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Professeur P.-A. Carrupt

Development of New Experimental Tools for Fast Determination of Solubility and Lipophilicity

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention Sciences pharmaceutiques

par

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de
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Thèse *de Monsieur Bruno BARD*

intitulée :

"Development of New Experimental Tools for Fast Determination of Solubility and Lipophilicity"

La Faculté des sciences, sur le préavis de Messieurs P.-A. CARRUPT, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques, Laboratoire de pharmacochimie), J.-L. VEUTHEY, professeur ordinaire (Section des sciences pharmaceutiques, Laboratoire de chimie analytique), Madame S. MARTEL, docteur (Section des sciences pharmaceutiques, Laboratoire de pharmacochimie) et de Messieurs A. TCHAPLA, professeur (Institut Universitaire de Technologie d'Orsay - Paris XI - Groupe de chimie analytique, Orsay, France) et R. ROZOT, docteur (L'Oréal, Aulnay-sous-Bois, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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Abbreviations and symbols

α	H-bond donor acidity
β	H-bond acceptor basicity
ACN	Acetonitrile
ADMET	Absorption, distribution, metabolism, excretion and toxicity
BCS	Biopharmaceutics classification system
CE	Capillary electrophoresis
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
DS	Degree of saturation
FaSSIF	Fasted State Simulated Intestinal Fluid
FeSSIF	Fed State Simulated Intestinal Fluid
HILIC	Hydrophilic interaction chromatography
HTS	High throughput screening
I	Ionic strength
ITIES	cyclic voltammetry at the interface between two immiscible electrolyte solutions
LOD	Limit of detection
log k	Logarithm of the retention factor
$\log k_{90}$	Logarithm of the retention factor measured in presence of 90% acetonitrile in the mobile phase
$\Delta\log k_{95\text{-}0}$	Difference between retention factors measured in presence of 95 and 0% of acetonitrile in the mobile phase
$\text{Log } P^{I}$	Logarithm of the partition coefficient of an ionized form
$\log P_{oct}$	Logarithm of the n-octanol/water partition coefficient
$\log P^{N}_{oct}$	Logarithm of the partition coefficient of the neutral form in octanol/water system
$\log P^{C}_{oct}$	Logarithm of the partition coefficient of the cationic form in octanol/water system
$\log P_{alk}$	Logarithm of the alkane/water partition coefficient
LSERs	Linear solvation energy relationships

m	Slope of the correlation
NCEs	New chemical entities
NPLC	Normal phase liquid chromatography
PC	Polycarbonate
PES	Polyethersulfone
PD	Pharmacodynamic
${}^{w}_{w}pH$	pH measured and calibrated in water
${}^{S}_{w}pH$	pH measured in a mixture organic solvent/water and calibrated in water
pK_a	Ionization constant in water
PES	Polyethersulfone
PK	Pharmacokinetic
PTFE	Polytetrafluoroethene
PVDF	Polyvinyldifluoride
$\pi*$	Solvatochromic parameter standing for the solute's dipolarity/polarizability
RP(LC)	Reverse phase (liquid chromatography)
(Q)SAR	(Quantitative) structure activity relationship
S	Apparent solubility
S_0	Intrinsic solubility
$S_0^{x\%}$	Intrinsic solubility measured in presence of x% DMSO
SD	Standard deviation
SPME	Solid phase microextraction
t_r	Retention time of the solute
t_0	Retention time of an unretained compound
SF	Shake-flask
TLC	Thin layer chromatography
UPLC	Ultra Performance Liquid Chromatography
V	Volume

Chapter 1 : Résumé

Introduction

La recherche de nouveaux médicaments est un processus complexe et fastidieux. De la découverte de nouvelles entités chimiques (NCEs) à la mise sur le marché d'un nouveau médicament, une période de 12 à 20 ans est en effet nécessaire. Ce processus peut être divisé en deux grandes phases communément appelées phases de découverte et de développement d'un médicament. La phase de découverte a pour but de découvrir et d'optimiser des composés chefs de files (candidats médicaments ayant des propriétés pharmacocinétiques et pharmacodynamiques suffisantes pour progresser vers un programme de développement complet [1]). La phase de développement regroupe les phases d'essais précliniques et cliniques. Le nombre de composés rejetés durant le processus de recherche de nouveaux médicaments est extrêmement élevé. Pour plus de 100'000 à 1'000'000 entités chimiques synthétisées, à peu près 10 atteignent le stade des essais cliniques chez des volontaires sains et une seule sera commercialisée [2,3].

Des propriétés pharmacocinétiques inappropriées sont considérées comme une des principales causes de retrait des NCEs lors de la phase de développement de médicaments [4]. De réels efforts ont donc été entrepris pour évaluer ces propriétés dès que possible pendant la phase de découverte, afin d'éviter que des candidats médicaments inadaptés n'entrent dans le processus de développement [5].

Certaines propriétés physicochimiques peuvent être utilisées pour prédire les propriétés ADMET (Absorption, Distribution, Métabolisme, Elimination et Toxicité) des candidats médicaments [6,7]. De ce fait, le développement de méthodes de criblage à haut débit pour déterminer le profil physicochimique des composés a connu un essor considérable. Parmi les propriétés physicochimiques couramment utilisées, la solubilité et la lipophilie ont montré être des paramètres clés dans la prédiction des comportements pharmacocinétiques des médicaments.

Solubilité

Une solubilité insuffisante est une des principales causes d'échec durant l'ensemble des phases de découverte et de développement d'un médicament. Une faible solubilité peut fausser les résultats des différents essais *in vitro*, et interférer avec les propriétés pharmacodynamiques et pharmacocinétiques d'une molécule.

Les tests de solubilité en phase de découverte et de développement doivent être adaptés aux caractéristiques et aux besoins de chaque phase. En développement, des données de solubilité de haute qualité sont requises afin de prendre les décisions appropriées pour surmonter les potentiels problèmes de solubilité. La solubilité thermodynamique (à l'équilibre) y est généralement mesurée. Les composés doivent être disponibles en grandes quantités, les tests de solubilités sont longs, à faibles débits et faiblement automatisables. Les essais de solubilité sont aussi exécutés dans des milieux caractéristiques des liquides biologiques (intestino-mimétiques) pour prédire l'absorption gastro-intestinale des médicaments administrés par voie orale conformément au système BCS (Biopharmaceutics Classification System) [8,9]. La solubilité des médicaments dans l'intestin peut effectivement être fortement augmentée par rapport à la solubilité en milieu aqueux en raison (entre autre) de la présence de sels biliaires [10-12].

Au stade de découverte, le nombre important de composés à tester ainsi que les faibles quantités disponibles, font que les méthodes doivent être pleinement automatisées, peu coûteuses, économes en produits, rapides et à haut débit [13]. A ce stade de recherche, les informations les plus pertinentes en terme de solubilité viseraient deux buts principaux. Le premier but serait d'évaluer si les composés généralement dissous dans du DMSO restent en solution après dilution dans le milieu de criblage. Une solubilité incomplète pourrait en effet si non identifiée, mener à des résultats douteux, à une sous-estimation de l'activité, et à des relations structure activité (SAR) inexactes [14]. Dans ce but, les conditions expérimentales sont adaptées aux conditions rencontrées dans les différents tests biologiques *in vitro* et la solubilité cinétique qui mesure la précipitation d'un soluté prédissous, est mesurée. Même si ces méthodes de criblage sont particulièrement utiles dans ce contexte, la solubilité cinétique est fortement dépendante des conditions expérimentales et les valeurs de solubilité ne sont pas comparables entre des méthodes cinétiques impliquant des conditions expérimentales différentes [15]. La solubilité observée en utilisant les méthodes de criblage à haut débit est généralement surestimée de manière composé dépendante par rapport à la solubilité thermodynamique. C'est pourquoi la solubilité cinétique n'est pas la plus adéquate pour prédire les propriétés ADMET d'un candidat médicament. Il y a donc de nos jours un réel besoin de développer des méthodes à haut débit permettant d'obtenir des valeurs de solubilité en accord avec la solubilité thermodynamique ou caractéristiques de la solubilité *in vivo* [8,16].

Lipophilie

La lipophilie est une propriété fondamentale pour prédire à la fois le comportement pharmacocinétique (perméabilité membranaire, distribution tissulaire, liaison aux protéines plasmatiques, métabolisme) et pharmacodynamique (affinité de liaison aux récepteurs) d'un médicament. Elle est généralement exprimée par le logarithme du coefficient de partage (P) entre l'eau et un solvant organique non miscible. Le système biphasique le plus utilisé est le système *n*-octanol/eau en raison de l'analogie structurale entre le *n*-octanol et les phospholipides qui composent les membranes cellulaires (un groupement polaire et une longue chaîne alkyle). La méthode de référence pour mesurer la lipophilie est la méthode « shake-flask », et permet la mesure de log P allant de –3 to +3 [17,18]. Cette approche est longue, fastidieuse et requiert des quantités importantes de produits.

La chromatographie en phase inverse RPLC (reversed phase liquid chromatography) est également largement utilisée pour mesurer la lipophilie. Par rapport au shake-flask, cette technique requiert de plus petites quantités de produits, peut être facilement automatisée et permet d'obtenir des mesures à un plus haut débit. De plus, cette méthode permet la séparation des éventuelles impuretés ou produits de dégradation qui pourraient altérer les mesures de coefficients de partage. Cependant cette technique n'est pas appropriée à la détermination de lipophilie de composés polaires ($\log P < -1$). De plus, les solutés, en particulier les composés basiques, peuvent interagir avec les silanols libres résiduels des phases stationnaires traditionnelles à base de silice. Ces interactions « parasites » peuvent mener à des estimations de la lipophilie incorrectes [19,20]. L'autre désavantage des phases traditionnelles à base de silice est leur instabilité chimique à pH supérieur à 8, ce qui rend impossible la détermination de coefficients de partage de la forme neutre de composés basiques ayant un $pK_a > 7$ [21-24].

Parmi les nombreuses approches décrites permettant de mesurer la lipophilie à un plus ou moins haut débit, il n'y a toujours pas de méthodes pour obtenir des valeurs fiables de lipophilie de composés polaires non ioniques ($\log P < -3$).

But de la thèse

Ce travail a donc été consacré au développement d'outils rapides et fiables pour mesurer la solubilité et la lipophilie au stade de découverte d'un nouveau médicament. Une méthode de criblage à haut débit 96 puits et détection UV a tout d'abord été adaptée pour obtenir des valeurs de solubilité proches des valeurs thermodynamiques obtenues par « shake-flask ». Cette méthode optimisée a ensuite été adaptée pour mesurer la solubilité directement dans 2 milieux *in vitro* intestino-mimétiques (FaSSIF et FeSSIF).

Dans la deuxième partie de ce travail, la technique HILIC (hydrophilic interaction chromatography) qui retient les composés polaires et chargés, a été évaluée comme une nouvelle approche pour mesurer la lipophilie des solutés non ioniques polaires et des composés basiques, deux catégories de composés difficilement mesurables par les méthodes conventionnelles de mesure de la lipophilie.

Méthode UV de criblage à haut débit pour l'estimation de la solubilité thermodynamique et de la solubilité dans des milieux caractéristiques des milieux biologiques

Pour obtenir des mesures de solubilité de haute qualité lors de la phase de découverte une méthode UV de criblage de solubilité à haut débit (HTS) a été adaptée. Des paramètres expérimentaux comme l'intensité de l'agitation, le temps d'incubation et le pourcentage de DMSO, ont été optimisés afin d'obtenir des valeurs de solubilité proches de la solubilité thermodynamique obtenue par « shake-flask ». Les résultats ont montré que l'augmentation de solubilité généralement enregistrée avec les méthodes HTS était due principalement à la formation de solutions sursaturées ayant des cinétiques de précipitation lentes. Il a également été montré que la sursaturation pouvait être minimisée par application d'une agitation plus vigoureuse (1200 rpm) et par l'augmentation des temps d'incubation à 24 h. La solubilité mesurée dans ces conditions a donné des résultats en bon accord avec la solubilité mesurée par la méthode du « shake-flask » sans DMSO. En comparaison, l'augmentation de solubilité due à la seule présence de DMSO (co-solvant) était modérée et moins « composé dépendante » en présence de 0.5 et 1.0% (v/v) de DMSO (Fig. 1-1).

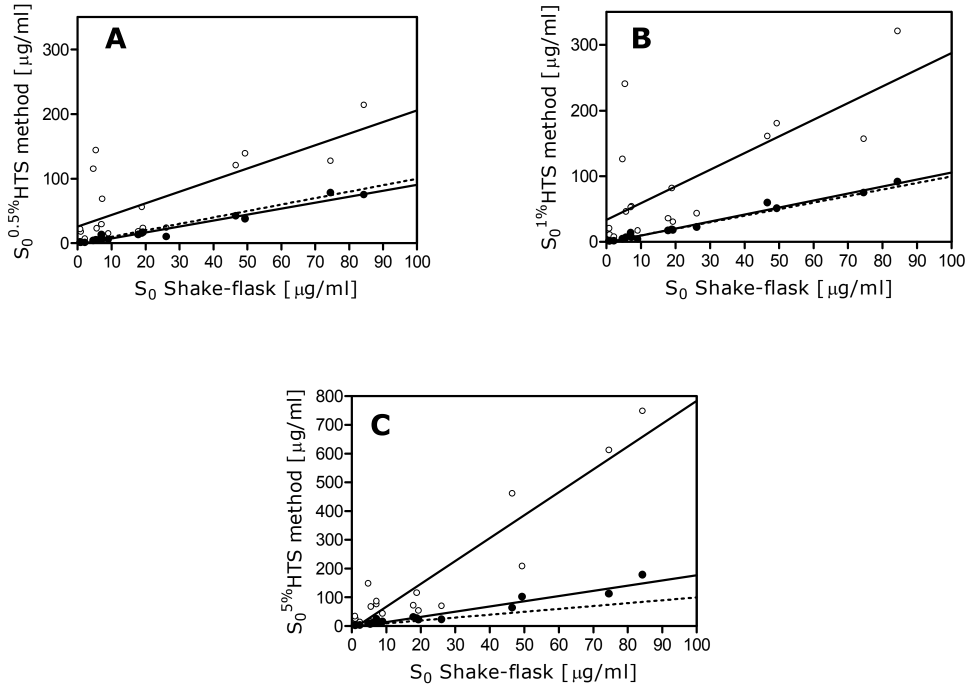

Fig. 1-1 : Corrélations (lignes continues) entre S_0 (solubilité intrinsèque = de la forme neutre) mesurée par shake-flask (sans DMSO) et S_0 mesurée avec la méthode HTS UV en présence de (A) 0.5 %, (B) 1 % et (C) 5 % de DMSO, après des temps d'incubation de 1.5 h et une agitation de 300 rpm (○) et un temps d'incubation de 24 h et une agitation de 1200 rpm (●). Les lignes discontinues représentent l'identité.

Afin d'évaluer à un stade très précoce la biodisponibilité et l'absorption des médicaments après administration par voie orale, la méthode HTS UV optimisée a été adaptée à la mesure de solubilité dans des milieux caractéristiques des milieux biologiques, à savoir les deux milieux intestino-mimétiques FeSSIF et FaSSIF [25] qui simulent respectivement les conditions postprandiales et à jeun dans l'intestin grêle. Ces deux milieux, contiennent notamment des sels biliaires (acide taurocholique, lécithine), connus pour augmenter la solubilité des composés les plus lipophiles.

Le DMSO comme seul adjuvant à tout d'abord été évalué comme substitut des sels biliaires contenus dans les milieux FaSSIF et FeSSIF ce qui permettrait de diminuer les coûts des mesures HTS.

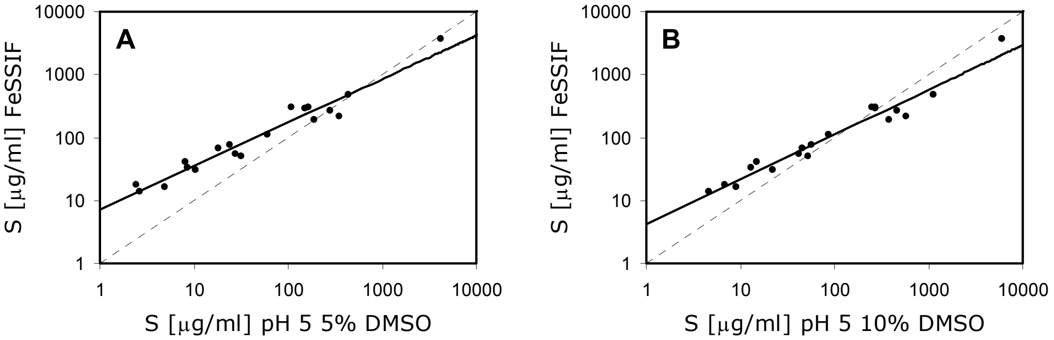

Fig. 1-2 : Corrélations (lignes continues) entre log S mesuré en milieu FeSSIF et log S mesuré dans les tampons aqueux pH 5 correspondants, en présence de A. 5%, B. 10% (v/v) de DMSO. Les lignes discontinues représentent l'identité.

Les résultats ont montré (Fig. 1-2) que, bien que le DMSO joue un rôle solubilisant, il augmente de manière globalement équivalente la solubilité de tous les médicaments testés, contrairement aux sels biliaires qui jouent un rôle solubilisant très différent sur les composés hydrophiles et lipophiles [10,11]. Par conséquent, le DMSO comme seul adjuvant ne peut pas remplacer de manière avantageuse les sels biliaires.

La méthode HTS UV optimisée a donc été adaptée pour mesurer la solubilité directement dans les milieux FaSSIF et FeSSIF. Pour ce faire, les solutions mères de DMSO ont tout d'abord été diluées dans une solution tampon et incubées pendant 24 h afin de permettre la précipitation des solutions sursaturées. A la fin de ce temps d'incubation, une solution concentrée en taurocholate de sodium et en lécithine a été ajoutée pour donner la concentration finale en sels biliaires caractéristique des milieux FaSSIF et FeSSIF. Après cette adjonction, les solutions ont encore été incubées pendant 8 h pour s'assurer que l'équilibre de micellisation soit atteint. D'excellentes corrélations ont été obtenues entre les mesures effectuées avec la méthode HTS UV et celles obtenues par shake-flask dans les milieux FaSSIF et FeSSIF (Fig. 1-3).

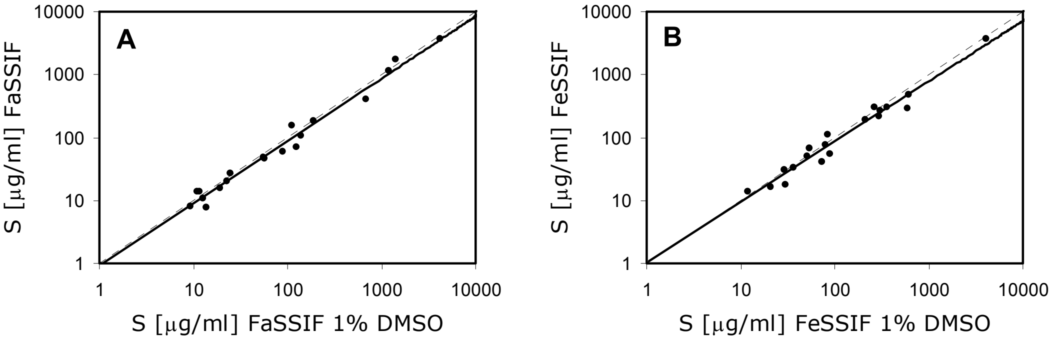

Fig. 1-3 Corrélations entre: (A) log S mesuré en milieu FaSSIF avec la méthode shake-flask et log S mesuré en milieu FaSSIF en présence de 1% de DMSO à l'aide de la méthode HTS UV. (B) log S mesuré en milieu FeSSIF avec la méthode shake-flask et log S mesuré en milieu FeSSIF en présence de 1% de DMSO à l'aide de la méthode HTS UV.

Cette méthode offre donc un moyen unique et fiable de mesurer la solubilité dans des milieux intestino-mimétiques à des stades précoces de découverte d'un nouveau médicament, à un débit relativement élevé et à un coût amoindri.

Sources d'erreurs sur les mesures de solubilité en utilisant la méthode HTS UV

L'influence de plusieurs facteurs analytiques sur l'exactitude des résultats obtenus avec la méthode HTS UV a été évaluée.

Le précipité qui apparait après le temps d'incubation peut être éliminé par centrifugation, cependant, ce procédé est long et quelques fois inefficace [26]. C'est pourquoi, la filtration sur microplaques filtrantes reste la technique de choix pour la détermination de solubilité par la méthode HTS UV. Toutefois, l'adsorption « non spécifique » sur les supports ou les membranes filtrantes de ces microplaques est un paramètre qui peut altérer l'exactitude des mesures de solubilité, car il en résultera une baisse significative et artificielle de la concentration du filtrat. Le choix de la membrane filtrante est donc crucial en vue d'obtenir des résultats fiables de solubilité. Deux différentes microplaques filtrantes, ayant des membranes connues pour être parmi les membranes montrant le moins de rétention [27,28], à savoir des membranes en polycarbonate (PC) ou en polyvinyldifluoride (PVDF) ont donc été directement comparées et évaluées. Les membranes en PC ont montré globalement peu de rétention et leur utilisation est donc recommandée pour la mesure de solubilité par la méthode HTS UV.

L'exactitude des systèmes de pipetage a également été évaluée par des études gravimétriques et les résultats ont mis en évidence la présence d'une erreur systématique dans les volumes dispensés par le système « Precision 2000 Pipetting System ». Les volumes ont donc été adaptés pour corriger cette erreur. Une étude détaillée de la source de variabilité a ensuite été réalisée. Il a été montré que la principale source de variabilité en utilisant la méthode HTS UV se situait dans la variabilité entre les puits (CV<4.3%), dépendant des imprécisions de pipetage, de la cinétique de précipitation et de facteurs liés au matériel. A l'opposé, la reproductibilité de la détection UV était excellente (CV<0.5%). Il a aussi été démontré que la limite de détection (LOD) dépendait fortement de la longueur d'onde de détection et que la sensibilité de la méthode était fortement diminuée à 230 nm (pic d'absorbance du DMSO), par rapport à des longueurs d'ondes en dessus de 240 nm, où une LOD inférieur à 0.010 unité optique peut être atteinte.

Détermination des coefficients de partage alcane/eau de composés polaires par HILIC (Hydrophilic Interaction Chromatography)

Il n'y a actuellement toujours pas de méthodes pour obtenir des valeurs fiables de lipophilie de composés polaires non ionisés (log P < -3) [29] et le développement de telles méthodes seraient donc d'une grande utilité. La technique HILIC retenant les composés polaires et chargés a donc été évaluée comme une nouvelle méthode permettant de mesurer la lipophilie des composés polaires non ionisés.

Les facteurs de rétention (log k) de 46 composés polaires ont été mesurés sur une colonne ZIC®-*p*HILIC en utilisant de grandes proportions (90%) d'acetonitrile (ACN) dans la phase mobile, et une étude LSERs (Linear Solvation Energy Relationship) a été réalisée pour déterminer les forces intermoléculaires impliquées dans la rétention sur cette phase stationnaire.

