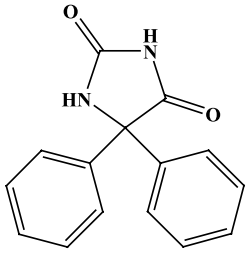
Scheme IX. Ibuprofen [15687-27-1]; non-steroidal anti-inflammatory agent.



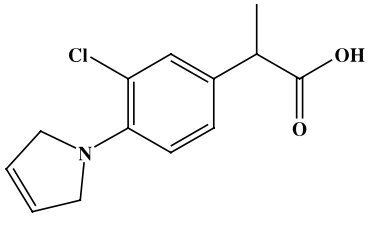
Scheme X. Isradipine [75695-93-1]; calcium channel blocker with antihypertensive and platelet antiaggregatory properties.



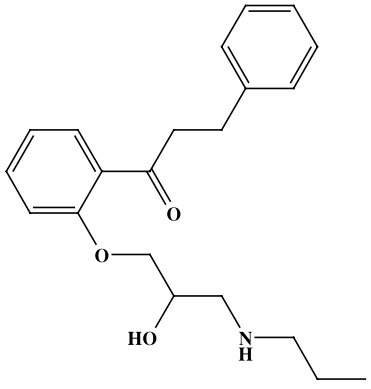
Scheme XI. Phenobarbital [50-06-6]; non-selective central nervous system depressant.



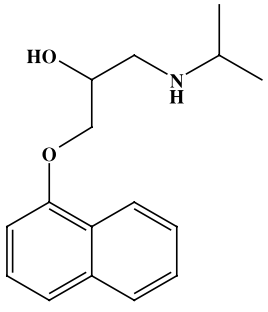
Scheme XII. Phenytoin [57-41-0]; anticonvulsant and antiepileptic.



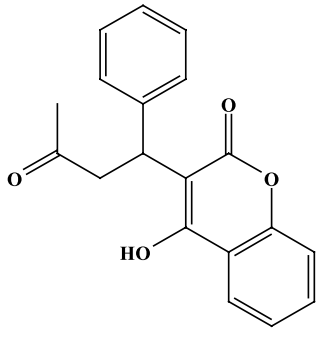
Scheme XIII. Pirprofen [31793-07-4]; non-steroidal anti-inflammatory agent.



Scheme XIV. Propafenone [54063-53-5]; antiarrhythmic.



Scheme XV. Propranolol [525-66-6]; non-selective β-adrenergic receptor blocking agent.



Scheme XVI. Warfarin [81-81-2]; anticoagulant.