Les résultats ont montré que les principaux facteurs qui régissent la rétention dans ces conditions sont le pouvoir donneur de liaison hydrogène (α), ainsi que le pouvoir accepteur de liaison hydrogène (β). Ces paramètres sont suivis par le volume (V) alors que la contribution de la dipolarité/polarisabilité (π^*) est de moindre importance et statistiquement non significative. Ces résultats ont été comparés à des modèles LSERs publiés pour le log P_{oct} [30]. Il a été montré que la principale différence entre ces deux systèmes résidait dans l'expression du pouvoir donneur de liaison hydrogène (α), peu impliqué dans le log P_{oct}, et au contraire très important en HILIC. Cela signifie que la lipophilie dans le système *n*-octanol/eau ne peut pas être déterminée en utilisant cette méthode.

Cependant, les forces intermoléculaires impliquées dans la rétention en mode HILIC, en particulier les importantes contributions des paramètres α et β, sont similaires à celles régissant le coefficient de partage dans des systèmes comprenant un solvant inerte tel que le système alcane/eau [31]. Les résultats de cette analyse LSER ont été confirmés par la bonne corrélation obtenue dans la Fig. 1-4 représentant la relation entre log P_{alk} et log k_{90} (facteur de rétention mesuré en présence de 90% d'ACN dans la phase mobile). Ceci implique, que cette méthode peut être utilisée pour la détermination de la lipophilie dans le système alcane/eau de composés polaires jusqu'ici difficilement mesurables.

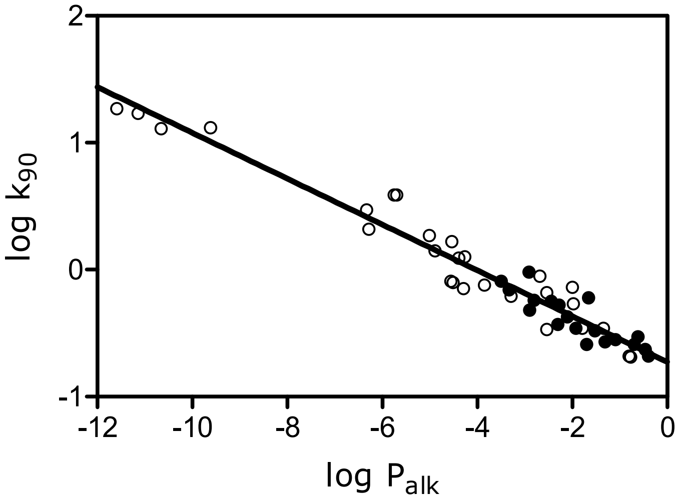

Fig. 1-4 Corrélation entre log k_{90} et log P_{alk} ● Valeurs provenant de [32] ○ Valeurs calculées par le modèle LSER présenté dans [31], la ligne continue représente la régression linéaire donnée par: log k_{90} = -0.18 (± 0.01)· log P_{alk} -0.73 (±0.03); n = 46 ; r^2 = 0.94; s = 0.12; F = 720

Détermination des coefficients de partages dans le système octanol/eau de composés basiques par HILIC

Dans ce chapitre, la technique HILIC a été utilisée pour déterminer la lipophilie de composés basiques, une catégorie de composés difficilement mesurables en phase inverse (RP) avec des phases stationnaires conventionnelles à base de silice. Des études ont montré que la rétention de solutés chargés en mode HILIC est une combinaison complexe de différentes interactions comprenant de la rétention de type hydrophile [33-37], de type RP (hydrophobe) [38,39] et de type échange d'ions [40], en fonction des conditions expérimentales rencontrées. Comme la lipophilie n'est autre qu'une expression de la balance entre hydrophobie et polarité, la particularité de la rétention en mode HILIC a été exploitée pour obtenir des mesures de lipophilie. La différence entre deux valeurs de log k isocratiques (Δlog k_{0-95}) de composés basiques mesurés sous leur forme cationique a été utilisée pour la détermination de coefficients de partage dans le système *n*-octanol/eau.

Une régression linéaire a été obtenue (Fig. 1-5) entre Δlog $k_{0\text{-}95}$ et log $P^N{}_{oct}$ (coefficient de partage de la forme neutre dans le système octanol/eau)

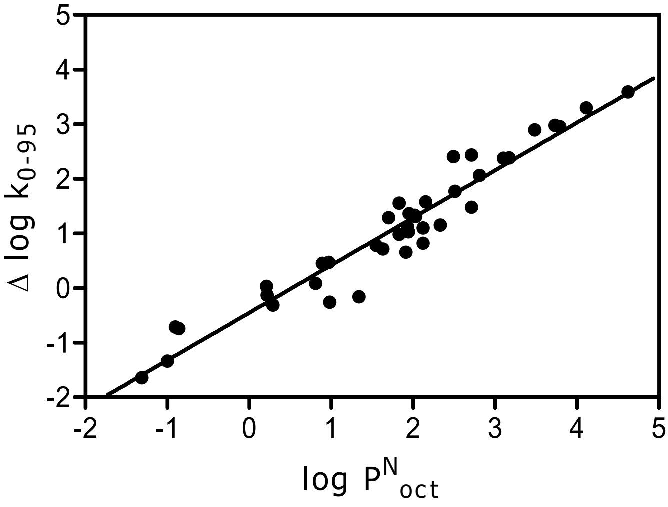

Fig. 1-5: Corrélation entre Δlog $k_{0\text{-}95}$ (phase mobile = acetonitrile-tampon TFA ammonium pH 2 (I = 100 mM) et log $P^N{}_{oct}$.

Il y a cependant quelques précautions à prendre pour mesurer ces facteurs de rétention. A cause du mécanisme d'échange d'ions qui entre en jeu, la mesure des facteurs de rétention a montré être influencée par la composition du tampon aqueux utilisé (type, pH et force ionique). En conséquence, ces résultats impliquent que log k_{95} et log k_0 doivent être mesurés en utilisant un tampon rigoureusement identique pour obtenir des mesures fiables de lipophilie. De plus, une droite de calibration spécifique (Δlog $k_{0\text{-}95}$ *vs* log $P^N{}_{oct}$) sera aussi requise pour chaque type de tampon, pH et force ionique. Cette méthode offre, lorsque la composition de la phase mobile est strictement contrôlée, un moyen prometteur d'obtenir le log P_{oct} de la forme neutre de composés basiques. En outre, le fait de mesurer le temps de rétention de la forme cationique de ces bases, permet d'éviter de travailler à des pH extrêmement élevés, ce qui endommage souvent les phases stationnaires.

Conclusion

Dans ce travail, des solutions intéressantes ont été trouvées pour obtenir des mesures de solubilité proches de la solubilité thermodynamique ainsi que la solubilité dans des milieux intestino-mimétiques au stade de découverte de nouveaux médicaments. Il faut relever que la simplicité de la méthodologie utilisée laisse envisager son extension à la mesure de solubilité dans d'autres milieux caractéristiques des milieux biologiques ou dans des milieux intestino-mimétiques plus complexes, afin de toujours mieux mimer les conditions *in vivo*.

Des solutions ont également été trouvées pour mesurer la lipophilie de composés polaires non ionisés et de composés basiques ayant un $pK_a > 7$ par la technique HILIC. Dans ce cas aussi de nombreux développements sont envisageables notamment pour augmenter la vitesse des mesures en utilisant un développement chromatographique récent, la technique UPLC (Ultra Performance Liquid Chromatography).

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Chapter 2 : Introduction

Pharmaceutical research

Drug research is a complex and time consuming process. From the discovery of new chemical entities (NCEs) to the marketing approval of a new effective drug a period of 12 to 20 years will pass. This process can be divided in two main phases, drug discovery which aims at the discovery of lead compounds and optimization of their properties, and drug development which gathers preclinical and clinical phases. A lead can be defined as a compound with sufficient pharmacokinetic (PK) and pharmacodynamic (PD) potential to progress to a full development program [1]. As represented in Fig. 2-1, the compounds attrition during the whole drug research is extremely high. For about 100'000 to 1'000'000 chemical entities synthesized, about 10 reach the level of clinical testing in healthy volunteers and a single one reaches the market [2,3].

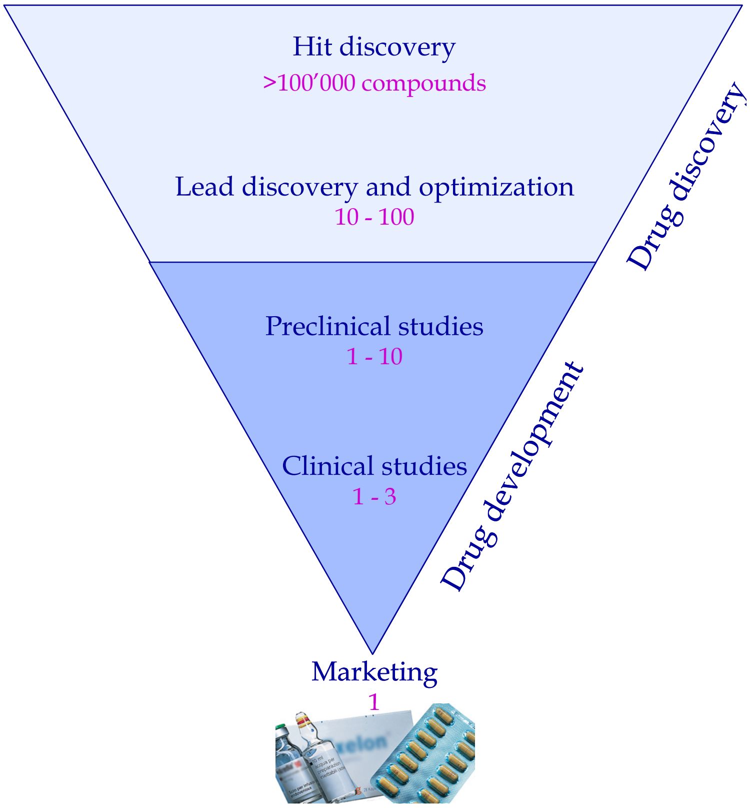

Fig. 2-1 : Drug research process in the 21st century

As inappropriate PK has been recognized as being one of the major factors leading to the retirement of NCEs from drug development [4], there has been great efforts to perform these studies as early as possible in the discovery process, in order that only compounds with high potency and suitable PK properties are selected for development thus avoiding costly late failure [5]. Physicochemical properties can be used as predictors of ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties [6,7]. Compared to *in vivo* ADMET procedures, these properties can indeed be screened at reduced expense, time, and animal usage for an important number of compounds.

Therefore, there has been an increasing interest to develop HTS methods for physicochemical profiling [8]. Despite the well-known advantages of these approaches, the simplicity of these models is getting far from *in vivo* situation. After a background of several years of high throughput physicochemical profiling, many pharmaceutical companies have realized that there was a need for higher quality data, to take more relevant decisions in compounds selection [9]. Among these physicochemical properties, solubility and lipophilicity have shown to be key parameters in the prediction of PK drug behaviour.

Solubility

Solubility is a generic term which is used to describe many different phenomena. In a general sense, solubility can be defined as the amount of a substance that dissolves in a given volume of solvent at a specified temperature. For ionizable compounds, distinction can be done between solubility in unbuffered solvent (water), apparent solubility (at a given pH) and intrinsic solubility (solubility of the neutral form) [9], while for unionizable compounds there are no distinction between these three definitions.

Solubility is a key property for the characterization of NCEs during the whole discovery and development process. Among the commonly used physicochemical filters, poor solubility seems to be the more undesirable compound properties [9] and is one of the major causes of failure during discovery and development, since insufficient solubility can affect results of other *in vitro* assays and interfere with both PD and PK properties.

Experimentally, solubility can be measured either thermodynamically or kinetically. Thermodynamic solubility can be defined as the concentration in solution of a compound in equilibrium with excess solid at the end of the dissolution process, and is often considered as the “true” solubility of a compound. In this process, crystallinity plays an important role, because crystal lattice has to be disrupted for compounds’ solubilization [10].

In contrast, kinetic solubility measures the precipitation of a predissolved compound (in a co-solvent or for ionizable compounds in aqueous media by pH-adjustment) after dilution in a given media. Kinetic solubility measurement is not a substitute for thermodynamic solubility evaluation, because crystal lattice disruption is neglected in this process. Moreover, the solubility is not measured at equilibrium, and the appearance of a precipitate is strongly time dependent [10]. Kinetic solubility values are then likely to overpredict the thermodynamic solubility [9]. The choice of kinetic or thermodynamic solubility measurement will then be dictated by the type of information that is needed and by the quantity of compound available.

Solubility assays in discovery and development must be adapted to phase properties and requirements (Table 2-1). In drug development, high quality solubility data are needed to take appropriate decisions to overcome potential solubility liabilities. Solid state properties (purity, degree of crystallinity, particle size and polymorphism) of the tested compounds are studied and characterized. Compounds are available in large quantities, solubility assays are slow, low throughput and only partially automated. In-depth assays are performed in a few selected solvents to elaborate preformulation strategies for further PK, PD and toxicological studies [11]. Solubility measurements are also performed in biorelevant media to identify *in vitro*/*in vivo* correlations in accordance with the Biopharmaceutics Classification System (BCS) [12,13]. This classification has been developed to identify the fundamental rate-limiting biopharmaceutical factors of intestinal drug absorption, and classifies drug compounds into four classes based on their solubility and intestinal permeability (Fig. 2-2).

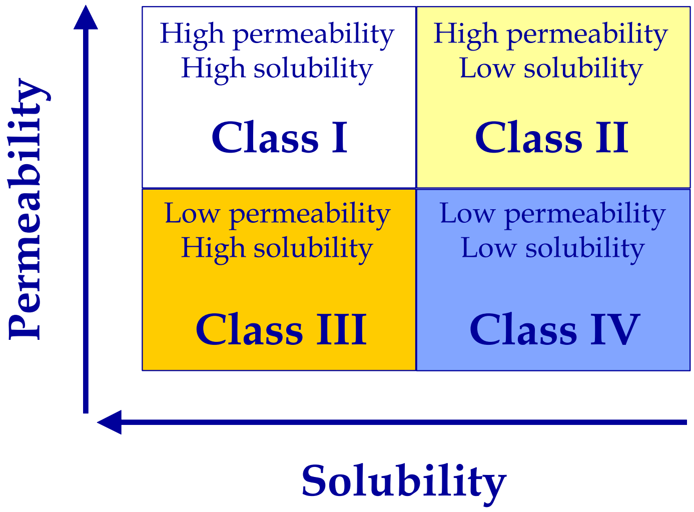

Fig. 2-2 : Schematical representation of the Biopharmaceutics Classification System

This system typically takes as input aqueous solubility measured in buffer media within the whole pH range 1–7.5. In this system, drug compounds are classified as highly soluble, when the highest clinical dose is dissolved in 250 ml aqueous buffer. It has been shown that in the intestine, drug solubility is increased compared to solubility in aqueous buffers due to the presence of bile salts and lecithin. These amphiphilic components can self assemble to form micelles which increases solubility of lipophilic compounds [14,15], but they also act as wetting agents which enhances the dissolution rate of drugs [16]. Moreover, after food intake, the levels of bile salts are considerably increased, which could importantly enhance absorption of poor soluble drugs. Therefore, to evaluate dissolution and solubility in the small intestine, two different *in vitro* intestino-mimetic media have then been developed to simulate the fed (FeSSIF) and fasted (FaSSIF) states conditions, using biorelevant pH values [17] and bile salts concentrations [18]. In these media, sodium taurocholate was chosen as a model bile salt, while lecithin, always cosecreted with bile salts in a ratio of 4:1 [15], was added to better mimic *in vivo* conditions. The use of these media is expensive and dissolution studies of drugs candidates in these *in vitro* intestino-mimetic conditions is generally carried out at advanced stages of drug development.

In discovery, because of the numerous compounds to test and to the weak amount of compounds available, methods must be fully automatable, inexpensive, compound sparing, rapid and high throughput [19]. At this stage useful solubility information would aim two different purposes. The first one would be to evaluate if the compounds generally dissolved in DMSO stay in solution after dilution in the screening media. Incomplete solubility could indeed lead to unreliable results, underestimated activity, reduced HTS-hit rates and inaccurate structure activity relationship (SAR) when not identified [20]. In this purpose, experimental conditions are adapted to bioassays conditions, and solubility values measured are kinetic solubility, and are highly dependant on experimental conditions.

Even if these HTS methods are very useful in this context, solubility data are not intended to be comparable between different kinetic methods involving different experimental conditions [21]. Solubility values generally observed using HTS methods are generally overestimated in a highly compound dependent way compared to thermodynamic solubility. This phenomena has shown to be mainly due to co-solvent effect [22] and formation of supersaturated solutions [23]. Therefore taking kinetic solubilities as input data for ADMET prediction tools should be avoided [9], and HTS methods to obtain solubility data in good agreement with thermodynamic solubility or biorelevant media (as recommended by the BCS) remain also highly desired [12,24].

Table 2-1: Differences between methods used for solubility measurement in drug discovey and drug development processes

	Discovery	Development
Compounds tested		
Number	100-1000	10
Quantity available	Few mg	> g
Purity	Limited	Improved
Solid state	Amorphous or partially crystalline(not characterized)	Stable crystalline material (characterized)
Distribution	Generally DMSO stock solutions	Generally solid form
Methods		
Type of solubiliy measured	Kinetic solubility (dependent on exp. conditions)	Thermodynamic solubility
Throughput	High	Low (25-50 cpds a week)
Automation	Fully	Only partially automated
Format	96-well microplates	Small scale (single tube)
Incubation time	Minutes scales	Hours or days
Detection	UV, turbidity	HPLC-UV, HPLC-MS
Media	aqueous	>20 (aqueous, organic, biorelevant, formulations, excipients,…)
Data generated and intended purpose	Solubility in screening bioassays media (to avoid misinterpretation)	Solubility and dissolution in biorelevant media
	Rank-order hits	Evaluation of formulations
		Characterization and optimization of solid state
		Selection of promising cpds
		Development of adequate strategies to overcome solubility problems

Experimental methods to measure solubility

The shake-flask method is considered as the gold standard procedure in term of solubility measurement. An excess of solid sample is added to the medium in a flask, and the resulting suspension is shaken until equilibrium solubility is reached (generally 24 hours). Thus thermodynamic solubility is obtained by this process. Undissolved sample is then removed by filtration and the concentration of the compound in the filtrate is quantified. Effect of crystal lattice and polymorphic forms, which could significantly affect solubility behaviour, are considered in this process [10]. However, this method is time consuming, requiring large amounts of sample and long equilibration times and cannot be fully automated [25]. Therefore, this method is rather used in advanced studies on the final optimum forms of lead compounds which are often different from the crystal form used at early discovery stage [10].

Pharmaceutical companies are now looking for a HTS version of this method. Recently miniaturized shake-flask methods to determine equilibrium solubility have been described [26,27] aiming to measure thermodynamic solubility using minimum amount of sample (<1 mg). However, the throughput of these methods (up to 30 compounds a week), remains relatively low, especially because accurate distribution of solid compounds into vials or wells of a microplate is still a time consuming and hardly automatable process.

Potentiometric method is the reference approach to determine kinetic solubility [28-30] and is based on pH-metric titration of a saturated aqueous drug solution, using two commercially available systems pSol and CheqSol. Solubility is calculated using the difference between apparent pK_a (pK_a^{app}) determined under precipitation's conditions and aqueous pK_a measured in absence of solid phase. Because potentiometric method allows the measurement of a reliable kinetic solubility without co-solvent, this technique is often used to calibrate high throughput solubility methods and computational procedures [5]. This approach use low amount of compounds, provides a complete solubility pH-profile with a limited number of experiments and therefore allows a better understanding of solubility behaviours throughout the gastro-intestinal tract. However, this technique, suitable for ionisable compounds, needs an accurate knowledge of the pK_a of the molecule to obtain a solubility value and is too slow for screening applications.

Stuart et al. [21] have proposed a simplified version of this methodology named “Chasing Equilibrium”. In this procedure, the rate of change of pH due to compounds precipitation or dissolution while adding strong acid and base titrants is monitored, and the intrinsic solubility of the compounds is determined instead of the entire pH/solubility profile, which is only available using Henderson-Hasselbach relationships.

HTS screening methods for solubility measurement, were designed to early evaluate compounds solubility under screening bioassays conditions, to avoid potential misinterpretation due to incomplete solubility. Considering the large number of compounds to test and the weak amount of sample available at this stage of discovery, small scale (generally 96-well), compound sparing and fully automated methods are necessary. Several HTS methods, in which compounds are introduced as dimethyl sulfoxide (DMSO) stock solutions into aqueous media, have been described. The solubility measured is a kinetic solubility. Two different approaches are commonly used, one by detecting apparition of a precipitate as an indicator of the solubility limit (turbidimetric methods) and the other by removing the precipitates and determining the concentration of compound remaining in solution.

In the turbidimetric method first introduced by Lipinski [10], small aliquots of DMSO stock solutions are added at 1 min intervals to buffer media. The formation of precipitate is determined by measuring light scattering using a nephelometric turbidity detector in the 620-820 nm range. Further adaptations of this methodology were then proposed to keep the DMSO concentration in the buffer constant in the course of addition and to scale down the method in the 96-well plate format [31-34]. The main drawbacks of these approaches are the relatively low sensitivity of the detection, which doesn’t allow the ranking of compounds having very low aqueous solubility (< 20 μM) [22] and the sensitivity to impurities.

In the second approach, small aliquots of DMSO stock solutions are diluted on a filter microplate with aqueous buffer to obtain a final DMSO percentage varying from 1 to 5% (v/v). When compounds present a poor aqueous solubility, precipitation occurs and precipitates are removed by filtration or centrifugation. Solubilities are then determined by measuring compounds' concentrations remaining in solution using UV detection [22,34,35] or HPLC [22,34]. This technique has shown to be more sensitive and less subject to erroneous results caused by impurities than turbidimetric method [22]. Undissolved material can also be removed by centrifugation instead of filtration. According to Alsenz et al. [9], both methods give comparable results. Nevertheless, divergent results can be observed for hydrophobic compounds due either to non specific adsorption on filter material or, in the case of centrifugation, to sample floating on the surface of the solvent. However, filtration through filter microplates is faster than centrifugation [9].

The limitations associated with the determination of kinetic solubility have encouraged searchers to find solutions to measure thermodynamic solubility during discovery process to obtain high quality solubility data early on [9,36]. A solution was to evaporate the solvent before the start of the assay, thus giving the possibility to measure thermodynamic solubility, with the advantages of compounds distribution via standardized stock solutions. Various protocols using DMSO [37], ACN [38], MeOH/DME [39] as solvent for stock solutions have been described. Another approach would be to adapt standard protocols of kinetic solubility measurements to minimize effect of predissolution in DMSO. This methodology will be exhaustively developed in chapter 3.

Lipophilicity

Lipophilicity has shown to be a fundamental property to predict both PK (membrane permeability, tissue distribution, protein binding, metabolism) and PD (binding affinity) properties of drugs.

Lipophilicity is commonly expressed as the logarithm of the partition coefficient (P) between water and an immiscible organic solvent. The most commonly used biphasic system is *n*-octanol/water first introduced by Hansch [40]. The octanol/water partition coefficient ($\log P_{oct}$) was indeed one of the first model of biological membrane permeation, due to the structural analogy of *n*-octanol with the phospholipids found in membranes (a polar group and a long alkyl chain). However, $\log P_{oct}$ appeared to be not sufficient to model permeation through the numerous types of biological membranes [41]. It has been suggested that simultaneous analysis of four different biphasic systems could give more complete information for SAR studies [41]. Those four solvents composed of an amphiprotic solvent (*n*-octanol), an inert one (*n*-alkane), a hydrogen-bond donor (chloroform) and a hydrogen-bond acceptor (di-*n*-butyl ether) have been called "critical quarter" [42] and has shown to be a valuable descriptor to model the partitioning of solutes into membranes [43,44], even if some authors consider that, alongside the standard octanol/water partition coefficient, the alkane/water system is the only system that can be successfully used in ADMET predictions, because of its completely different nature from octanol/water [45].

The gold standard procedure to measure lipophilicity is the shake-flask method. Accuracy of partition coefficient measurements using this technique strongly depends on experimental conditions such as precision of phase volume ratio, mutual saturation of the phases, nature of the buffers and ionic strength, temperature, solute solubility and purity, formation of microemulsions [46]. Moreover, the shake-flask method suffers from its relatively high time and compound consumption and from a number of practical limitations such as solute self association or degradation. However, this method can be used to measure partition coefficients in different biphasic solvent systems and the log P range measurable by this technique is approximately –3 to +3 [47,48].

Due to practical disadvantages and the limitation of the range of log P measured by the shake-flask method [47,48], reversed-phase liquid chromatography (RPLC) was largely used as an alternative to shake-flask for the measurement of log P_{oct}, as illustrated by many well documented reviews published on this subject [49-54]. This approach is based on the partitioning of the solute between a polar mobile phase and an apolar stationary phase. The retention is then expressed as the retention factor (log k) given by Eq. 2.1 :

$$\log k = \log\left(\frac{t_r - t_0}{t_0}\right) \qquad \text{Eq. 2-1}$$

where t_r and t_0 are the retention time of the solute and of an unretained compound respectively. Depending on the stationary phase used, retention parameters can be correlated to log P_{oct} values by Eq. 2.2 :

$$\log P_{oct} = a \cdot \log k + b \qquad \text{Eq. 2-2}$$

where log k is the retention factor of the solute, log P_{oct} the partition coefficient of the solute, a and b the linear regression constants.

The main advantages of these methods are their better throughput compared to shake-flask method, the small amounts of compounds required, and their ability to separate impurities or degradation products which might affect partition results.

However, these methods are not appropriate for the determination of partition coefficients of polar compounds (log P < -1) and are generally limited to the determination of log P in octanol/water even if a stationary phase was reported to be able to determine log P in alkane/water systems [55]. Furthermore, solutes, in particular basic compounds can interact with free residual silanols using conventional silica-based stationary phases, and can cause peak tailing and incorrect estimation of lipophilicity [56,57]. Another drawback of traditional silica based stationary phases is their chemical instability at pH above 8, making impossible the determination of partition coefficients of basic compounds with pKa > 7 under their neutral form [58-61], even if new strategies allow modern stationary phases to be resistant to a broader pH-range.

The potentiometric method also allows the determination of the lipophilicity profile of ionizable compounds from two acid-base titrations, one in a biphasic sytem (water/organic solvent) and the other in pure aqueous phase [62,63]. The main advantages of the potentiometric method is the possibility to use a large variety of partition solvents and to measure an extensive range of log P values (-0.5 to +8) [64]. The major limitations of potentiometry concern unionizable compounds and hydrophilic solutes and its sensibility to impurities. The throughput of this method also remains relatively low.

Finally the lipophilicity of drug ions can be determined by cyclic voltammetry at the interface between two immiscible electrolyte solutions (ITIES) [65-69]. In this method, a cyclic potential is imposed by the use of two reference electrodes between two immiscible electrolyte solutions. The polarization of the interface induces the movement of ions. The resulting current is measured with two counter-electrodes. Analysis of the current response can give information about the thermodynamics, kinetics, and mechanisms of the ion-transfer reactions across the liquid-liquid interface. This method requires the presence of a polarizable interface, characterized by the absence of ion exchange between the two phases. Therefore, the *n*-octanol/water system is inadequate. In a polarizable interface, at least one of the ions is present simultaneously in both phases and can then transfer easily through the interface. Given its interesting properties, the system usually used is 1,2-dichloroethane/water [70].

At thermodynamic equilibrium, the distribution of an ion is determined by the equality of its electrochemical potentials in the two phases and is given by the Nernst equation at the ITIES, and the standard Gibbs energy of transfer is related to the partition coefficient in a straightforward manner. Then log P^I (logarithm of the partition coefficient of an ionized form) of drugs can be deduced [69]. The major drawback of cyclic voltammetry is the limited number of solvent systems to which it can be applied.

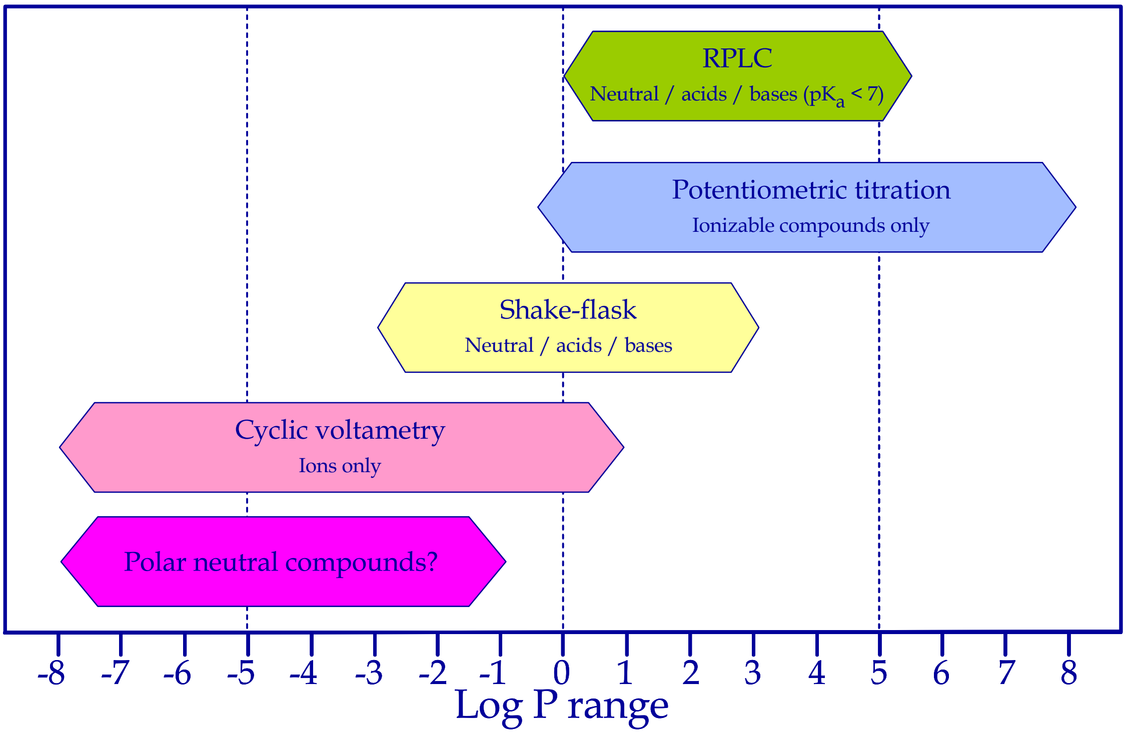

Fig. 2-3 : Approximate log P range of experimental methods to measure lipophilicity

An overview of the log P range measurable by the most commonly used techniques to determine lipophilicity is represented in Fig. 2-3. The only reported approach to obtain experimental partition coefficients of polar neutral compounds was a method mixing experimental lipophilicity measurements and fragmental addition (by structure analogy) [71]. Therefore, methods to measure with a good confidence level the lipophilicity of very polar non ionic compounds (log P < -3) are still missing [71] and would be of great utility.

The methods mentioned above generally suffer from a relatively low/medium throughput. In response to the growing interest to screen lipophilicity as early as possible in drug discovery process, different strategies have been elaborated to speed up lipophilicity measurements using RPLC methods. Valko et al. [72] have for example developed a fast gradient RPLC procedure based on a chromatographic hydrophobicity index (CHI). With this approach, log P could be determined in the range -0.45 < log P < +7.3. However, relatively poor correlations with log P_{oct} were obtained.

Attempts were also made to use short chromatographic columns [73-76]. Despite the resulting degradation of chromatographic performance registered, this approach resulted in significant decrease in analysis times. Furthermore, the use of shorter columns in combination with compatible polar embedded stationary phases also enabled the direct measurement of retention factors in pure aqueous mobile phases instead of a mixture of water and cosolvent [77]. This direct determination allowed a considerable time saving by the measurement of a single retention factor by solute as opposed to the minimum four measures necessary when the extrapolation method is employed.

RPLC methods to measure lipophilicity have also benefit from the recent emergence of UPLC (Ultra Performance Liquid Chromatography) which combines the use of short columns packed with small particles (<2 μm) and very high-pressure conditions (>5800 psi) [78]. These particular conditions allowed a significant analysis time reduction without compromising chromatographic performance and have shown to be a fast reliable way to screen lipophilicity [79].

Concurrently to these developments in chromatographic methods, there has also been attempts to develop automated shake-flask procedure in 96-well plates [80-82]. Despite the small volumes required, the use of automated liquid handler allow a similar accuracy than the traditional shake-flask method within an improved throughput compared to traditional shake-flask method.

Another promising high throughput technique first described by Faller et al. [83] was designed to measure octanol/water partition coefficients in 96-well microplates using immobilized artificial membranes. In this technique based on the PAMPA technology [84], octanol/water partition coefficients are derived from compounds diffusion between two aqueous compartments separated by a thin octanol liquid layer. This methodology has shown to be able to measure log P_{oct} in good agreement with shake-flask method within the range of -2 to +8 and can be extended to other solvent systems.

Another approaches based on solid phase microextraction (SPME) have been applied by several authors to estimate octanol/water partition coefficients [85-90]. These techniques typically performed in a 96-well format, have achieved satisfactory correlations with $\log P_{oct}$. An innovative strategy was also developed by Gao et al [91] using porous magnetic nanoparticules able to absorb *n*-octanol, and measure the partition coefficients of organic compounds between the solid supported *n*-octanol nano-droplets and the bulk aqueous phase. The small sample requirements using this miniaturized method make it suitable for high throughput log P screening.

For an explicit enumeration of methods applicable at high throughput level, capillary electrophoresis (CE) have to be mentioned using 96-capillary multiplexed instruments [92-94], but will not be covered here considering the recent excellent review published by Martel et al. [54].

Aim of thesis

This work was devoted to the development of rapid and reliable experimental tools for the measurement of solubility and lipophilicity. Considering the growing interest for having high quality solubility data in the early stages of drug discovery [9], a 96-well HTS UV method was adapted to obtain solubility values close to thermodynamic solubility measured by shake-flask method. Therefore, experimental parameters such as shaking intensity, incubation time and DMSO percentage were studied and optimized in this purpose.

Biorelevant solubility values are also more and more required in discovery to early evaluate compounds bioavailability and absorption after oral administration. Thus the optimized HTS UV method was also adapted to measure solubility directly in two different *in vitro* intestino-mimetic media commonly employed to simulate the fed (FeSSIF) and fasted (FaSSIF) states conditions.

In the second part of this work, hydrophilic interaction chromatography (HILIC), a technique retaining polar and charged compounds, was evaluated as a new approach to measure lipophilicity of polar non ionic and basic compounds, two categories of solutes hardly measurable by conventional methods to measure lipophilicity. For polar compounds, the retention factors (log k) were measured using high proportions of ACN in the mobile phase and a linear solvation energy relationship (LSER) analysis was performed to elucidate the intermolecular forces controlling the retention in HILIC. The model obtained was then compared to LSER models of partition coefficients in different biphasic solvent systems. For basic compounds measured under their cationic forms, the particularity of HILIC retention (a complex combination of hydrophilic, hydrophobic and ion exchange retention) was exploited to obtain octanol/water partition coefficients using two diametrically opposite mobile phase compositions (i.e. 0% and 95% (v/v) of ACN in the mobile phase)

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Chapter 3 : High Throughput UV Method for the Estimation of Thermodynamic Solubility and the Determination of the Solubility in Biorelevant media

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Abstract

The growing interest for high quality solubility data in the early stages of drug discovery suggested a detailed optimization of experimental conditions for a 96-well HTS UV method in order to obtain solubility values close to thermodynamic solubility measured by shake-flask method. Results have shown that solubility data obtained by the HTS approach were highly dependent on shaking intensity and incubation times due to the formation of supersaturated solutions resulting from the dilution of DMSO stock solutions in aqueous buffer. Thus careful experimental set-up was developed to improve the quality and the reproducibility of the HTS method. Moreover, the early qualitative prediction of bioavailability and absorption of orally administered drugs require more and more biorelevant solubility values in drug discovery programs. Thus the optimized HTS method was also adapted to measure solubility directly in FaSSIF and FeSSIF media. The versatile HTS UV approach presented in this paper provides an unique and reliable way to determine solubility in various experimental conditions.

Introduction

Solubility is an essential physicochemical property that has to be evaluated during the drug discovery and development process [1-3]. Experimentally, solubility can be measured either thermodynamically or kinetically. Thermodynamic solubility can be defined as the concentration in solution of a compound in equilibrium with an excess of solid material at the end of the dissolution process, and is often considered as the "true" solubility of a compound. The thermodynamic solubility is generally measured using the shake-flask method which is considered as the gold standard procedure. However, this labour intensive method require large amounts of sample and long equilibration times and cannot be fully automated [4]. Kinetic solubility considers the precipitation after dilution in a suitable solution of a compound predissolved in a co-solvent or in aqueous media by pH-adjustment for ionizable compounds. In drug discovery, kinetic solubility is the more relevant parameter to rapidly measure, to evaluate if the compounds, generally predissolved in dimethyl sulfoxide (DMSO), stays in solution after dilution in specific screening media. If unidentified, incomplete solubility could indeed reduce HTS-hit rates and lead to unreliable results, such as underestimated activity or inaccurate structure activity relationship (SAR) [5]. Therefore, several high throughput screening (HTS) methods for kinetic solubility measurements, have been developed to evaluate solubility in a manner as close as possible to the experimental conditions encountered in screening bioassays.

One pioneering approach is the turbidimetric titration first introduced by Lipinski [6] and further adapted by other authors [7-9]. With this method, the precipitation point is determined turbidimetrically by a laser nephelometer. The main drawback of this approach is its relatively low sensitivity, which does not allow the ranking of compounds having very low aqueous solubility (< 20 μM) [10].

Recently an alternative HTS approach for solubility determination was described using 96-well filter microplates [9-11]. Small aliquots of

DMSO stock solutions were diluted on a filter microplate with aqueous buffer. The precipitate which appeared for poorly soluble compounds was removed by filtration and the amount of compound still in solution was quantified by UV detection. This technique has shown to be more sensitive and less subject to erroneous results caused by impurities compared to the turbidimetric method [10]. However, methods requiring compound predissolution in a co-solvent, lead to solubility values higher than thermodynamic ones, this increment being highly compound dependent, whereas thermodynamic solubility data are more and more required during discovery process to obtain high quality solubility data to prevent unsuitable compounds to enter development process [2,12]. Solubility enhancement using HTS methods has shown to be mainly due to both co-solvent effect [10] and formation of supersaturated solutions [13]. The supersaturation phenomenon is due to the dissolution of a high quantity of compound in DMSO before its dilution in aqueous media, while the co-solvent effect is only due to the changes of the media physicochemical properties. Then minimizing both of these effects could provide more pertinent solubility data for drug development. In this purpose, experimental parameters such as shaking intensity, incubation time and DMSO percentage were studied and optimized.

Biorelevant solubility values are also more and more required in the early discovery to predict qualitatively bioavailability and absorption of orally administered drugs in accordance with the Biopharmaceutics Classification System" (BCS) [14-16]. A valuable strategy was recently proposed to enhance BCS ranking of drug candidates using the solubility [17]. In this approach aqueous solubility was coupled to the dose (dose/solubility ratio) in order to predict key steps involved in gastro-intestinal absorption like mean dissolution time and food effect [18]. In relation with dissolution and solubility in the small intestine, two different *in vitro* intestino-mimetic media containing bile salts and lecithin were already employed to simulate the fed (FeSSIF) and fasted (FaSSIF) states conditions [19]. It has been shown that in these conditions drug solubility was increased compared to solubility in aqueous buffers since these amphiphilic components can self assemble to form micelles which increases solubility of lipophilic compounds [20-22] and also may act as wetting agents which enhances the dissolution rate of drugs [22]. Thus the derivation of dose/solubility ratio using FeSSIF or FaSSIF media may offer enhanced BCS predictions especially if a fast method to perform such measurements is available. Indeed these media are expensive, usually used with the tedious shake-flask method and solubility measurements in such biomimetic media were generally carried out at advanced stages of drug development.

A HTS UV method would provide an opportunity to provide reliable intestino-mimetic solubility in drug discovery. First, DMSO as unique adjuvant was evaluated as a substitute to taurocholic acid and lecithin contained in FaSSIF and FeSSIF media in order to lower the costs of HTS experiments. However DMSO and bile salts play a different solubilizing role, the use of FeSSIF and FaSSIF media in the optimized HTS UV method was then proposed as an alternative to the traditional shake-flask in intestino-mimetic solubility measurements.

Materials and methods

Chemicals

All compounds were purchased from commercial sources. Carbamazepine, β-estradiol, diclofenac sodium, digitoxin, dienestrol, flurbiprofen, glybenclamide, ibuprofen, ketoconazole, ketoprofen, naproxen, nifedipine, phenytoin, pindolol, piroxicam, prednisone, propranolol hydrochloride, spironolactone, terfenadine and warfarin were purchased from Sigma (Buchs, Switzerland). Chloramphenicol, chlorpromazine hydrochloride, estrone, indomethacin, nalidixic acid, testosterone and verapamil hydrochloride, were obtained from Fluka (Buchs, Switzerland). Progesterone was purchased from Acros (New Jersey, USA), diazepam from Bufa (Uitgeest, Netherlands) and promethazine hydrochloride from ICN (Aurora, USA).

Acetonitrile, phosphoric acid and formic acid were of analytical grade and were supplied by Merck (Darmstadt, Germany). DMSO was supplied by Aldrich (Buchs, Switzerland). Boric acid, acetic acid, sodium hydroxide, potassium dihydrogen phosphate and potassium chloride were supplied by Fluka. Sodium taurocholate was purchased from Sigma. Egg-phosphatidylcholine lipid EPC was supplied by Lipoid (Ludwigshafen, Germany).

HTS UV solubility measurements

General method

The pH of buffer solutions were chosen according to the pK_a of studied compounds (Table 3-1), to measure the intrinsic solubility S_0 (solubility of neutral solutes and of the neutral form of ionisable compounds). Phoebus software v1.0 (Sedere, Centre Analyse, Orléans, France) was used to prepare standard buffers varying from pH 2 to 12 with a fixed ionic strength of 20 mM (phosphoric acid / sodium hydroxide pH 2-3, formic acid / sodium hydroxide pH 3.5-4, acetic acid / sodium hydroxide pH 4.5-5.5, phosphoric acid / sodium hydroxide pH 6-8, boric acid / sodium hydroxide pH 8.5-10 and phosphoric acid / sodium hydroxide pH 10.5-12).

The pipetting operations described below were all automated using a Precision 2000® 96 Well Automated Microplate Pipetting System (Witec AG, Littau, Switzerland) except for small volumes of DMSO stock solutions, which were dispensed using an Eppendorf Multipette plus system (Vaudaux – Eppendorf, Schönenbuch, Switzerland).

Two different 96-well filter plates were tested for non specific sample adsorption: Multiscreen solubility filter plate (MSSLBPC10, Millipore AG, Volketswil, Switzerland) with an hydrophilic polycarbonate (PC) filter membrane (pore size 0.45 μm, thickness 17-22 μm), and Multiscreen GV (MAGVN2210, Millipore AG, Volketswil, Switzerland) with an hydrophilic PVDF filter membrane (pore size 0.22 μm, thickness 125 μm).

To obtain saturated solutions after dilution in aqueous buffer, 50 mM stock compound solutions in DMSO were prepared. For the most soluble compounds, DMSO stock solutions having concentrations up to 200 mM were needed under certain conditions to obtain a quantitative value of solubility. Two 96-well microplates were then prepared namely a filter plate for the solubility assay and a 96-well home-made Teflon microplate for compound standard calibration. Small aliquots of DMSO stock solutions (1 µl (0.5%) 2 µl (1.0%) 10 µl (5.0%) and 20 µl (10.0% (v/v) of DMSO)) were then dispensed directly on filter and Teflon microplates and diluted with respectively aqueous buffer and acetonitrile to a final volume of 200 µl. Therefore, nominal compound concentrations varied from 500 to 3000 µM in both plates.

Filter plates were shaken at room temperature on an orbital shaker (Heidolph Titramax 1000®, Schwabach, Germany) at varying shaking rates to evaluate the influence of shaking on the solubility values. Effect of incubation time was assessed by shaking during 1.5 and 24 h. Microplate's content was then filtered on Multiscreen Vacuum Manifold, (MAVM0960R, Millipore AG, Volketswil, Switzerland) by application of a weak vacuum of 10 mm Hg, and collected in a 96-well home-made Teflon microplate.

Filtrate and standard solutions were diluted according to Table 3-2. Finally, 200 µl of these solutions were transferred in two different 96-well quartz microplates (Hellma, Basel, Switzerland) and full blank-corrected absorption spectra (200-400 nm) were recorded with a microplate Scanning Spectrophotometer (Powerwave, Witec AG, Littau, Switzerland). Then compounds were quantified at their λ_{max}. For each compound, solubility assay was repeated 6 times in the same plate.

Table 3-1:Physicochemical properties of the studied compounds

Compounds	**log P_{oct}**	**pK_a**		**pH of measure**	**λ detection [nm]**	**ε [$mol^{-1} \cdot cm^{-1}$] a)**
Chlorpromazine	5.20 b)	9.24 h)	B	12	256	31811
Diclofenac	4.40 c)	3.99 h)	A	2	276	10252
Estradiol	4.01 c)	-	N	3.7	280	1778
Flurbiprofen	3.81 d)	4.21 d)	A	2	246	17701
Ibuprofen	3.87 d)	4.31 d)	A	2	264	267
Indomethacine	4.27 d)	4.42 d)	A	2	260	14485
Ketoprofen	2.77 d)	4.25 d)	A	2	256	3871
Nalidixic acid	1.86 e)	6.41 j)	A	2	256	22748
Naproxen	3.06 d)	4.18 d)	A	2	272	4402
Phenytoin	2.68 f)	7.94 f)	A	2	252	745
Pindolol	1.83 g)	9.54 g)	B	12	270	4899
Piroxicam	1.98 h)	2.33 ; 5.07 h)	AB	3.7	360	3964
Progesterone	3.87 c)	-	N	3.7	244	13864
Promethazine	4.14 b)	8.62 b)	B	12	306	3800
Propranolol	3.48 b)	9.53 h)	B	12	292	5403
Testosterone	3.32 c)	-	N	3.7	244	13813
Verapamil	3.79 i)	9.07 i)	B	12	280	4929
Warfarin	2.70 g)	4.82 g)	A	2	282	10584

a) Measured molar extinction coefficients at the selected wavelength in 50% ACN / 50% aqueous buffer (v/v), average value N = 6; b) Taken from [23]; c) Taken from [24]; d) Taken from [25]; e) Taken from [26]; f) Taken from [27]; g) Taken from [28]; h) Taken from [29]; i) Taken from [30]; j) Taken from [31].

Table 3-2 : Dilutions of filtrates and standard solutions prior to UV analysis

	Filtrate		Standard	
	Dilution 1	**Dilution 2**	**Dilution 1**	**Dilution 2**
Filtrate	100 µl	50 µl	–	–
Standard solution	–	–	100 µl	50 µl
Aqueous buffer [a)]	–	50 µl	100 µl	100 µl
Acetonitrile [a)]	100 µl	100 µl	–	50 µl

[a)] Containing the corresponding DMSO fraction (0.5, 1.0 or 5.0% v/v)

HTS solubility measurement in intestino-mimetic media

DMSO stock solutions were first diluted in aqueous buffers without bile salts and were shaken on an orbital shaker at 1200 rpm during 24 h. After this incubation time, 1% and 5% (v/v) of a concentrated solution of sodium taurocholate (0.3 M) and lecithin (0.075 M) were added to give final bile salt concentrations present in respectively FaSSIF and FeSSIF media as indicated in Table 3-3. Saturated solutions were further incubated during 8 h before filtration, to ensure the micellization process to be completed. Filtrate was then diluted with 50% (v/v) of methanol and 200 µl of each solution were transferred in a 96-well quartz microplate for UV analysis.

Solubility measurement by shake-flask method

An excess of sample was added to 2 ml of aqueous buffers (Table 3-3) and the resulting suspension was shaken at room temperature for 24 h on a rotary shaker to reach equilibrium solubility. Saturated solutions were filtered on PC filter plates. Finally, 100 µl of filtrates were transferred in the quartz microplate and were diluted by adjunction of 1 µl of DMSO and 99 µl of acetonitrile (or methanol when bile salts were present). The solubility was determined using UV detection and comparison with calibration standards prepared as previously described for the 96-well method. Experiments were done in triplicate for each compound.

Table 3-3: Composition of intestino-mimetic media and their corresponding aqueous buffer without bile salts

Intestino-mimetic media			
FaSSIF		**FeSSIF**	
pH	6.5	pH	5
Sodium taurocholate	3 mM	Sodium taurocholate	15 mM
Lecithin	0.75 mM	Lecithin	3.75 mM
KH2PO4	3.9 g	KCl	15.2 g
KCl	7.7 g	Acetic Acid	8.65 g
NaOH	qs pH 6.5	NaOH	qs pH 5
Purified water	qs 1 l	Purified water	qs 1 l
Aqueous media			
Aqueous medium pH 6.5		**Aqueous medium pH 5**	
pH	6.5	pH	5
KH2PO4	3.9 g	KCl	15.2 g
KCl	7.7 g	Acetic Acid	8.65 g
NaOH	qs pH 6.5	NaOH	qs pH 5
Purified water	qs 1 l	Purified water	qs 1 l
HTS intestino-mimetic media			
HTS FaSSIF		**HTS FeSSIF**	
Aqueous medium pH 6.5	196 µl	Aqueous medium pH 5	188 µl
DMSO stock solutions	2 µl	DMSO stock solutions	2 µl
Solution 0.3 M sodium taurocholate, 0.075M lecithin	2 µl	Solution 0.3 M sodium taurocholate, 0.075M lecithin	10 µl

Results and discussion

General considerations

PC and PVDF filters were tested in solubility measurements, and microplates with PC membranes were then chosen since the non specific adsorption of analytes was significantly lower than with PVDF filter (data not shown).

Standard solutions were prepared in mixtures of acetonitrile and buffer (50/50, v/v) to ensure complete solubilization of compounds at tested concentrations. To compare standard solutions and filtrates in the same conditions, dilutions of filtrates with an equal volume of acetonitrile were realized. The original protocol [11] recommended to prepare standard solutions and dilute filtrates with a final volume of 20% of acetonitrile. However, to increase the upper solubility limit of the method keeping the same DMSO stock solutions concentrations for all tested compounds, more concentrated stock solutions than in original protocol were used. Therefore, for very poorly soluble compounds as chlorpromazine, 20% of acetonitrile was not sufficient to avoid precipitation of standard solutions in the tested conditions. The solubility of this compound has been tested using 20, 30, 40 and 50 % acetonitrile in buffer and results showed that 50% (v/v) of acetonitrile was the minimum required to solubilize the standard solution at the tested concentrations.

During the experiment, evaporation could occur. The general effect of the evaporation will be to generate more concentrated solutions but the outcome in term of solubility will not be altered. For the highly soluble compounds, the measured concentration at the end of the incubation time will be higher than initial concentration, meaning that the compound is totally soluble. For poorly (weak) soluble compounds, the concentrated solution resulting from the evaporation is balanced by precipitation because solutions are already saturated. Futhermore, the loss of volume resulting from evaporation is also not a problem, because only half of the filtrate is pipetted after filtration and used for solubility determination.

Because an influence of storage was observed for some compounds DMSO stock solutions [32], they were never kept longer than 1 week.

By care of a better automation, some HTS methods using UV detection propose a dosage at one [9] or six fixed wavelengths [10]. If the compound of interest is not pure, which is often the case at early discovery stage of drug discovery [29,33], detection at fixed wavelengths increases the risk of considering impurity absorbance, which can affect accuracy of solubility determination. The detection at λ_{max} was then chosen in this study.

Optimization of experimental conditions for HTS solubility measurements

Solid phases generated by precipitation are often metastable and can lead to the formation of supersaturated solutions with slow precipitation kinetics [34]. Therefore, HTS solubility measurements should be dependent on shaking intensity, incubation time and DMSO percentages.

Influence of shaking and incubation time in solubility measurements

Effect of shaking intensity in presence of 1% (v/v) of DMSO and after an incubation time of 1.5 h was evaluated by comparing solubility values obtained for a series of compounds (with varying solubility) using a 300 rpm (recommended in original method [11]) and a 1200 rpm orbital shaking. As shown in Table 3-4, solubility values obtained using an orbital 300 rpm shaking were higher than the solubility measured using the more vigorous shaking, in a compound dependant way. For piroxicam and warfarin, 50-fold and 27-fold higher solubility values were respectively registered, whereas no significant change was observed for pindolol or propranolol. In a general way, it was observed that solubility of the most soluble compounds tend to slightly differ by increasing shaking rate during an incubation time of 1.5 h, and solubility values obtained using 300 rpm and 1200 rpm remained higher than thermodynamic values. At the opposite, solubility of the less soluble compounds was generally decreased in an important way when a vigorous shaking was applied. Using a 1200 rpm orbital shaking, thermodynamic solubility was already reached after 1.5 h for this class of compounds.

Effect of equilibration time in presence of 1% of DMSO (v/v) and using a 1200 rpm shaking was also assessed by comparing solubility measured after incubation times of 1.5 and 24 h (Table 3-4). A significant decrease in solubility between 1.5 and 24 h was observed especially for compounds having a high solubility (ibuprofen, ketoprofen, pindolol and propranolol), providing values closer to thermodynamic ones. As mentioned above, equilibrium solubility was apparently reached after 1.5 h for poor soluble compounds, therefore only slight changes were registered after 24 h.

HTS solubility data being reduced by application of vigorous shaking and long equilibration times, thus giving results closer to thermodynamic solubility, clearly indicates that dilution of DMSO stock solutions into aqueous buffer lead to the formation of unstable supersaturated solutions. Supersaturation has shown to be minimized by increasing orbital shaking rate to 1200 rpm. However, for high soluble compounds, an incubation time of 1.5 h, even under strong shaking, was not sufficiently effective to induce precipitation of supersaturated solutions, and an equilibration time of 24 h was necessary to obtain results closer to shake-flask solubility values. At the opposite, for poor soluble compounds only slight changes were registered when incubation time was enhanced to 24 h, because a period of 1.5 h strong shaking was generally sufficient to induce the precipitation. These results can be partially explained by the degree of saturation (DS: compound's effective concentration in solution at the beginning of the assay, divided by its solubility at equilibrium) of the solutions. Compounds with high shake-flask solubility form solutions having a small degree of saturation and consequently lead to more stable supersaturated solutions. For the poor soluble compounds, dilution of concentrated DMSO stock solutions into buffer results in highly unstable supersaturated solutions having high DS.

Table 3-4 : Solubility measured by shake-flask method and influence of shaking intensity and incubation time in HTS solubility measurement in presence of 1 % DMSO. Data are presented as mean solubility [µg/ml] ± SD (N=6)

			$S_0^{1\%}$ (HTS), 1.5 h incubation time				**$S_0^{1\%}$ (HTS), 24 h incubation time**	
Compounds	**S_0 Shake-flask**		**Shaking 300 rpm**		**Shaking 1200 rpm**		**Shaking 1200 rpm**	
Chlorpromazine	0.9	± 0.1	1.0	± 0.1	0.5	± 0.2	0.6	± 0.1
Diclofenac	1.1	± 0.1	10.0	± 0.3	0.6	± 0.2	0.8	± 0.1
Estradiol	2.3	± 0.1	7.0	± 0.4	1.9	± 0.1	0.9	± 0.2
Flurbiprofen	7.4	± 0.5	53.4	± 1.1	10.0	± 0.8	6.6	± 0.5
Ibuprofen	49.5	± 1.3	179.7	± 1.8	115.5	± 4.1	50.2	± 0.8
Indomethacine	0.9	± 0.2	20.1	± 0.3	0.8	± 0.1	1.0	± 0.2
Ketoprofen	84.5	± 2.7	320.1	± 4.9	236.5	± 7.9	90.9	± 4.6
Nalidixic acid	19.4	± 0.8	29.9	± 0.3	25.8	± 0.8	17.5	± 0.6
Naproxen	18.0	± 0.6	35.2	± 0.9	19.4	± 0.9	16.4	± 0.7
Phenytoin	26.3	± 1.0	42.8	± 1.1	21.8	± 1.2	21.8	± 1.4
Pindolol	46.8	± 0.7	160.3	± 2.3	155.7	± 4.5	59.0	± 2.5
Piroxicam	5.5	± 0.4	240.0	± 5.7	4.6	± 0.5	4.2	± 0.4
Progesterone	5.8	± 0.2	45.4	± 0.5	11.4	± 0.6	6.4	± 0.6
Promethazine	9.2	± 0.6	16.4	± 0.6	11.2	± 0.1	3.5	± 0.4
Propranolol	74.7	± 4.3	156.3	± 15.9	167.0	± 18.3	74.2	± 0.7
Testosterone	19.1	± 1.4	81.3	± 1.4	32.1	± 8.1	18.2	± 1.2
Verapamil	7.2	± 0.2	52.2	± 0.8	25.1	± 1.1	13.3	± 0.4
Warfarin	4.8	± 0.3	125.6	± 1.3	4.7	± 0.9	3.8	± 0.7

The effect of DS on the precipitation of supersaturated solutions is illustrated for ketoprofen by diluting 1% (v/v) of DMSO stock solution at three different concentrations of ketoprofen (340, 170 and 80 mM) into aqueous buffer (Fig. 3-1). Solubility was then measured immediately after dilution (t = 5 min), to evaluate concentration at the beginning of the assay, and after an incubation time of 1.5 h and a 1200 rpm shaking. As expected, precipitation of supersaturated solutions was facilitated when degree of saturation increased. Therefore, to obtain solubility values as close as possible to thermodynamic ones, enhancing the degree of saturation should be a way to minimize incubation time. However, DS is not the more judicious parameter to change to make supersaturated precipitate, especially for highly soluble compounds, which would require very concentrated DMSO stock solutions, and consequently important amounts of compounds, which is not feasible in drug discovery process.

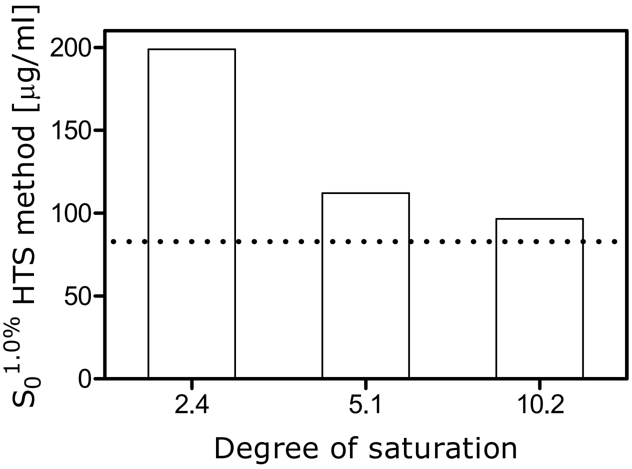

Fig. 3-1 : Effect of degree of saturation DS (concentration in solution at the beginning of the assay divided by the solubility measured after the incubation time) on ketoprofen's solubility measured with UV HTS method using 1% (v/v) of DMSO, an incubation time of 1.5 h and a 1200 rpm shaking.

Influence of DMSO percentage in solubility measurements

It has already been reported that the presence of as little as 0.5% (v/v) of DMSO can enhance aqueous solubility to an unknown extent [29,32,34-36] due to both co-solvent effect and formation of supersaturated solutions. In order to study the effect of both parameters on solubility, this study was conducted at varying commonly used DMSO percentages (0.5% [1], 1% [10,37,38], 5% [11,12,39] (v/v)) in both conditions, 1.5 h at 300 rpm and 24 h at 1200 rpm orbital shaking. The solubility values obtained were compared to solubility values measured by shake-flask method without DMSO (Fig. 3-2). The following correlations between S_0 obtained by shake-flask method and S_0 measured with HTS UV method using different experimental conditions were obtained by linear regression.

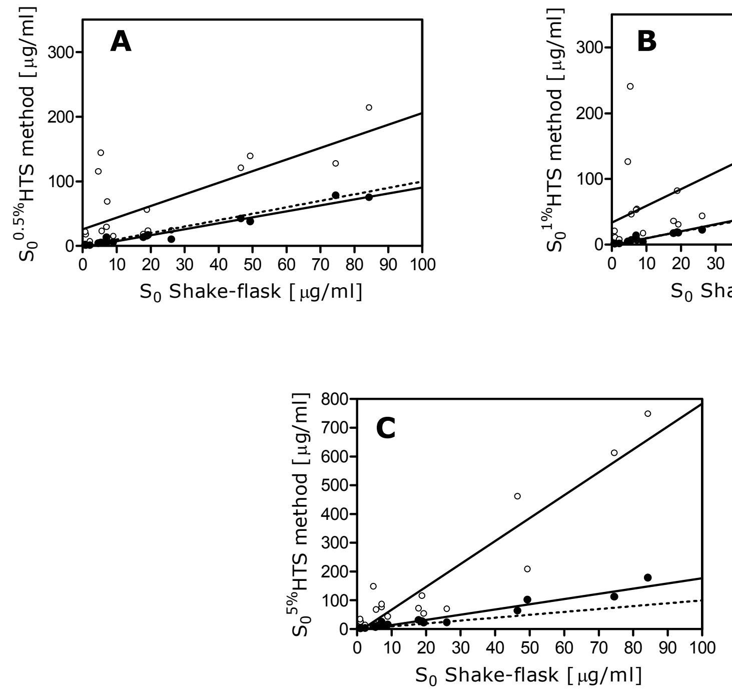

Fig. 3-2 : Correlations (plain line) between S_0 obtained by shake-flask method (without DMSO) after a 24 h equilibration time and S_0 measured with UV high throughput method with 0.5 % (A), 1 % (B) and 5 % (C) of DMSO, after incubation times of 1.5 h and 300 rpm shaking (O) and 24 h and 1200 rpm shaking (●). The dotted line represents the identity.

(A) Open circles, 1.5 h, 300 rpm shaking, 0.5% DMSO :

$$S_0^{0.5\%}\ (\mathrm{HTS}) = 1.80\ (\pm 0.41) \cdot S_0\ (\mathrm{SF}) + 25.8\ (\pm 13.4) \qquad \text{Eq. 3-1}$$

$n = 18$; $r^2 = 0.55$; $s = 43.3$; $F = 19.3$

(A) Dark circles, 24 h, 1200 rpm shaking, 0.5% DMSO :

$$S_0^{0.5\%}\ (\mathrm{HTS}) = 0.92\ (\pm 0.05) \cdot S_0\ (\mathrm{SF}) - 1.8\ (\pm 1.5) \qquad \text{Eq. 3-2}$$

$n = 18$; $r^2 = 0.96$; $s = 4.9$; $F = 392.2$

(B) Open circles, 1.5 h, 300 rpm shaking, 1.0% DMSO :

$$S_0^{1.0\%}\ (\mathrm{HTS}) = 2.54\ (\pm 0.61) \cdot S_0\ (\mathrm{SF}) + 33.4\ (\pm 20.0) \qquad \text{Eq. 3-3}$$

$n = 18$; $r^2 = 0.52$; $s = 64.4$; $F = 17.5$

(B) Dark circles, 24 h, 1200 rpm shaking, 1.0% DMSO :

$$S_0^{1.0\%}\ (\mathrm{HTS}) = 1.07\ (\pm 0.04) \cdot S_0\ (\mathrm{SF}) - 1.1\ (\pm 1.2) \qquad \text{Eq. 3-4}$$

$n = 18$; $r^2 = 0.98$; $s = 3.9$; $F = 851.2$

(C) Open circles, 1.5 h, 300 rpm shaking, 5.0% DMSO :

$$S_0^{5.0\%}\ (\mathrm{HTS}) = 7.96\ (\pm 0.74) \cdot S_0\ (\mathrm{SF}) - 12.2\ (\pm 24.3) \qquad \text{Eq. 3-5}$$

$n = 18$; $r^2 = 0.88$; $s = 78.4$; $F = 115.4$

(C) Dark circles, 24 h, 1200 rpm shaking, 5.0% DMSO:

$$S_0^{5.0\%}\ (\mathrm{HTS}) = 1.81\ (\pm 0.12) \cdot S_0\ (\mathrm{SF}) - 4.0\ (\pm 4.0) \qquad \text{Eq. 3-6}$$

$n = 18$; $r^2 = 0.93$; $s = 12.9$; $F = 222.1$

In these and subsequent equations 95% confidence limits are in parentheses, n is the number of compounds, r^2 is the coefficient of determination (squared correlation coefficient), s is the SD, and F is the Fisher value.

After a 1.5 h incubation time and a 300 rpm shaking, conditions where supersaturation is favorized, HTS UV S_0 was significantly increased, in a compound dependent way compared to shake-flask S_0. The slope (m) of the correlation, a good indicator of global solubility enhancement, was highly increased by enhancing DMSO percentage ((m=1.80, 2.54 and 7.96 for a DMSO percentage of respectively 0.5%, 1% and 5%). The poor regression coefficients obtained between the two methods (r^2 =0.55; 0.52 and 0.88 for respectively 0.5%, 1% and 5% of DMSO) confirm the already mentioned highly compound dependent solubility behaviour in such conditions.

After a 24 h incubation time and a 1200 rpm shaking, conditions where the formation of supersaturated solutions is minimized, solubility enhancement can be principally imputed to co-solvent effects. In such conditions, S_0 was less influenced by the presence of DMSO since the global enhancement compared to shake-flask solubility was moderate for 5% of DMSO (m=1.81), almost null for 1% (m=1.07) and a decrease was even observed for 0.5% (m=0.92). Reduction of solubility observed, compared to equilibrium solubility, with 0.5% of DMSO could partially be explained by non specific adsorption resulting from a prolonged storage of samples solutions on filter microplates. Actually, a test was carried out (data not shown) and showed that non specific adsorption may occur on this filter microplates, even if adsorption on PC filter plates has proved to be less important than on PVDF filter plates. The quality of the correlations was also much better than with 1.5 h incubation times in such conditions, (r^2 =0.96; 0.98 and 0.93 for respectively 0.5%, 1% and 5% of DMSO) meaning that co-solvent effects are less compound dependent than supersaturation effects.

Determination of Drug Solubility in intestino-mimetic Media

The solubility of some poorly soluble drugs at pH 5 and pH 6.5 was examined using shake-flask method in two intestino-mimetic media to simulate the proximal small intestine conditions in the fasted state (FaSSIF) (pH 6.5) and fed state (FeSSIF) (pH 5) and compared to their corresponding solubility values in aqueous media pH 6.5 and pH 5 containing no bile salts (composition inTable 3-3). Experimental values are reported in Table 3-5 and Table 3-6. As already shown in previous works [20-22], solubility in 100 % aqueous media does not represent intestinal solubility. In fact, solubility in 100 % aqueous medium pH 6.5 results in an underestimation of FaSSIF solubility in particular for the less soluble compounds, which are also the more lipophilic ones. The same observations can be done comparing solubility measured in FeSSIF medium and in the corresponding aqueous buffer pH 5. Moreover, the concentration of bile salts being 5-fold more concentrated in FeSSIF than in FaSSIF medium, underestimation is even more important.

Table 3-5 : S [µg/ml] measured in FaSSIF medium and in aqueous buffer (pH 6.5) using shake-flask and HTS method

	Shake-flask		**HTS**		
Compounds	**S (FaSSIF)**	**S Aqu. buffer**	**$S^{1\%}$ (FaSSIF)**	**$S^{5\%}$ Aqu buffer**	**$S^{10\%}$ Aqu.buffer**
Carbamazepine	156.1	96.3	110.5	201.1	349.5
Chloramphenicol	3625.6	2566.7	4162.7	3796.5	5615.4
Diazepam	59.5	39.3	90.0	58.1	80.3
Diclofenac	399.5	303.1	694.2	1003.5	2002.2
Dienestrol	27.3	12.7	24.9	28.5	68.5
Digitoxin	13.9	3.9	11.1	8.4	16.0
Estradiol	7.9	3.6	13.7	5.0	6.2
Estrone	13.9	1.0	11.8	4.3	4.7
Glybenclamide	8.0	2.1	9.2	3.1	4.6
Ibuprofen	1755.8	1187.0	1427.1	1755.8	1715.8
Ketoconazole	19.7	5.8	22.7	10.4	19.3
Naproxen	1154.0	578.4	1208.4	1005.1	1746.7
Nifedipine	104.6	77.5	137.9	151.2	330.7
Phenytoin	47.0	29.0	56.5	29.6	49.2
Piroxicam	70.8	55.0	126.0	120.3	166.1
Prednisone	179.6	133.2	188.1	236.8	542.5
Progesterone	10.9	6.4	12.5	10.7	12.5
Spironolactone	48.9	15.8	56.2	32.3	43.6
Terfenadine	15.6	2.7	19.2	12.1	54.2

Table 3-6 : S [µg/ml] measured in FeSSIF medium and in aqueous buffer (pH 5) using shake-flask and HTS method

	Shake-flask		**HTS**		
Compounds	**S (FeSSIF)**	**S Aqu. buffer**	**$S^{1\%}$ (FeSSIF)**	**$S^{5\%}$ Aqu. buffer**	**$S^{10\%}$ Aqu. buffer**
Carbamazepine	192.0	108.0	210.6	192.0	383.2
Chloramphenicol	3625.6	2879.9	4068.0	4162.7	6017.0
Diazepam	110.8	40.2	84.0	60.9	86.0
Diclofenac	67.8	11.0	53.9	18.3	45.9
Dienestrol	75.1	10.6	80.4	24.3	56.9
Digitoxin	41.1	3.8	73.1	8.2	14.9
Estradiol	16.4	2.7	20.7	5.0	9.0
Estrone	17.9	2.7	30.3	2.4	6.9
Glybenclamide	13.9	1.9	11.9	2.7	4.6
Ibuprofen	483.6	216.0	608.8	441.0	1133.6
Ketoconazole	298.8	111.0	359.3	164.2	254.4
Naproxen	270.5	142.0	303.5	283.3	459.4
Nifedipine	288.1	91.1	601.9	151.2	275.1
Phenytoin	50.3	28.3	51.5	31.8	52.7
Piroxicam	30.2	7.6	29.5	10.5	22.4
Prednisone	216.0	133.2	298.1	350.3	581.3
Progesterone	33.7	6.0	36.9	8.5	13.1
Spironolactone	53.7	17.0	89.1	27.5	42.6
Terfenadine	297.6	43.0	265.2	108.1	271.4

As bile salts, DMSO plays a solubilizing role, therefore DMSO as unique adjuvant was evaluated as a potential substitute to bile salts to estimate intestinal solubility using HTS UV method and experimental conditions allowing to minimize supersaturation. Correlations between HTS solubility in presence of DMSO and solubility values obtained in intestino-mimetic media are represented in Fig. 3-3 and reported in Eq. 3-7, 3–8 for FaSSIF medium.

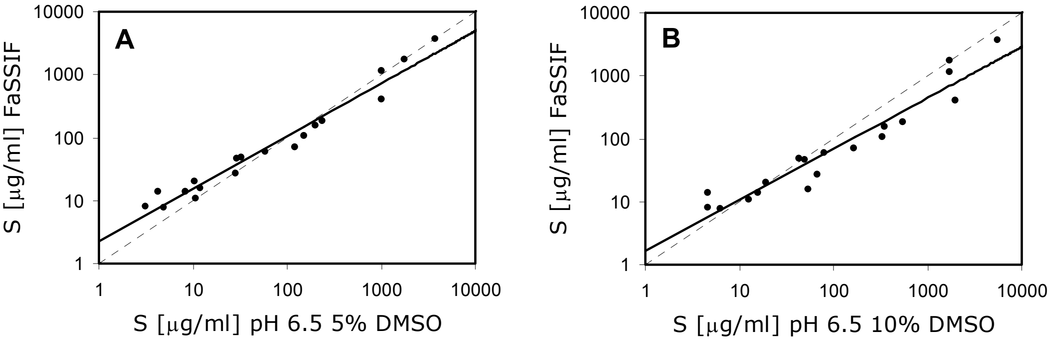

Fig. 3-3 : Correlation (plain line) between log S measured in FaSSIF medium and log S measured in the corresponding aqueous buffer pH 6.5 in presence of: A. 5%, B. 10% (v/v) of DMSO.

(A) 5% DMSO

$$S\ (SF) = 2.25 \cdot S^{5\%}\ (HTS)^{0.84} \qquad \text{Eq. 3-7}$$

$n = 19\ ;\ r^2 = 0.96$

(B) 10% DMSO

$$S\ (SF) = 1.65 \cdot S^{10\%}\ (HTS)^{0.81} \qquad \text{Eq. 3-8}$$

$n = 19\ ;\ r^2 = 0.92$

And in Fig. 3-4 and Eq. 3–9, 3–10 for FeSSIF medium:

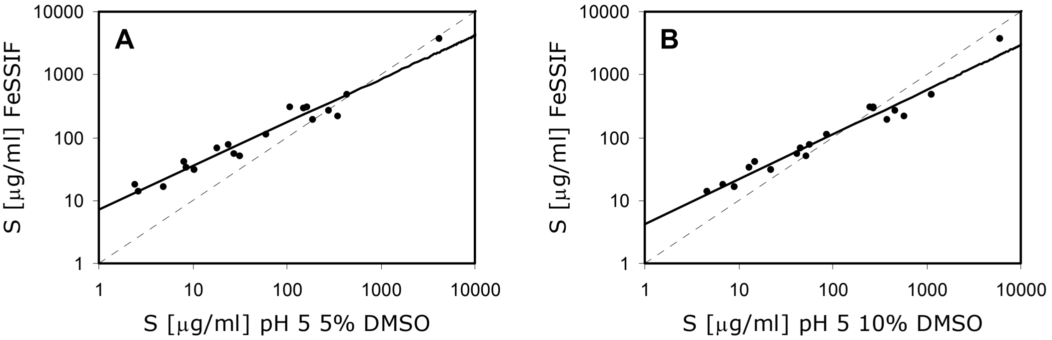

Fig. 3-4 : Correlation (plain line) between log S measured in FeSSIF medium and log S measured in the corresponding aqueous buffer pH 5 in presence of: A. 5%, B. 10% (v/v) of DMSO.

(A) 5% DMSO

$$S\ (SF) = 7.08 \cdot S^{5\%}\ (HTS)^{0.69} \qquad \text{Eq. 3-9}$$

$n = 19$; $r^2 = 0.95$

(B) 10% DMSO

$$S\ (SF) = 4.20 \cdot S^{10\%}\ (HTS)^{0.71} \qquad \text{Eq. 3-10}$$

$n = 19$; $r^2 = 0.95$

The quality of the correlations obtained were acceptable (r^2 ranging from 0.92 to 0.96), but the slopes of the correlations were poorly influenced by increasing DMSO percentages (m = 0.84 and 0.81 for respectively 5 and 10% of DMSO Fig. 3-3 A. and B ; m = 0.69 and 0.71 for respectively 5 and 10% of DMSO in Fig. 3-4 A. and B.). In terms of solubility, these results indicate that, although DMSO plays a solubilizing role towards drugs, it increases in a globally equivalent way the solubility of all the tested compounds. At the opposite, bile salts play a different solubilizing effect on lipophilic and hydrophilic compounds [20,21]. Consequently, DMSO as unique adjuvant cannot advantageously replace bile salts to obtain *in vitro* intestinal solubility.

To obtain biorelevant solubility data in discovery process, HTS UV method was adapted to screen solubility directly in FaSSIF and FeSSIF media. The percentage of DMSO was set to 1% (v/v) to minimize co-solvent effects. The presence of surfactants in the buffer lead to solubility values increased in a compound dependent way compared to shake-flask intestino-mimetic solubility, meaning that surfactants enhanced the stability of supersaturated solutions. To remedy this problem, DMSO stock solution were first diluted in aqueous buffers without bile salts and were shaken on an orbital shaker at 1200 rpm during 24 h to make supersaturated solutions precipitate. After this incubation time, a concentrated aqueous solution containing sodium taurocholate (0.3 M) and lecithin (0.075 M) was added to give final bile salt concentrations present in respectively FaSSIF and FeSSIF media (respectively 1% and 5% (v/v)). This adjunction had no significant effect on the pH of the solution. Saturated solutions were then further incubated during 8 h before filtration, to ensure micellization process to be completed. Because lecithin was denaturated by adjunction of acetonitrile prior to UV analysis, filtrates and calibration standards were diluted with methanol in this experiment.HTS intestino-mimetic solubility data were compared to shake-flask solubility measurements in the same media (without DMSO). Results are reported in Fig. 3-5 and Eq. 3–11, 3–12.

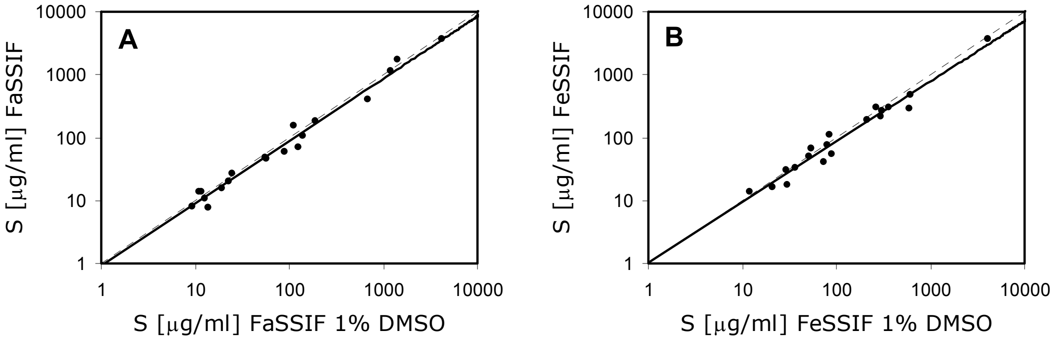

Fig. 3-5 : Correlation between: A. log S measured in FaSSIF medium using shake-flask method and log S measured in FaSSIF medium in presence of 1% DMSO using UV HTS method; B. log S measured in FeSSIF medium using shake-flask method and log S measured in FeSSIF medium in presence of 1% DMSO using UV HTS method.

(A) FaSSIF

$$S\ (SF) = 0.90 \cdot S^{1\%}\ (HTS)^{0.99} \qquad \text{Eq. 3-11}$$

$n = 19$; $r^2 = 0.98$

(B) FeSSIF

$$S\ (SF) = 1.04 \cdot S^{1\%}\ (HTS)^{0.96} \qquad \text{Eq. 3-12}$$

$n = 19$; $r^2 = 0.96$

Excellent correlations were obtained between the two methods with $r^2 = 0.98$ and 0.96 for FaSSIF and FeSSIF respectively. The slopes of the correlations were very close to identity ($m = 0.99$ and 0.96 for FaSSIF and FeSSIF respectively), which reflects the little effect of 1% of DMSO on final solubility values.

It must be noted that HTS UV approach using bile salts requires very small amounts of sample and small volumes of buffers. It also drastically reduce consumption of FaSSIF and FeSSIF media containing expensive components (sodium taurocholate and lecithin). Incubation times required for this method (32 h) are certainly quite long but the throughput of the method remains relatively high, because 96 samples are measured simultaneously in each microplate and several microplates can be prepared simultaneously. This method can then be used with a good confidence level and provides an unique and reliable way to measure intestino-mimetic solubility in the early stages of drug discovery.

Conclusion

High throughput solubility measurements obtained by diluting DMSO stock solutions into aqueous buffer lead to solubility data that can be increased in an highly compound dependent way compared to shake-flask solubility values. This phenomenon has shown to be mainly due to the formation of supersaturated solutions having slow precipitation kinetics, and in a less extent to co-solvent effect. Supersaturation, being a metastable state, has shown to be minimized by increasing orbital shaking rate to 1200 rpm and by enhancing incubation time to 24 h. Solubility measured in these conditions, gave results that were in good agreement with shake-flask solubility without DMSO. Comparatively to supersaturation, solubilization due to the single effect of DMSO was moderate using 0.5 and 1.0 % (v/v) of co-solvent and less compound dependent.

The main advantage of this HTS UV method is the possibility to easily adapt experimental conditions according to the requirements. Even if the UV method remains limited for poor soluble low absorbing compounds, a more sensitive detection method can easily be adapted. If the purpose is to evaluate whether compounds predissolved in DMSO stay in solution after dilution in the screening bioassays media, relevant solubility data are obtained using the same experimental conditions (supersaturation, shaking rate, incubation time, DMSO percentage) than in the screening test. To get solubility values in good agreement with thermodynamic aqueous solubility, supersaturation have to be reduced. To obtained more relevant data for the solubility in intestino-mimetic medium, DMSO as unique adjuvant failed to mimic micellar solubilization occurring in intestino-mimetic media containing bile salts.

The HTS UV method was then adapted to measure solubility in FaSSIF and FeSSIF media directly. The results were well correlated with FaSSIF and FeSSIF solubility data obtained by shake-flask method. Therefore, this method provides an unique and reliable way to measure intestino-mimetic solubility in the early stages of drug discovery within a relatively high throughput. Moreover this approach requires very small amounts of sample and small volumes of the expensive FaSSIF and FeSSIF media.

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Chapter 4 : Accuracy of Solubility Determination Using the HTS UV method

Introduction

Several analytical factors can alter accuracy of solubility measurement using HTS UV method. Even if solid material can be removed by centrifugation without the use of a filtration membrane, this method is more time consuming (30 to 40 min) and is sometimes ineffective because undissolved sample of lipophilic compounds can float on the surface of the solution [1]. Therefore filtration on filter microplates remain the method of choice in high throughput UV solubility measurement [1]. However, non specific adsorption on the microplate support or on the filter membrane is a parameter that can affect accuracy of solubility measurements because it will result in a significant decrease of the filtrate concentration. The selection of the filtrate membrane is then crucial to obtain reliable solubility data. Nylon filter membranes have already shown to be strong retentive membranes [2-4] and have better be avoided in solubility measurements. Many other filter membranes, like cellulose acetate [2,4], regenerated cellulose, glass fiber [4], polypropylene, polyethersulfone (PES) [2], polytetrafluoroethene (PTFE) [3], polyvinyldifluoride (PVDF) [1,2] and polycarbonate (PC) [1,5] were evaluated for sample non specific adsorption. The two less retentive and therefore the most recommended filter membranes for solubility measurements were reported to be PVDF and polycarbonate membranes. A direct comparison of non specific adsorption on these two filter membranes was then performed in this study.

To evaluate the quality of the data generated by the HTS UV method, parameters like accuracy, reproducibility and sensitivity of the analytical method have to be determined. The reproducibility of another HTS UV method to measure solubility was assessed by Pan et al. [3] by the examination of the coefficient of variation (CV %) obtained from a series of solubility measurements. No attempts were done to identify the source of variability i.e. analytical or related to the kinetics of solubility equilibration. Chen et al. [2] have made the distinction between these 2 parameters. The reproducibility of UV detection was assessed by measuring several times absorbance in the same well, and have shown to be excellent. At the opposite, the well-to-well reproducibility, accounting for liquid handling and material factors, was the major factor of variability in solubility measurement using HTS UV method.

A detailed study of the source of variability was performed for the method described in chapter 3. The limit of detection of the method was also determined.

Material and methods

The general procedure to obtain solubility values close to thermodynamic solubility data using the HTS UV method has already been described in chapter 3.

Two different 96-well filter plates were tested for non-specific adsorption: Multiscreen solubility filter plate (MSSLBPC10, Millipore AG, Volketswil, Switzerland) with an hydrophilic PC filter membrane (pore size 0.45 µm, thickness 17-22 µm), and Multiscreen GV (MAGVN2210, Millipore AG, Volketswil, Switzerland) with an hydrophilic PVDF filter membrane (pore size 0.22 µm, thickness 125 µm).

To evaluate analyte adsorption, saturated solutions containing 1% (v/v) of DMSO were first prepared by diluting DMSO stock solutions with aqueous buffer in small pyrex® vials and were shaken at room temperature for 24 h on a rotary shaker to reach equilibrium solubility. After a centrifugal step (14000 rpm for 20 min) to remove the precipitates, 200 µl of each saturated solutions were transferred on microplates having either PC or PVDF filters, and the complete HTS solubility assays were carried out on these solutions. Non-specific adsorption was then assessed by comparing the concentrations of the filtrates to samples concentrations after centrifugation.

Accuracy of solubility determination was assessed by the following procedure. For DMSO handling using Eppendorf Multipette Plus, 3 different volumes (10, 2, 1 µl) of pure DMSO were dispensed in the 96 wells of a pre-weighed Teflon microplate. After filling, microplates were weighed again, average volumes dispensed per well were determined and compared to expected volume. For liquid handling with Precision 2000 Pipetting System, distinction was done between liquid dispensing using manifold and plate-to-plate liquid transfer. Average volumes dispensed per well (300, 200, 100 and 50 µl) and volumes transfer (100, 50 and 25 µl) was assessed for pure water and ACN using the method described above.

Results and discussion

Non-specific adsorption of the drug on the microplate

The results presented in Fig. 4-1 are ranked from high to low log P_{oct} data (left to right), in order to evaluate if non specific adsorption is influenced by compounds lipophilicity.

The results have shown that the extent of analyte adsorbed on PC filter microplates, in particular for lipophilic compounds, was generally lower then on PVDF filter plates. Even if differences were for some compounds within the variability of the analytical method, adsorption of chlorpromazine, progesterone and verapamil was significantly higher on PVDF filter than on PC filter plates. At the opposite, only one compound, estradiol, showed a more important adsorption on PC plates compared to PVDF plates. Although PC seems to be globally less retentive than PVDF, the comparison of filtrates concentrations with concentrations after centrifugation revealed that the loss of compounds on PC filters, could for some compounds (indomethacine) represent an important part of the solubilized dose.

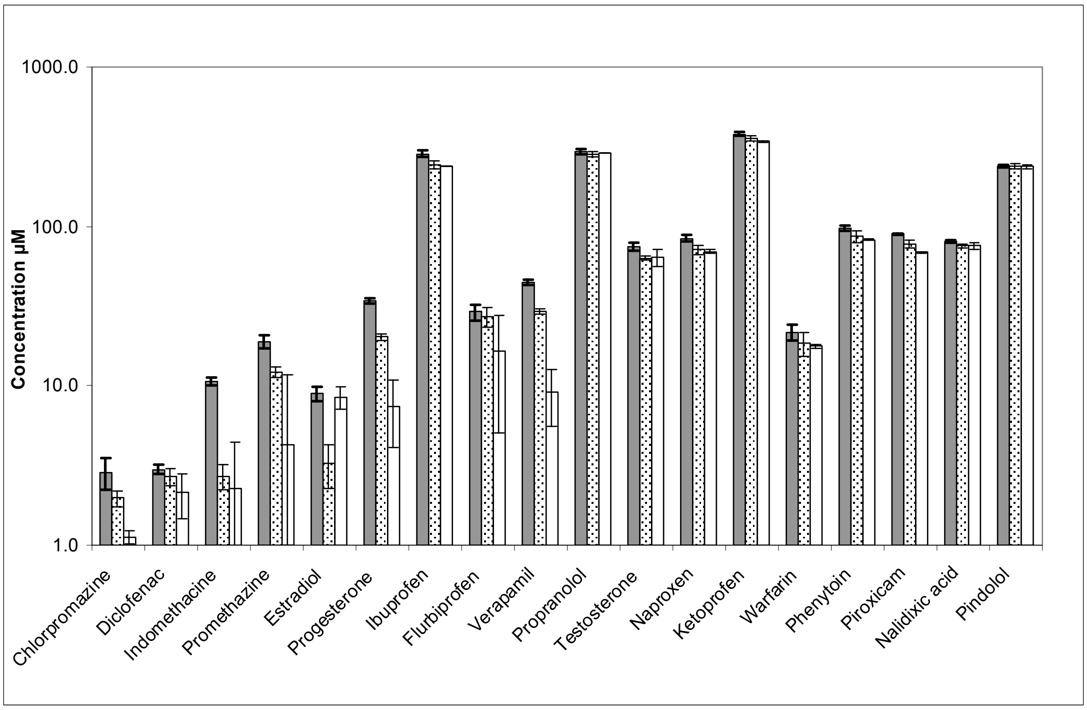

Fig. 4-1: Evaluation of sample recovery after filtration through PC (dotted) and PVDF (white) filter membranes and comparison with initial concentrations after centrifugation (dark grey). Data are presented as mean concentration [µM] ± SD (N=6) and the compounds are ranked from high to low log P_{oct} data (left to right)

Accuracy of liquid handling

Detailed gravimetric results are presented in Table 4-1. Volume accuracy of DMSO dispensing using Eppendorf Multipette Plus was within 98% of the expected volumes, which is satisfactory. For Precision 2000 Pipetting System, volumes dispensed and volumes transferred were always lower than requested volumes. For water dispense, the dispensed volumes using this system were ranging from 94.6% to 98.7% of the requested volume, and for water and ACN transfer, volumes dispensed were ranging from 93.3% to 97.9% of the requested volume. These pipetting errors could result in a systematic error in solubility determination if not corrected. A linear regression between requested volumes and volumes effectively dispensed was therefore established and gave a perfect line (r^2 =1, data not shown). To eliminate this systematic error, volumes were corrected using the regression line equation below:

$$V_R = 0.9826 \cdot V_D - 1.3139 \qquad \text{Eq. 4-1}$$

Where V_R = volume requested and V_D = volume effectively dispensed.

To obtain an effective volume of 50 µl, a volume of 52 µl was thus requested. This correction ensured a good accuracy for liquid dispense and liquid transfer and was applied to all pipetting operations using this system.

Table 4-1 : Accuracy of liquid dispensing using Eppendorf Multipette plus and Precision 2000 manifold and liquid transfer using Precision 2000 pipette system. Volume dispensed per well is the average volume dispensed in the 96 wells of the same plate.

Dispensing DMSO solution using Eppendorf Multipette Plus

Requested volume µl	Volume dispensed per well µl	Overall Accuracy %
10	10.01	99.9%
2	2.02	99.0%
1	1.02	98.0%

Accuracy of liquid dispensing with Precision 2000 using Manifold

Requested volume µl	Volume dispensed per well µl	Overall Accuracy %
307	302.9	98.7%
205	201.5	98.3%
103	99.9	97.0%
52	49.2	94.6%

Accuracy of water transfer plate-to-plate with Precision 2000 Pipette

Requested volume µl	Volume dispensed per well µl	Overall Accuracy %
103	100.8	97.9%
52	50.2	96.5%
27	25.2	93.3%

Accuracy of ACN transfer plate-to-plate with Precision 2000 Pipette

Requested volume µl	Volume dispensed per well µl	Overall Accuracy %
103	100.2	97.3%
52	50.0	96.2%
27	25.4	94.0%

Variability of the whole process

Variability in solubility determination with this 96-well HTS method was evaluated by examining the coefficient of variation (CV %) obtained from a series of solubility measurements carried out on two compounds, naproxen and testosterone. For each compound, solubility measurement procedure was applied in six wells using six different DMSO stock solutions (obtained from six independent weighing) at three different DMSO percentages (0.5%, 1% and 5% (v/v)). Sources of variability in solubility determination depend on pipetting inconstancy, material factors (significant differences between wells), variability in UV measurements and factors related to the kinetic apparition of a precipitate. To evaluate the UV variability only, UV absorption was measured three times in the same well, while other imprecision was assessed by measuring well-to-well variability. Detailed results of the variability study are shown in Table 4-2. The variability in the same well, which is only due to UV lecture error, was low (< 1%) for the two compounds tested, which is in accordance with the results published by Chen et al. [2]. Well-to-well CV in solubility measurement were within 3.1% and 4.3% for naproxen and testosterone respectively. These results seems to confirm that the UV detection process, is only a minor part of the whole process variability. The main source of error in solubility determination lies in the well-to-well variability. In all cases, the well-to-well CV were within 4.3% and results of this study are similar to those obtained by Pan et al. (<5%) [3] and Millipore (4,8%) [5].

Table 4-2 : Evaluation of the variability of the HTS process to determine solubility at 0.5%, 1.0% and 5.0% of DMSO, for naproxen and testosterone. Data are presented as mean ± SD (N=6)

Compound	% DMSO	Variability in the same well		Well-to-well variability	
		S_0 [μM]	CV [%]	S_0 [μM]	CV [%]
Naproxen	0.5	55.4 ± 0.1	0.2%	55.3 ± 1.1	1.9%
	1.0	71.6 ± 0.2	0.3%	71.3 ± 2.2	3.1%
	5.0	128.4 ± 0.2	0.2%	128.3 ± 3.3	2.6%
Testosterone	0.5	1.2 ± 0.2	0.5%	1.1 ± 1.2	2.3%
	1.0	2.6 ± 0.2	0.3%	2.7 ± 2.7	4.3%
	5.0	4.2 ± 0.2	0.2%	4.2 ± 2.3	2.5%

Sensitivity – Limit of detection (LOD)

The limit of detection (LOD) is defined as the smallest measure, that can be detected with reasonable certainty for a given analytical procedure [6]. Statistically, LOD is obtained by multiplying the standard deviation (SD) of blanks by a factor 3 [7]. LOD in UV detection is compound dependent since it depends on the chromophores present in the molecule. Therefore, it is difficult to give a general LOD guideline in terms of concentration. The LOD was estimated using the optical density SD of 16 blanks at several wavelengths (Table 4-3). Due to the presence of DMSO, SD of blanks reach its maximum at 230 nm and decrease above 240 nm, to progressively come near zero. Consequently, to determine the solubility of a compound at 230 nm, its background corrected absorbance needs to be higher than 0.073, but it only needs to be higher than 0.006 at 320 nm. Conversion of absorbance LOD to concentration is calculated by multiplying sensitivity (S=Δconcentration/Δintensity) determined on standard solutions, by SD of blanks at the selected wavelength. LOD of indomethacine (λ_{max} 320 nm) and diclofenac (λ_{max} 270 nm) was then calculated to be 4.5 μM (1.3 μg/ml) and 3.9 μM (1.4 μg/ml) respectively. Comparatively, a compound dosed at 230 nm with the same sensitivity than indomethacine, will have a LOD of 54 μM.

Table 4-3 : Evaluation of the limit of detection (LOD) of the UV detection by measuring the SD of blanks as a function of the wavelength. Absorbance of blanks are presented as mean absorbance ± SD (N=16)

Wavelengths [nm]	Absorbance of blanks (background)	LOD [Abs] (Background corrected)
200	2.416 ± 0.011	0.033
210	2.454 ± 0.012	0.035
220	2.166 ± 0.009	0.026
230	0.380 ± 0.024	0.073
240	0.053 ± 0.004	0.012
250	0.042 ± 0.004	0.011
260	0.040 ± 0.004	0.011
270	0.038 ± 0.004	0.011
280	0.037 ± 0.003	0.010
290	0.035 ± 0.003	0.008
300	0.033 ± 0.003	0.008
310	0.032 ± 0.002	0.007
320	0.031 ± 0.002	0.006
330	0.030 ± 0.002	0.006
340	0.030 ± 0.002	0.006
350	0.030 ± 0.002	0.006
360	0.029 ± 0.002	0.006
370	0.029 ± 0.002	0.006
380	0.029 ± 0.002	0.006
390	0.028 ± 0.002	0.006
400	0.028 ± 0.002	0.005

Conclusion

In this study, importance of several analytical factors on accuracy of solubility determination using HTS UV method were evaluated. Non-specific adsorption was tested on 2 different filter microplates (with PC or PVDF filter membranes). PC have shown to be the less retentive filter membranes and is then recommended for HTS solubility measurements.

Accuracy of pipetting systems was also assessed by gravimetric studies and results revealed the presence of a systematic error in liquid handling using Precision 2000 Pipetting System. The volumes were adapted to correct this error. It was then shown that the main source of variability using HTS UV method lies in the well-to-well variability depending on pipetting imprecision, material factors and the kinetic of precipitation of predissolved compounds. At the opposite, the reproducibility of the UV detection was excellent. It was also demonstrated that the LOD was strongly dependent on the detection wavelength and the sensitivity of the method was highly decreased at 230 nm (high absorbance of DMSO)compared to wavelength above 240 nm, where a LOD of 0.010 AU (absorbance unit) can be achieved.

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Chapter 5 : Determination of Alkane/Water partition coefficients of Polar Compounds using Hydrophilic Interaction Chromatography

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Abstract

Retention factors (log k) of 45 polar neutral compounds were measured using hydrophilic interaction chromatography (HILIC) and a linear solvation energy relationship (LSER) analysis was performed to elucidate the intermolecular forces controlling the retention. The relative contribution of each parameter, showed that the H-bond donor acidity parameter (α) was particularly more important in HILIC than *n*-octanol/water system partitioning. Conversely, intermolecular interactions involved in HILIC retention were similar to those responsible for alkane/water partitioning. This method could then represent an alternative and reliable way to characterize the lipophilicity of polar compounds in alkane/water system, a useful parameter for SAR studies.

Keywords: Hydrophilic interaction chromatography, Alkane/water partition coefficients, Linear solvation energy relationship

Introduction

Lipophilicity is one of the most used physicochemical parameters to build structure activity relationships (SAR). The gold standard procedure to measure lipophilicity, is the shake-flask method. In addition to experimental restrictions, it is limited to a range of log P varying from –3 to +3 [1,2]. Other methods like reverse-phase liquid chromatography

(RPLC) [3-8] are nowadays considered as true alternatives to the traditional shake-flask method and permit to extend the range of lipophilicity to more lipophilic compounds [9,10].

However, methods to measure, with a good confidence level, the lipophilicity of very polar non ionic compounds ($\log P < -3$) are still missing. Nevertheless, many biologic targets (proteins or enzymes) include highly polar active sites, notably all enzymes involving carbohydrates and sugars derivatives substrates, and a precise characterization of the lipophilicity of these polar compounds could provide useful data for SAR studies.

Conceptually, measuring the lipophilicity of polar compounds by liquid chromatographic methods could be achieved using polar stationary phases and apolar mobile phases, the retention being inversely proportional to the lipophilicity. However, normal phase liquid chromatography (NPLC), can't be used in this purpose, because its mechanism of retention seems to be principally governed by surface adsorption [11], which involves important contributions of dipolar interactions. A confirmation of this phenomenon is brought by the solvatochromic study realized by Park et al. [12], which showed that the relative contribution of dipolarity/polarisability (π^*) parameter in NPLC retention was much more important than its contribution to the partition between common solvent systems [13-15].

Hydrophilic interaction chromatography (HILIC) is a variant of normal phase chromatography [16] where the separations occurs on polar stationary phases using mobile phase containing a part of water and a miscible solvent (generally acetonitrile). For non ionic compounds, using high proportions of acetonitrile, the retention mechanism is considered as being partition between a water-enriched layer of stagnant eluent on the polar stationary phase and the mobile phase [11,16-19]. There are in principle no direct interaction between analytes and the stationary phases for uncharged compounds [19], although contradictory results were observed by other authors [18,20].

The forces responsible for the retention in chromatographic methods can be determined by solvatochromic analysis, a method based on linear solvation-energy relationships (LSERs). This method was used to evaluate and identify the intermolecular interaction forces underlying the partitioning mechanisms of solutes in various organic/aqueous biphasic systems [21,22] and retention in various chromatographic modes [23-25] in order to characterize retention mechanisms. LSERs can be expressed by the following multilinear equation:

$$S_p = a \cdot \alpha + b \cdot \beta + p \cdot \pi^* + \nu \cdot V + c \qquad \text{Eq. 5-1}$$

S_p is a given molecular property of a neutral organic solute (for example log P or log k). The structural parameters represent a steric term (volume V) and polar terms (dipolarity/polarizability π^*, hydrogen-bond donor acidity α, and hydrogen-bond acceptor basicity β). The steric term accounts for solvophobic/hydrophobic and dispersive forces, and the polar terms account for polar interactions between solutes and solvents. The regression coefficients a, b, p, and ν reflect the absolute contribution of each solute parameter to S_p and c is a constant term.

In this study, the retention factors (log k) of 46 polar model compounds were first measured using HILIC technique, and a LSER analysis was performed to elucidate the intermolecular forces controlling the retention on ZIC®-*p*HILIC. Then, log k values were evaluated as lipophilicity indices to determine partition in octanol/water and alkane/water solvent systems.

Materials and methods

Chemicals

All solutes were obtained from commercial sources Aldrich (Steinheim, Germany); Fluka (Buchs, Switzerland); Boehringer-Mannheim (Mannheim, Germany) and Sigma (St-louis, MO, USA) in the highest available purity.

Acetonitrile of superpure quality for HPLC was purchased from VWR (Dietikon, Switzerland), water was obtained with the Milli-Q Water Purification System from Millipore (Bedford, MA, USA), trifluoroacetic acid and sodium hydroxide were provided by Fluka (Buchs, Switzerland), ammonium carbonate and formic acid were obtained from Acros (New Jersey, USA) and Reactolab (Servion, Switzerland) respectively.

LSERs analysis

Solvatochromic parameters were calculated using Absolv module integrated in ADME Boxes software (V. 3.5) (Pharma Algorithms, Vilnius, Lithuania). LSERs models were generated by multivariate regression using TSAR 3.3 software (Oxford Molecular, Oxford, GB). The relative contributions of each variable to the LSERs models were obtained by Mager's standardization [26].

Measurements of retention factors

In chromatographic methods, the lipophilicity index is characterized by the retention factor log k given by:

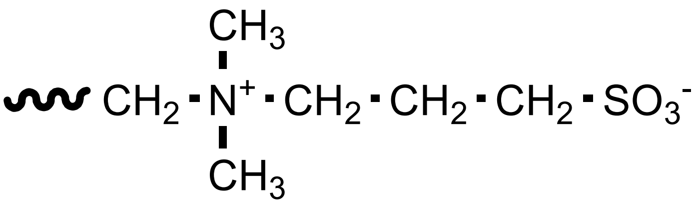
$$\log k = \log \left(\frac{t_r - t_0}{t_0} \right) \qquad \text{Eq. 5-2}$$

where t_r and t_0 are the retention times of the solute and an unretained compound, respectively. In RPLC, log k is generally correlated to log P_{oct}. All retention factors were measured using a Merck Hitachi EliteLaChrom liquid chromatograph (Merck, Darmstadt, Germany and Hitachi Instrument, Inc., San Jose, CA, USA) equipped with a L-2200 auto sampler, a L-2130 pump, a L-7614 degasser. Detection was performed using a L-2400 UV detector operating at 210 nm for all tested compounds. The chromatographic system was controlled by a EzChrom Elite System Manager software version 3.1.7 (Merck Hitachi). Retention measurements were performed on a ZIC®-*p*HILIC column (Sulfoalkylbetaine on a polymeric support, 10 cm x 4.6 mm, 5 μm) from SeQuant (Umeå, Sweden).

Depending on the tested compounds, mobile phases composed of Acetonitrile/Trifluoroacetic acid-sodium hydroxide buffer pH 2 (ionic strength = 20 mM) 90/10 or acetonitrile/Ammonium acetate-formic acid pH 7 (ionic strength = 20 mM) 90/10 were used to have compounds under their neutral forms. Composition of buffers was calculated by the Phoebus software 1.0 (Sedere, Centre Analyse, Orleans, France). Using buffer having a constant ionic strength is important, because buffer salt concentration has shown to have an influence on the retention [20]. All chromatographic experiments were performed at 22°C using a flow rate of 1 ml/min.

Results and discussion

In HILIC, there are in principle no direct interactions between analytes and the stationary phases for uncharged compounds [11,16-19], However. according to Guo et al. [20], which have tested four different polar stationary phases in HILIC mode, compounds retention and elution order can vary from column to column. These results suggest that direct interactions may take place between solutes and the polar functional groups of the stationary phases. For accurate lipophilicity measurement, the choice of the stationary phase becomes a crucial parameter. The ZIC®-*p*HILIC column (Fig. 5-1), made of a zwitterionic sulfoalkylbetaine phase on a polymeric support, has been chosen in this study. The use of zwitterionic stationary phases provides to this column a high osmotic strength and consequently a high ability of binding water to surface, which improve compounds retention. Another appreciable advantage of this column is that the ionization state of the stationary phase does not change over the whole recommended pH-range, because it is constituted of a permanent cation and a strong acid (sulfonic acid). Moreover, a polymeric support was also preferred to traditional silica based columns to avoid influence of free silanol deprotonation at high pH. Therefore, retention times of neutral compounds are not intended to vary on ZIC®-*p*HILIC column by changing the mobile phase pH.



$$\sim CH_2 - \overset{CH_3}{\underset{CH_3}{N^+}} - CH_2 - CH_2 - CH_2 - SO_3^-$$

Fig. 5-1 : ZIC®-pHILIC stationary phases

The measure of the void volume of the column is evaluated by the retention time of an unretained compound. As lipophilic compounds are presumed to be unretained in HILIC, assays were carried out to evaluate the use of toluene and naphthalene ($\log P_{oct} = 2.73$ and 3.30 [27] respectively) as void volume markers. Fig. 5-2 represents the retention time t_r as a function of acetonitrile percentage in the eluent and shows that retention increases with compounds lipophilicity.

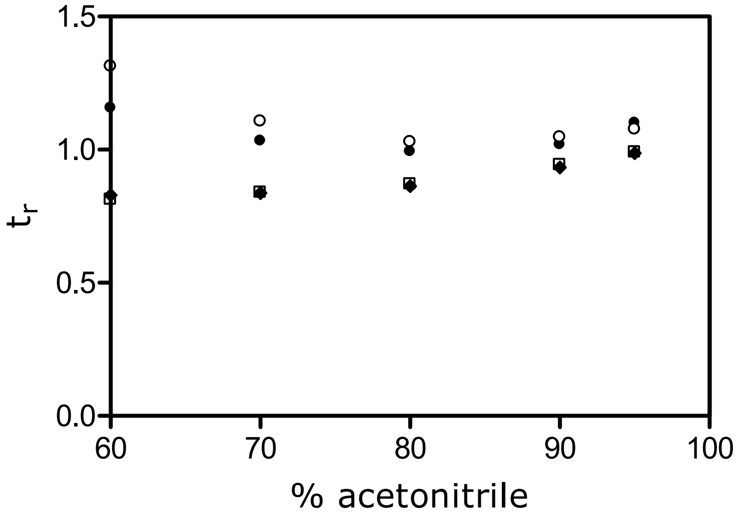


Fig. 5-2 : Evaluation of void volume markers. The graphs represent the retention time t_r as a function of % of acetonitrile in the eluent. ○ naphthalene, ● toluene, ♦ 1,2-dimethoxyethane, □ acetonitrile.

Retention times of lipophilic compounds (naphthalene and toluene) are also increased when the polarity of the eluent is enhanced, which demonstrates the existence of hydrophobic interactions. This "reverse-phase (RP)-like" retention (i.e. a decreased retention by increasing organic modifier proportion) has already been observed by other groups [28,29] in

HILIC mode on bare silica with mobile phases containing high proportions of water. Among tested compounds, acetonitrile and 1,2-dimethoxyethane were the least retained compounds and for detection reasons, 1,2-dimethoxyethane was chosen as void volume marker in this study.

Another important question that arises for the measurement of lipophilicity by HILIC is the choice of the retention factor. In RPLC, log k are measured in mobile phases composed of various mixtures of aqueous buffers and organic modifiers (typically methanol or acetonitrile) and log k_w (extrapolated to a 100% aqueous mobile phase) are generally used as lipophilicity indices instead of isocratic log k obtained at a single selected mobile phase concentration [10]. The use of the extrapolated value log k_w, generally give better correlations with log P_{oct} than isocratic log k.

In HILIC, considering the retention mechanism involved, maximal retention (and also highest discrimination) is obtained using high proportions of acetonitrile in the mobile phase. However, extrapolated values to 100% acetonitrile would not be judicious, because the mechanism involved in HILIC mode is the partition of the solute between the water-enriched layer on the stationary phase and the mobile phase. Therefore, isocratic log k containing 95 and 90% of acetonitrile could represent suitable parameters to measure lipophilicity indices using HILIC technique. In this work, log k obtained using 90% (v/v) of acetonitrile (log k_{90}) were chosen, because they were considered as the best compromise between discrimination and analyse times.

In this study, a set of 45 polar model compounds, having varied molecular structures were chosen. In order to elucidate the structural properties governing retention mechanisms on the ZIC®-*p*HILIC column and to evaluate their potential ability to determine lipophilicity, LSER analyses was applied to log k_{90} (Eq.5-3):

$$\log k_{90} = 0.74\ (\pm 0.20)\cdot\alpha + 1.17\ (\pm 0.20)\cdot\beta + 0.03\ (\pm 0.14)\cdot\pi^* - 1.03\ (\pm 0.24)\cdot V - 0.48\ (\pm 0.16) \qquad \text{Eq. 5-3}$$

$$n = 45\ ;\ q^2 = 0.91\ ;\ r^2 = 0.93\ ;\ s = 0.14\ ;\ F = 136$$

In this and subsequent equations, 95% confidence limits are in brackets, n is the number of compounds, r^2 is the coefficient of determination (squared correlation coefficient), s is the standard deviation, and F is the Fisher value. Solvatochromic parameters (α, β, π^* and V) and experimental log k_{90} are reported in Table 5-1. A quite good LSER model was obtained for log k_{90} (see correlation between measured and predicted log k_{90} in Fig. 5-3).

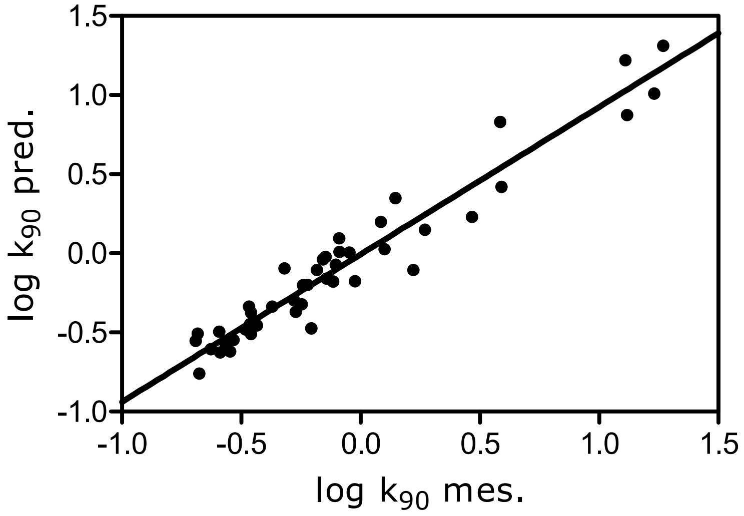


Fig. 5-3 Correlation between log k_{90} pred. (predicted by the LSER model) and log k_{90} mes. (measured).

The relative contributions of each solvatochromic descriptor to the measured retention factors obtained by data standardization showed that the principal factors governing the retention on the ZIC®-*p*HILIC column were hydrogen-bond donor acidity (α) and hydrogen-bond acceptor basicity (β), with almost equal importance (Table 5-2). They were followed by the volume (V), while the contribution of dipolarity/polarizability (π^*) was of minor importance and statistically not significant.

Table 5-1: Solvatochromic parameters (hydrogen-bond donor acidity α, and hydrogen-bond acceptor basicity β, dipolarity/polarizability term, π* and volume, V), isocratic retention factors measured on ZIC®-*p*HILIC stationary phase and measured and calculated partition coefficients in octanol/water and alkane/water systems.

Compounds	α [a)]	β [a)]	π* [a)]	V [a)]	log k_{90} [b)]	log P_{oct} [c)]	ClogP [e)]	log P_{alk} [f)]
1,2-phenylenediamine	0.47	0.71	1.42	0.916	-0.22	0.09	0.15	-2.44
1,4-phenylenediamine	0.45	0.74	1.50	0.916	-0.02	-0.31	-0.30	-2.57
1-butanol	0.31	0.31	0.46	0.731	-0.59	0.82	0.88	-0.23
1-pentanol	0.31	0.32	0.46	0.872	-0.68	1.35	1.56	0.45
1-propanol	0.31	0.31	0.45	0.590	-0.48	0.29	0.25	-0.98
2-aminophenol	0.51	0.55	1.21	0.875	-0.25	0.62	0.62	-1.76
2-butene-1,4-diol	0.63	0.65	0.83	0.747	-0.05	-0.81	-1.25	-3.35
2-mercaptoethanol	0.31	0.42	0.64	0.613	-0.27	–	-0.33	-1.57
2-propanol	0.31	0.34	0.43	0.590	-0.46	0.07	0.05	-1.16
2-pyrrolidinone, 1-(2-hydroxyethyl)-	0.23	1.11	1.18	1.020	-0.15	–	-0.82	-3.47
2-pyrrolidone	0.26	0.68	0.95	0.679	-0.14	-0.85 [d)]	-0.97	-2.71
3-aminophenol	0.73	0.65	1.28	0.875	-0.16	0.25	0.17	-3.11
3-nitroaniline	0.30	0.53	1.65	0.990	-0.63	1.26	1.37	-0.45
4-aminophenol	0.73	0.69	1.32	0.875	-0.09	0.25	0.04	-3.36
4-nitroaniline	0.28	0.53	1.65	0.990	-0.55	1.26	1.39	-0.38
Acetanilide	0.41	0.67	1.42	1.114	-0.59	1.16	1.16	-0.95

Table 5-1: continued

Compounds	α a)	β a)	π a)	V a)	log k_{90} b)	log P_{oct} c)	ClogP e)	log P_{alk} f)
Acetic acid	0.57	0.36	0.61	0.465	-0.32	-0.19	-0.17	-2.84
Allantoin	1.03	1.63	2.13	1.010	1.11	–	-2.24	-9.53
Ammeline	0.81	1.73	1.31	0.851	1.27	–	-3.07	-10.06
Antipyrine	0.00	1.28	1.75	1.485	-0.43	0.20	0.38	-1.42
Azodicarbonamide	0.97	1.33	1.76	0.778	0.32	–	-1.33	-1.93
Benzamide	0.49	0.66	1.55	0.973	-0.28	0.66	0.64	-0.68
Benzyl alcohol	0.31	0.53	0.84	0.916	-0.53	1.10	1.10	-2.04
Caffeine	0.00	1.27	1.90	1.363	-0.47	-0.07	-0.04	-2.49
Dithiothreitol	0.54	0.87	1.08	1.117	-0.12	–	-0.18	-1.73
Ethanol	0.31	0.31	0.45	0.449	-0.37	-0.24	-0.31	-4.45
Formamide	0.49	0.56	1.14	0.365	0.09	-1.51	-1.51	-0.85
gamma-butyrolactone	0.00	0.46	1.40	0.638	-0.69	-0.64	-0.80	-7.89
Glycoluril	0.75	1.40	2.18	0.886	1.12	–	-2.13	-5.14
Lactamide	0.66	0.88	1.13	0.706	0.15	–	-1.27	-7.52
Malonamide	0.97	1.12	2.00	0.762	0.59	–	-2.18	-2.41
Methanol	0.31	0.30	0.44	0.308	-0.24	-0.76	-0.77	-1.06
N,N-dimethylacetamide	0.00	0.64	1.06	0.788	-0.46	-0.77	-0.80	-1.76

Table 5-1: continued

Compounds	α a)	β a)	π a)	V a)	log k_{90} b)	log P_{oct} c)	ClogP e)	log P_{alk} f)
N-methylformamide	0.26	0.57	1.10	0.506	-0.18	-0.97	-0.97	-1.04
Oxazole	0.00	0.39	0.83	0.495	-0.68	0.12	-0.18	-2.98
Purine	0.37	0.77	1.47	0.823	0.22	-0.37	-0.22	-0.46
tert-butanol	0.31	0.35	0.39	0.731	-0.57	0.47	0.35	-3.27
Theobromine	0.24	1.22	1.89	1.222	-0.10	-0.78	-0.67	-4.08
Theophylline	0.35	1.29	1.99	1.222	-0.09	-0.03	-0.02	-1.10
Thioacetamide	0.21	0.39	0.76	0.611	-0.21	-0.26	-0.25	-4.62
Thiourea	0.42	0.82	0.77	0.570	0.47	-1.08	-1.02	-3.61
Thymine	0.44	0.90	1.41	0.893	0.10	-0.62	-0.56	-4.25
Uracil	0.44	0.88	1.47	0.752	0.27	-1.07	-1.06	-5.47
Urea	0.72	0.69	1.17	0.465	0.59	-2.11	-1.66	-8.59
Uric acid	0.80	1.58	2.36	1.000	1.23	-2.17	-1.45	-2.44

a) Values calculated using ADME Boxes software (V 3.5); b) log k measured using mobile phases containing 90% acetonitrile and 10% of trifluoroacetic acid-sodium hydroxide buffer pH 2 (ionic strength = 20 mM) or ammonium acetate-formic acid pH 7 buffer (ionic strength = 20 mM) ; c) Values taken from [27]; d) Values taken from [30]; e) Values calculated with CLOGP software (V. 4.0) (Daylight Chemical Information System, Inc., Irvine, CA, 2005); f) Values calculated by the LSER model presented in Eq. 5-6.

Table 5-2 : Relative contributions of solvatochromic descriptors to the LSERs models obtained by standardization of the data presented in Eq. 5-3 to 5-6

	n	% α	% β	% π*	% V
log k_{90}	45	21	49	2	29
ClogP	45	3	53	1	43
log P_{oct}	37	2	49	1	48
log P_{alk}	74	29	32	3	36

The contribution of the different solvatochromic descriptors to log k_{90} were compared to their contributions to experimental or predicted partition coefficients in octanol/water system (Eq. 5-4 to 5-5).

$$\log P_{oct} = 0.25\ (\pm\ 0.51)\cdot\alpha - 3.54\ (\pm\ 0.61)\cdot\beta + 0.03\ (\pm\ 0.39)\cdot\pi^* + 4.13\ (\pm\ 0.60)\cdot V - 1.24\ (\pm\ 0.39) \qquad \text{Eq. 5-4}$$

$n = 38$; $q^2 = 0.85$; $r^2 = 0.89$; $s = 0.32$; $F = 67$

$$\text{ClogP} = 0.29\ (\pm\ 0.49)\cdot\alpha - 3.16\ (\pm\ 0.49)\cdot\beta + 0.02\ (\pm\ 0.35)\cdot\pi^* + 3.80\ (\pm\ 0.59)\cdot V - 1.27\ (\pm\ 0.40) \qquad \text{Eq. 5-5}$$

$n = 45$; $q^2 = 0.84$; $r^2 = 0.89$; $s = 0.35$; $F = 84$

The comparison between the relative contributions of each solvatochromic descriptor to the measured log P_{oct} and log k_{90} obtained by data standardization (Table 5-2) reveal that important differences exist between both properties. The principal factors governing the retention on the ZIC®-*p*HILIC column were hydrogen-bond donor acidity (α) and hydrogen-bond acceptor basicity (β), with almost equal importance. On the other side, the α term has only minor importance whereas β present a large contribution in octanol/water partition coefficient LSER model. Thus HILIC retention coefficients and log P_{oct} are characterized by different balances of intermolecular interactions, meaning that lipophilicity in octanol/water system cannot be determined using these conditions. As an indication, the determination coefficient obtained for the linear relationship between log k_{90} and experimental log P_{oct} was 0.55 (Fig. 5-4).

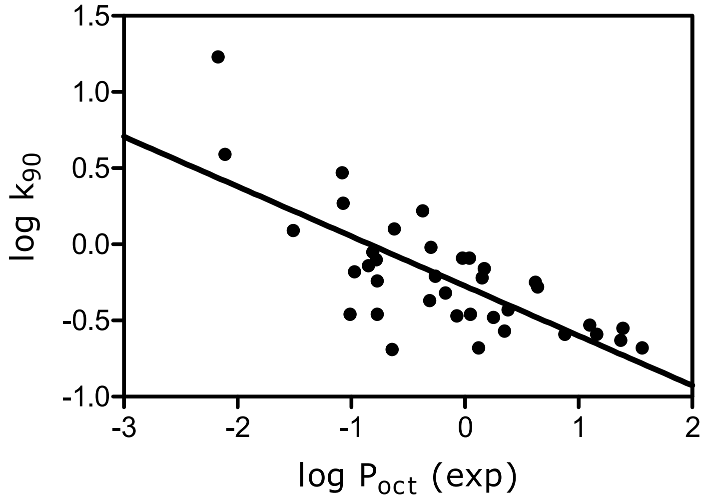


Fig. 5-4 : Relationship between log k_{90} and log P_{oct} measured by shake-flask method, plain line represents the linear regression given by :
log k_{90} = -0.33 (± 0.05)· log P_{oct} -0.27 (±0.05);
n = 37 ; r^2 = 0.55; s = 0.27; F = 43

However intermolecular forces involved in HILIC retention, especially the importance of α and β contributions are similar to those involved in the partition process in inert solvents/water systems such as alkane/water (Table 5-2 and Eq. 5-6).

$$\log P_{alk} = -3.28\ (\pm\ 0.58)\cdot\alpha - 6.07\ (\pm\ 1.08)\cdot\beta - 0.24\ (\pm\ 0.50)\cdot\pi^{*} + 5.30\ (\pm\ 0.90)\cdot V - 1.10\ (\pm\ 0.64) \qquad 5\text{-}6$$

$$n = 74\ ;\ q^2 = 0.87\ ;\ r^2 = 0.90\ ;\ s = 0.55\ ;\ F = 148$$

Eq. 5-6 was obtained using log P_{alk} values presented in [31] and solvatochromic parameters calculated using Absolv module integrated in ADME Boxes software.

Therefore, HILIC could provide an alternative way to obtain a lipophilicity indice related to log P_{alk} for polar neutral compounds. To be consistent and because experimental values were not available for all tested compounds, log P_{alk} values were calculated using LSER model presented in Eq. 6. To ensure the accuracy of log P_{alk} LSER model, 25 experimental log P_{alk} values taken from [32] and selected by cluster analysis were compared to their predicted log P_{alk} calculated with Eq. 5-6, and a good correlation was obtained (Fig. 5-5).

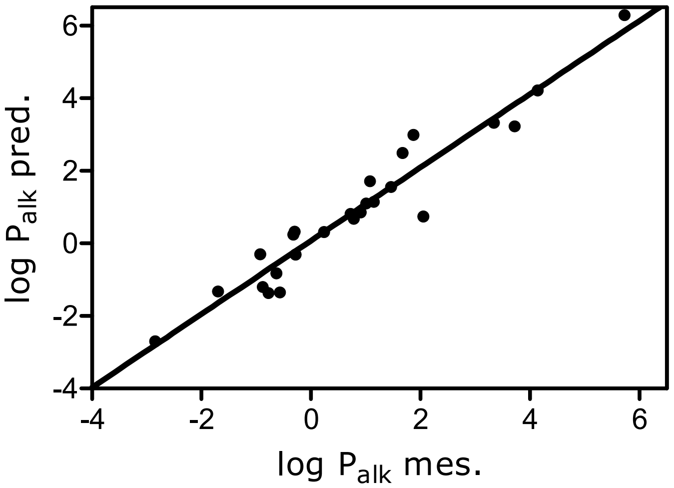


Fig. 5-5 Correlation between experimental log P_{alk} values taken from [32] and log P_{alk} values calculated by the LSER model presented in Eq. 6. Plain line represents the regression given by :

log P_{alk} pred. = 1.01 (± 0.06)· log P_{alk} mes. +0.07 (±0.12);

n = 25 ; r^2 = 0.93; s = 0.55; F = 310

Then the log k_{90} of tested compounds were compared to the calculated log P_{alk} (Fig. 5-6). The good correlation obtained also confirms the results of the LSER analysis. Even if the slope of the correlation is weak (-0.20), meaning that both phenomena are energetically different, relative contributions of solvatochromic parameters are similar between both processes.

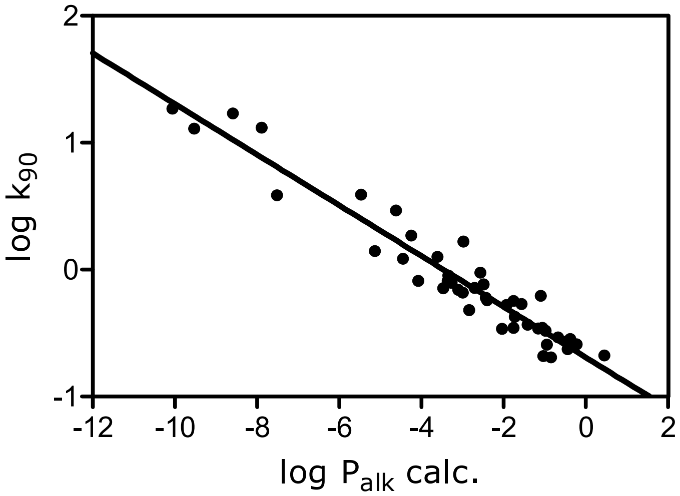


Fig. 5-6 : Correlation between log k90 and log Palk calculated by the LSER model presented Eq. 6, plain line represents the linear regression given by: log k90 = -0.20 (± 0.01) · log Palk -0.69 (±0.03);

n = 45 ; r2 = 0.93; s = 0.44; F = 570

Conclusion

The results of the LSER analysis showed that the principal factors governing retention in HILIC were hydrogen-bond donor acidity (α), hydrogen-bond acceptor basicity (β) and to a lesser importance volume, which are also the main factors governing partitioning in the alkane/water system. Determination of partition coefficients in alkane/water systems is often difficult due to the low solubility of many compounds in alkane. Log k HILIC provides an alternative method to obtain in a reliable way log P_{alk} for polar compounds with the well-known advantages of chromatographic methods: insensitivity to impurities, the small sample consumption and easy automatization.

Although the lipophilicity generally used in drug discovery is log P_{oct}, for polar compounds (log P < -3) no reliable experimental methods to measure partition coefficient are available and *in silico* model (also based on experimental values) are generally poorly predictive. Log P_{alk} has found to be useful for the calculation of the Δlog $P_{oct\text{-}alk}$ parameter (difference between partition coefficient in octanol/water and alkane/water systems) which has extensively been used to reveal intermolecular interactions [31-35] or to predict drugs permeation through numerous biorelevant membranes [36-40]. HILIC lipophilicity indices could then advantageously replace log P_{oct} data in (Q)SAR studies for such polar compounds.

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Chapter 6 : Determination of Partition Coefficient of Basic Drugs using Hydrophilic Interaction Chromatography

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Abstract

In this work, HILIC technique was used to measure lipophilicity indices of basic organic compounds. It was demonstrated that the differences between 2 isocratic log k values ($\Delta \log k_{95\text{-}0}$) of basic compounds measured under their cationic forms were well correlated with their $\log P^{N}_{oct}$ values. Thus, this method offers, when the composition of the mobile phase is strictly controlled (pH, buffer nature or buffer ionic strength), a new promising way of obtaining octanol/water partition coefficients data.

Introduction

Chromatographic methods and in particular RPLC (Reverse Phase Liquid Chromatography) methods are currently largely used and recognized for the determination of $\log P_{oct}$, as illustrated by the many well-documented reviews published on this subject [1-7]. The well known advantages of these methods are their good throughput and easy automation, the low sample consumption, and their general insensitivity to impurities. Another advantage of RPLC is the possibility to extend the range of log P to more lipophilic values compared to shake-flask method [8].

However, these methods are not appropriate for the determination of partition coefficients of polar compounds ($\log P < -1$). Furthermore, RPLC techniques, in particular using conventional silica-based stationary phases, still remains limited for the determination of partition coefficients of moderate to strong basic compounds ($pK_a > 7$) under their neutral form, because of the silica-based stationary phases instability at high pH conditions [9-13], even if new strategies allow modern stationary phases to be resistant to a broader pH-range {11318}.

An approach to overcome this problem could be the determination of a retention factor of the cationic forms, which should be related to lipophilicity of the neutral form. Hydrophilic interaction chromatography (HILIC) [14], is a separation technique using eluents containing appreciable amounts of water and a relatively hydrophobic miscible solvent (generally acetonitrile) and is particularly suitable for the retention of polar and charged compounds. This technique has already been successfully used for the determination of $\log P_{alk}$ of polar neutral compounds [15].

HILIC retention mechanism of charged compounds was reported to be a complex combination of different interactions [16], depending on the experimental conditions involved; an hydrophilic retention [14,17-20] when high proportions of organic modifier are used in the mobile phase, a reverse-phase (RP) type retention [21,22] (i.e. a decreased retention by increasing organic modifier proportion) when the mobile phase contains high proportions of water, and an additional ion exchange mechanism [23]. Since lipophilicity expresses the balance of polarity and hydrophobicity, the particularity of HILIC retention could be exploited to obtain octanol/water partition coefficients using two diametrically opposite mobile phase compositions (i.e. 0% and 95% (v/v) of ACN in the mobile phase) involving different balance of intermolecular interactions.

Because of the ion exchange mechanism involved for charged compounds, retention factors are likely to be influenced by aqueous buffer composition, pH and ionic strength. Effect of these parameters on partition coefficient measurement were then closely studied and “guidelines” were proposed to ensure accurate partition coefficient determination.

Material and methods:

Chemicals

Acebutolol HCl, alprenolol HCl, apomorphine HCl, atropine, bisoprolol, carbamoylcholine, methylhomatropine, metoprolol tartrate, moxisylyte HCl, oxprenolol, pindolol, propranolol HCl, pyridostigmine, S-butyrylthiocholine, scopolamine bromide, strychnine, timolol maleate and verapamil HCl were purchased from Sigma (Buchs, Switzerland).

Butylamine, dicyclohexylamine, ethanolamine, morpholine, MPTP HCl (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine HCl), octopamine HCl, serotonine HCl, tetra-N-ethylammonium, tetra-N-methylammonium and triethanolamine were obtained from Fluka (Buchs, Switzerland). Atenolol, tacrine HCl and tripropylamine were purchased from Aldrich (Steinheim, Germany). Carazolol, carvedilol and metipranolol were kindly offered by Boehringer-Mannheim (Mannheim, Germany). Penbutolol sulfate was offered by Hoechst Pharma (Zürich, Switzerland). Tyrosine methyl ester was obtained from Bachem (Bubendorf, Switzerland).

Acetonitrile of superpure quality for HPLC was purchased from VWR (Dietikon, Switzerland), water was obtained with the Milli-Q Water Purification System from Millipore (Bedford, MA, USA), trifluoroacetic acid and acetic acid were provided by Fluka (Buchs, Switzerland), ammonium hydroxide solution (25% in H_2O) and formic acid were obtained from Merck (Darmstadt, Germany) and Reactolab (Servion, Switzerland) respectively.

Measurements of retention factors

All experiments were performed using a Merck Hitachi EliteLaChrom liquid chromatograph (Merck, Darmstadt, Germany and Hitachi Instrument, Inc., San Jose, CA, USA) equipped with a L-2200 auto sampler, a L-2130 pump, a L-7614 degasser. Detection were performed

using both, a L-2400 UV detector operating at 230 nm for all compounds and a Sedex 85 LT-ELSD detector (Sedere, Alfortville, France).
ELSD conditions were as followed; for pure aqueous mobile phases, nebulization air pressure and temperature were set to 3.0 bar and 70°C respectively, while for ACN rich mobile phases these parameters were adjusted to 3.5 bar and 40°C.

The chromatographic system was controlled by a EzChrom Elite System Manager software version 3.1.7 (Merck Hitachi). Retention measurements were performed at 23°C on a ZIC®-*p*HILIC column (Sulfoalkylbetaine phase on a polymeric support, 10 cm x 4.6 mm, 5 µm) from SeQuant (Umeå, Sweden). Flow rate was set to 1 ml/min.

Phoebus software 1.0 (Sedere, Centre Analyse, Orleans, France) was used to prepare buffers at fixed pH and ionic strength (I). The pH of the mobile phase were measured both before (${}^{w}_{w}\mathrm{pH}$) and after the adjunction of organic solvent (${}^{s}_{w}\mathrm{pH}$), with the pH-meter calibrated in aqueous buffer in both cases, even if ${}^{s}_{w}\mathrm{pH}$ values have to be taken carefully [24].

The following buffers were then prepared, TFA (trifluoroacetic acid)/ammonium ${}^{w}_{w}\mathrm{pH}$ 2, $I = 20$ and 100 mM, formic acid/ammonium ${}^{w}_{w}\mathrm{pH}$ 3 and 4, $I = 20$ and 100 mM, acetic acid/ammonium ${}^{w}_{w}\mathrm{pH}$ 4 and 5, $I = 20$ and 100 mM.

Results and discussion

Retention mechanism involved for cationic solutes in HILIC mode has shown to be a complex combination of ion exchange, hydrophilic and “RP-like” mechanisms [16]. This HILIC particularity is generally expressed by opposite variations of log k at respectively high and low ACN percentages as illustrated in Fig. 6-1 for atenolol and pindolol.

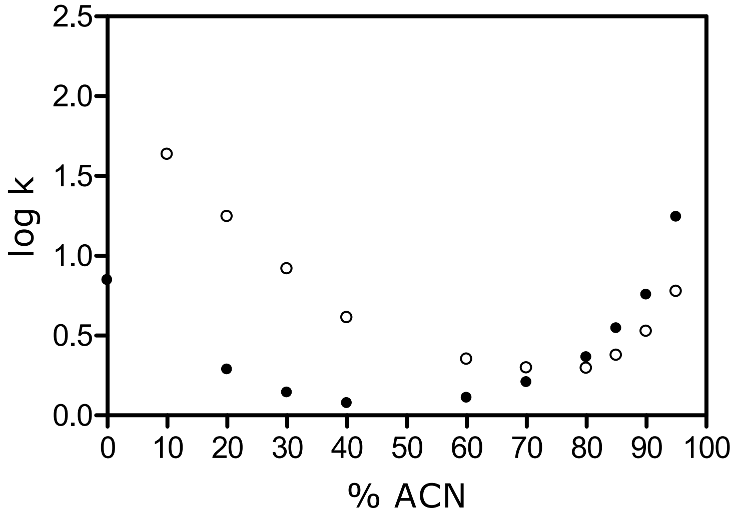

Fig. 6-1: Relationship between the measured log k in function of the percentage of acetonitrile in the mobile phase (mixtures of ACN - Ammonium acetate ${}_{w}^{w}pH$ 4, I = 20 mM). ● Atenolol ; ○ Pindolol

According to the work of Laurent et al. [25] influence of organic modifiers fraction on cation exchange chromatography using ammonium (having a positive free energy of transfer from water to ACN) as buffer cation, is not intended to vary importantly by increasing ACN fraction until quite high modifier concentrations, where a weak decrease should be observed. Therefore, the log k decrease registered at low ACN percentages can be attributed to RP-like retention retention, whereas at high ACN percentages, the log k increase can be ascribed to hydrophilic retention. The more lipophilic drug, Pindolol, showed a more marked RP-like retention than atenolol, while hydrophilic retention was higher for atenolol (i.e. the more hydrophilic solute).

Two diametrically opposite mobile phase compositions (i.e. 0% and 95% (v/v) of ACN) were tested on a set of 40 moderate to strong basic model compounds ($7.10 < pK_a < 10.95$) having log P^{N}_{oct} values ranging from -1.31 to 4.62, and the corresponding retention factors (log k_0 and log k_{95}) were compared with experimental log P^{C}_{oct} and log P^{N}_{oct} values, representing respectively the logarithm of the partition coefficient of the cationic and the neutral form in octanol/water system (Table 6-1).

Table 6-1: Physicochemical properties and data obtained for the 40 tested basic compounds

Compounds	**pK_a**	**log P^{C}_{oct}**	**log P^{N}_{oct}**	**log k_{95} l)**	**log k_0 m)**
Acebutolol	9.92 a)	-0.50 a)	2.02 a)	-0.071	1.260
Alprenolol	9.59 a)	0.25 a)	3.10 a)	-0.359	2.017 n)
Atenolol	9.54 a)	–	0.22 a)	0.556	0.424
Bisoprolol	9.57 a)	-1.22 a)	2.15 a)	-0.413	1.168
Carazolol	9.52 a)	0.77 a)	3.73 a)	-0.009	2.972 n)
Carvedilol	7.97 a)	1.92 a)	4.11 a)	-0.200	3.099 n)
Metipranolol	9.54 a)	-0.26 a)	2.81 a)	-0.498	1.565
Metoprolol	9.63 a)	-1.10 a)	1.95 a)	-0.268	1.097
Nadolol	9.67 a)	–	0.81 a)	0.503	0.587
Oxprenolol	9.57 a)	-0.13 a)	2.51 a)	-0.343	1.430
Penbutolol	9.92 a)	1.32 a)	4.62 a)	-0.511	3.078 n)
Pindolol	9.54 a)	-1.32 a)	1.83 a)	0.080	1.634
Propranolol	9.53 a)	0.78 a)	3.48 a)	-0.190	2.709 n)
Timolol	9.53 a)	-0.94 a)	2.12 a)	0.077	0.895
Lidocaine	7.9 b)	–	2.33 c)	-0.395	0.760
Mepivacaine	7.6 b)	–	1.94 c)	-0.177	0.854
Prilocaine	7.8 b)	–	2.12 c)	-0.171	0.930
Procaine	9.0 b)	–	2.03 c)	-0.259	1.053
Apomorphine	–	–	2.49 f)	0.332	2.742 n)
Atropine	9.43 g)	–	1.83 f)	-0.111	0.872
Butylamine	10.77 h)	–	0.97 f)	0.286	0.755

Table 6-1 : continued

Compounds	pK_a	$\log P^C_{oct}$	$\log P^N_{oct}$	$\log k_{95}$ l)	$\log k_0$ m)
Ethanolamine	8.92 h)	–	-1.31 f)	1.097	-0.542
Homatropine	9.81 c)	–	1.63 c)	-0.073	0.645
Methylephedrine	9.00 e)	–	1.55 e)	-0.140	0.637
Morpholine	8.49 h)	–	-0.86 f)	0.475	-0.267
Moxisylyte	8.72 g)	–	3.17 i)	-0.857	1.529
Nicotine	3.4 ; 8.2 d)	–	1.34 d)	0.137	-0.020
MPTP	–	–	2.71 f)	-0.181	1.296
N,N-dimethylbenzylamine	9.02 c)	–	1.91 c)	-0.226	0.429
Octopamine	8.81 g)	–	-0.90 h)	1.148	0.437
Pseudoephedrine	10.25 h)	–	0.89 j)	0.130	0.583
Rilmenidine	9.22 c)	–	1.70 c)	-0.412	0.877
Scopolamine	7.75 g)	–	0.98 g)	-0.542	-0.800
Serotonine	–	–	0.21 f)	1.154	1.186
Strychnine	–	–	1.93 f)	0.056	1.182
Tacrine	9.95 h)	–	2.71 f)	0.164	2.601 n)
Triethanolamine	7.76 h)	–	-1.00 f)	0.763	-0.574
Tyrosine methyl ester	–	–	0.29 k)	0.571	0.258
Verapamil	8.92 g)	–	3.79 f)	-1.265	1.689 n)

a) Taken from [26]; b) Taken from [27]; c) Taken from [28]; d) Taken from [29]; e) Taken from [30]; f) Taken from [31]; g) Taken from [32]; h) Taken from [33]; i) Taken from [34]; j) Taken from [35]; k) Taken from [36]; l) Mobile phase: acetonitrile / buffer pH 2 (TFA / ammonium I = 100 mM) 95/5; m) Mobile phase: buffer pH 2 (TFA / ammonium I = 100 mM); n) Extrapolated by quadratic relationship between log k values and ACN percentage

Aqueous fraction was constituted of a TFA-ammonium (I = 100 mM) acidic buffer pH 2 to have basic compounds fully under their cationic forms. Fig. 6-2 shows that no correlation was found between log P^{C}_{oct} or log P^{N}_{oct} and log k_{95} (r^2 = 0.05 and 0.59 respectively), while log k_0 was better correlated with log P^{C}_{oct} and log P^{N}_{oct} (r^2 = 0.84 and 0.71 respectively).

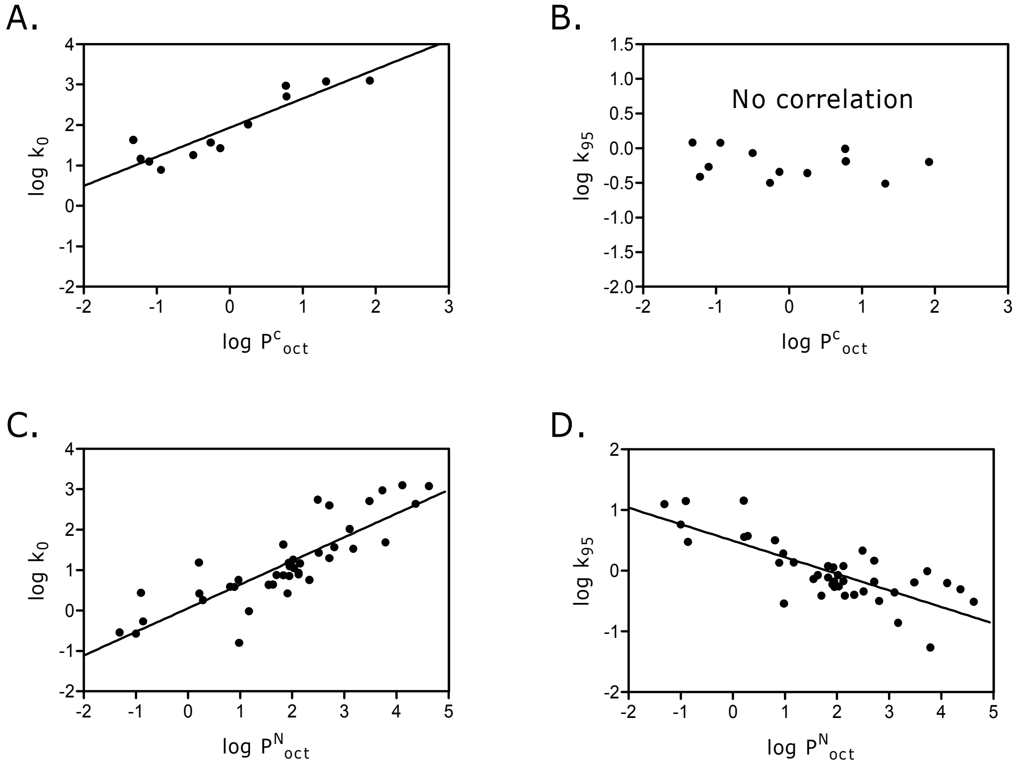

Fig. 6-2 Relationship between A. log k_0 versus log P^{C}_{oct} ; B. log k_{95} versus log P^{C}_{oct} ; C. log k_0 versus log P^{N}_{oct} ; D. log k_{95} versus log P^{N}_{oct}., where log k_0 was the isocratic log k obtained in a 100% buffer TFA-ammonium pH 2 (I = 100 mM) mobile phase and log k_{95} measured using a (95 :5, v/v) ACN - buffer TFA-ammonium pH 2 (I = 100 mM) mobile phase.

However, the use of an unique isocratic retention factor of charged compounds seems to be insufficient to accurately determine octanol/water partition coefficients, because notably of the cation exchange contribution involved over the whole range of ACN content in the mobile phase.

Moreover, except cation exchange contribution which is poorly influenced by changing ACN fraction in the mobile phase, log k_0 expresses almost hydrophobicity which involves important contribution of the volume, whereas log k_{95} is an expression of compounds polarity (hydrogen bond donor and acceptor abilities). Based on LSERs (Linear Solvation Energy Relationships) analysis, compounds lipophilicity in octanol/water partition coefficients is governed predominatly by the volume and hydrogen bond acceptor basicity [37]. Therefore both log k_0 and log k_{95} give information important for lipophilicity.

The difference between log k_0 and log k_{95} (Δlog $k_{0\text{-}95}$) was then performed and compared to log P^N_{oct} (Fig. 6-3 and Eq. 6-1).

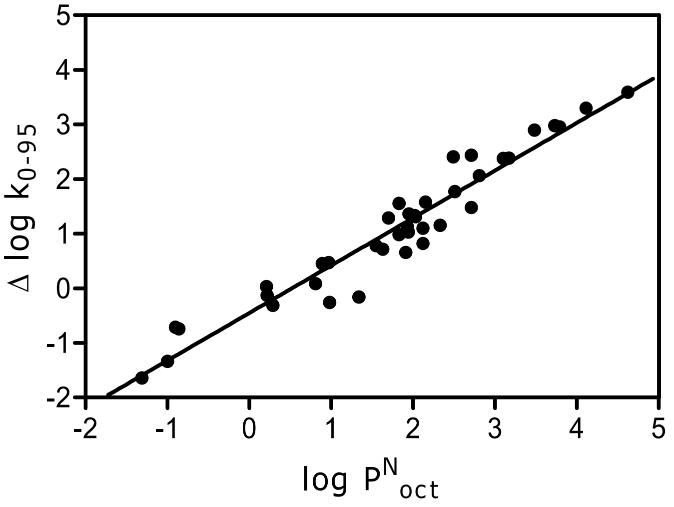

Fig. 6-3 Relationship between Δlog $k_{0\text{-}95}$ (phase mobile = acetonitrile - buffer TFA-ammonium pH 2 (I = 100 mM) and log P^N_{oct} .

A good linear regression was obtained between these two parameters :

$$\log k_{0\text{-}95} = 0.87\ (\pm\ 0.04) \cdot \log P^N_{oct} - 0.45\ (\pm\ 0.09) \qquad \text{Eq. 6-1}$$

$n = 40$; $r^2 = 0.93$; $s = 0.34$; $F = 480$

In these equations 95% confidence limits are in brackets, n is the number of compounds, r^2 is the coefficient of determination (squared correlation coefficient), s is the SD, and F is the Fisher value.

The $\Delta\log k_{0\text{-}95}$ parameter of cationic forms is then an efficient descriptor to obtain log P^{N}_{oct} of basic compounds.

However, the recent work of McCalley [16] has shown that contrary to neutral compounds, HILIC retention of charged compounds could be highly influenced by pH, buffer nature or buffer ionic strength of the mobile phase. These experimental parameters apparently acted on ion exchange retention mechanism by ionic competition.

To evaluate the effect of pH on retention, log k_{90} values of 13 β-blockers and 6 permanent cations were measured using the same buffer system (ammonium acetate I = 20 mM) at ${}^{w}_{w}pH$ 4 and 5. The relationship between log k_{90} values measured using these 2 different mobile phases compositions represented in Fig. 6-4 has shown to be linear, except for carvedilol which is outlier.

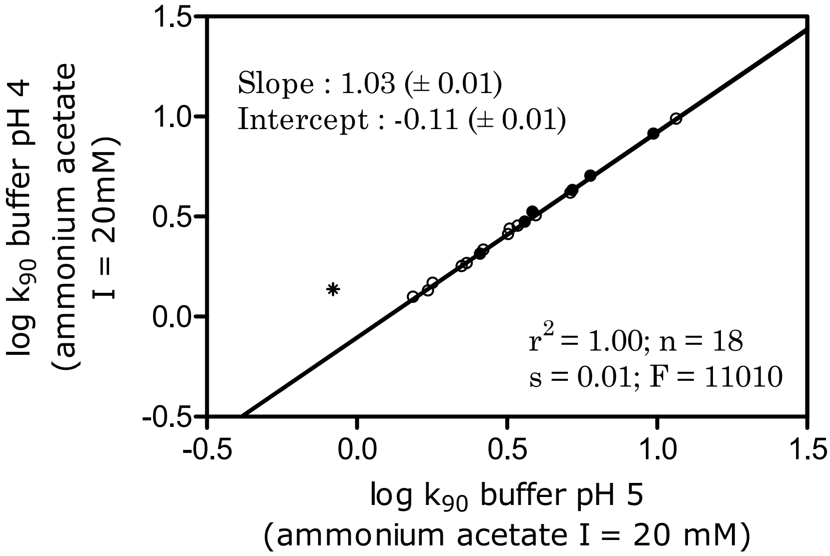

Fig. 6-4 Relationship between log k_{90} (ammonium acetate pH 5, I = 20 mM) and log k_{90} (ammonium acetate pH 4, I = 20 mM). ○ β-blockers, ● permanent cations, * carvedilol (outlier).

According to literature data (Table 6-1), this solute has a pK_a of 7.97, while all other tested β-blockers have a pK_a higher than or equal to 9.5. Considering the high amount of ACN in the mobile phase, ${}^{s}_{w}pH$ measured in presence of 90% ACN was highly increased, (${}^{s}_{w}pH$ = 6.9 and 7.6) compared to ${}^{w}_{w}pH$ values (4.0 and 5.0 respectively). A change of carvedilol ionization state between pH 4 and pH 5 is than likely to be responsible for the deviant behaviour of this compound. In Fig. 6-4 and subsequent figures, permanent cations were added as control solutes being entirely ionized independently of the mobile phase pH. Except carvedilol, all β-blockers were on the same regression line than permanent cations, confirming that ionizable compounds were 100% under their cationic form in the tested conditions.

To evaluate the effect of buffer ions on retention, log k_{90} values of 13 β-blockers and 6 permanent cations were measured using two different mobile phases, having the same pH (${}^{w}_{w}pH$ 4) and the same ionic strength (I = 20 mM), but using two different buffer types, namely ammonium formate or ammonium acetate. Results were reported in Fig. 6-5 and showed that a linear correlation was found between log k obtained in these two different mobile phases.

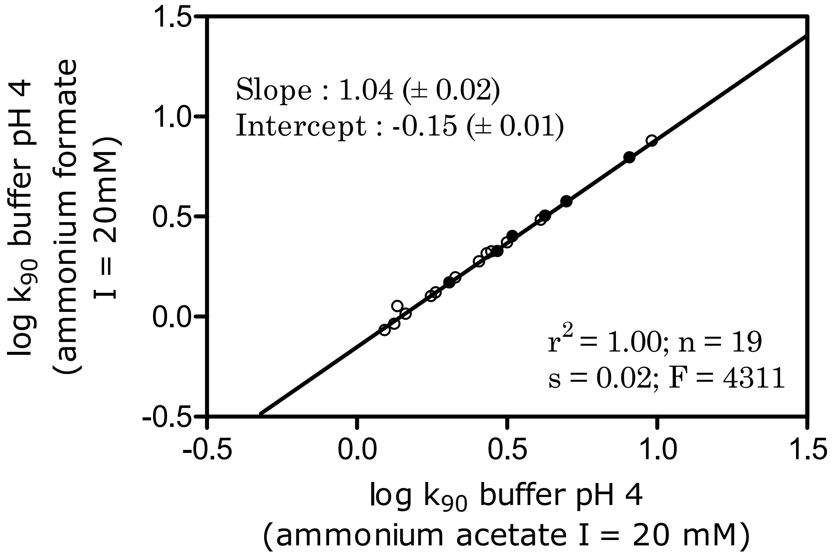

Fig. 6-5: Relationship between log k_{90} (ammonium acetate pH 4, I = 20 mM) and log k_{90} (ammonium formate pH 4, I = 20 mM). ○ β-blockers, ● permanent cations.

Even if the slope of the regression line was almost equal to 1, the y-intercept was significantly different from 0, indicating that the nature of buffer counter-ions can appreciably influence solutes retention, this effect being not compound dependent.

Influence of ionic strength (I) on retention was first assessed using buffers ammonium formate ${}^{w}_{w}\mathrm{pH}$ 3 (Fig. 6-6 A and B) having either I = 20 mM or I = 100 mM.

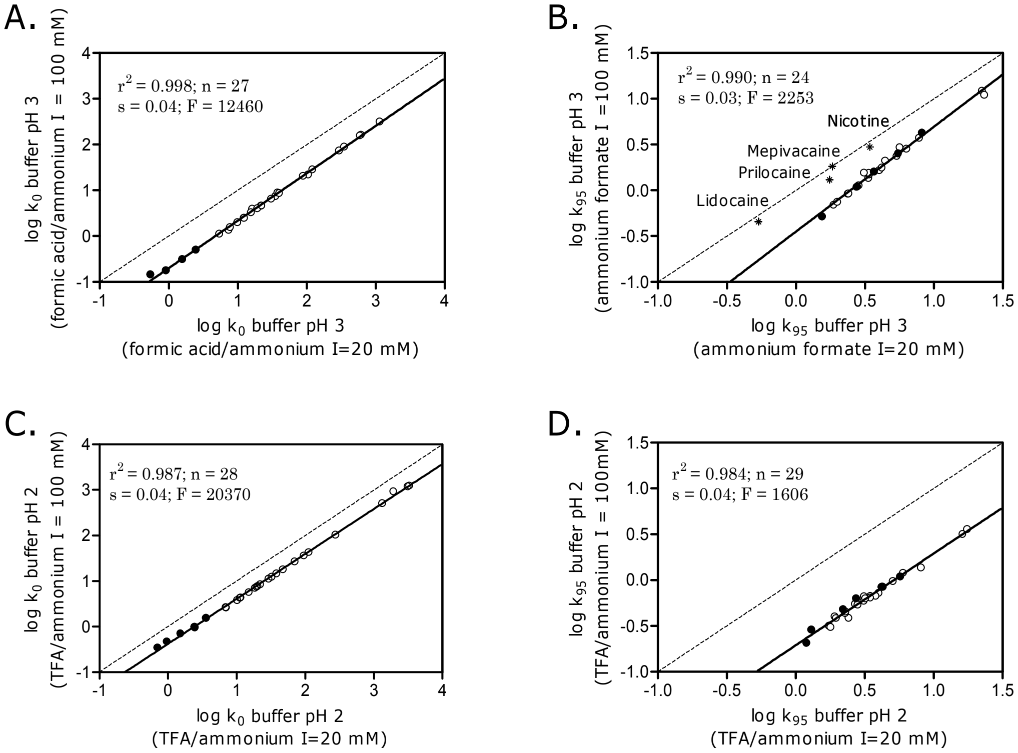

Fig. 6-6 Comparison of the effect of ionic strength on retention at pH 3 and pH 2. A. Mobile phase : buffer ammonium formate pH 3. B. Mobile phase (95:5, v/v) ACN / buffer ammonium formate pH 3. C. Mobile phase buffer pH 2 TFA/ammonium. D. Mobile phase (95:5, v/v) ACN / buffer pH 2 TFA/ammonium. ○ Ionizable compounds ● Permanent cations ∗ Outliers.

In Fig. 6-6 A, where the mobile phase was 100% aqueous, a perfect linear relationship was observed between the logarithm of the retention factor at the two tested ionic strengths, meaning that effect of ionic strength is not compound dependent in these conditions. Fig. 6-6 A also showed that, due to the contribution of ion exchange retention, increasing ionic strength of the mobile phase (especially ammonium cations) resulted in a decreased retention of cationic compounds, as already shown by McCalley et al. [38] on bare silica columns.

In Fig. 6-6 B where the mobile phase was composed of 95% ACN, the linear correlation was not as good as in pure aqueous mobile phase and 4 solutes (nicotine, mepivacaine, prilocaine, lidocaine) were clearly outliers. These 4 bases were also among the tested compounds having the lowest pK_a (Table 6-1) and an incomplete ionization was consequently suspected. Solutes having an incomplete ionization will show a competition with buffer cations (ammonium) that will not be equivalent to other compounds. This assumption was confirmed by the measure of the apparent ${}^{s}_{w}\mathrm{pH}$ of the mobile phase containing 95% ACN, which was highly increased (6.4 approximately) compared to ${}^{w}_{w}\mathrm{pH}$ (3.0). Moreover, apparent pK_a (${}^{s}_{w}\mathrm{pK_a}$) of basic compounds in hydro-organic mixtures, is known to be shifted to lower values compared to pure aqueous pK_a (${}^{w}_{w}\mathrm{pK_a}$), which is also likely to decrease the protonation of these compounds.

These results have shown the necessity to use buffers having ${}^{w}_{w}\mathrm{pH}$ of lower values to have moderate bases (pKa 7.5 – 8) entirely under their cationic forms. Measures of retention factors were then assessed in mobile phases composed of ACN and aqueous buffers pH 2 TFA/ammonium ${}^{w}_{w}\mathrm{pH}$ 2 (Fig. 6-6 C and D) having either I = 20 mM or I = 100 mM. Linear regressions were obtained in both Fig. 6-6 C and D, which showed that all solutes were 100% ionized using both mobile phases compositions.

On all graphs of Fig. 6-6, permanent cations were on the same regression line than other ionizable compounds (except the 4 outliers in Fig. 6-6 B), which confirm that tested compounds were totally ionized at the tested pH.

These results show that $\Delta\log k_{0\text{-}95}$ parameter measured by HILIC technique could be used to obtain $\log P^{N}_{oct}$ of basic compounds, thus offering a promising alternative to RPLC, in particular for polar basic solutes which are hardly retained under reverse phase conditions.

Guidelines for partition coefficients measurements

For lipophilicity measurement, these results involve that both $\log k_{95}$ and $\log k_{0}$ have to be measured using a rigorously identical buffer. Moreover, a specific calibration curve ($\Delta\log k_{0\text{-}95}$ vs $\log P^{N}_{oct}$) will also be required for each buffer nature, pH and ionic strength. Although these experimental restrictions can at first sight appear as drawbacks, it can also be an advantage, because it offers the possibility to adapt experimental conditions to the tested compounds. Ionic strength is for example a parameter that can be easily changed to modulate solutes retention. In this study a high ionic strength (100 mM) was used to minimize compounds retention times. Inversely, it is also conceivable to decrease ionic strength for a set of poorly retained solutes.

Conclusion

Although the retention mechanism of charged compounds in HILIC mode is much more complicated than for neutral solutes, it was demonstrated that the difference between 2 isocratic $\log k$ values ($\Delta\log k_{95\text{-}0}$) was well correlated with $\log P^{N}_{oct}$ values thus offering an innovative promising way of obtaining octanol-water partition coefficient data. This technique is particularly interesting for strong basic compounds, which are hardly measurable under unionized form by RPLC due to chemical instability of many stationary phases at high pH conditions. Results have also shown that pH, buffer nature or buffer ionic strength of the mobile phase were important parameters that could highly influence retention factors of cationic solutes in HILIC mode, and therefore needs to be rigorously controlled to ensure a good reproducibility between experiments.

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Chapter 7 : Conclusion

Physicochemical properties are well-known parameters to predict ADMET behaviour of drug candidates. The trends in pharmaceutical research, is to measure these properties earlier in the drug discovery process in a high throughput way. Despite the well-known advantages of these HTS approaches, there are still needs for further improvements in the quality of the data generated.

In this work, a HTS UV method for solubility measurement was first optimized to obtain solubility data related to thermodynamic solubility. The compound dependent increase of solubility generally registered using these HTS methods has been identified as being mainly due to the formation of supersaturated solutions. Supersaturation has shown to be minimized by enhancing shaking rate and incubation times, thus giving results in good agreement with thermodynamic solubility.

The optimized HTS UV method was then successfully adapted to measure biorelevant solubility in two different *in vitro* intestino-mimetic media (FaSSIF and FeSSIF) and excellent correlations were obtained with FaSSIF and FeSSIF solubility data measured by shake-flask method. Thus, this method provides a reliable high throughput and compound sparing way to measure intestino-mimetic solubility at the early stages of drug discovery. Moreover, the expensive components of these intestino-mimetic media (taurocholic acid and lecithin) are added as small volumes of a separated solution, which decrease the costs of these experiments.

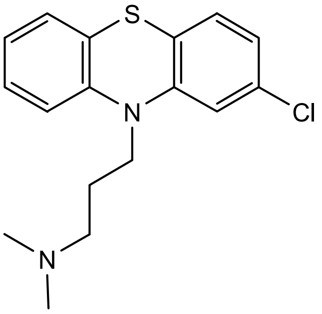
The general methodology presented in this work is promising, because it can easily be adapted to other biorelevant and more complex media that better mimic *in vivo* conditions, and there are still place for further progresses and developments towards the measurement of data adapted to the requirements of drug discovery within reasonable costs.

Furthermore, in spite of the progresses realized in lipophilicity measurements, the lipophilicity of some categories of compounds are still hardly measurable. Hydrophilic interaction chromatography (HILIC), a technique retaining polar and charged compounds, was evaluated as a new approach to measure lipophilicity of polar neutral and basic compounds. For polar neutral compounds, the LSER analysis performed on retention factors has shown that intermolecular forces involved in HILIC retention especially the importance of α and β contributions were similar to those involved in the partition process in alkane/water system. Therefore HILIC provide an alternative way to obtain $\log P_{alk}$ for polar neutral compounds.

For basic organic compounds, a category of compounds hardly measurable by conventional silica based RP stationary phases, a combination of retention information measured using two diametrically opposite mobile phase compositions (i.e. 0% and 95% (v/v) of ACN in the mobile phase) were used to obtain octanol/water partition coefficient. A good linear regression was obtained between $\Delta\log k_{0\text{-}95}$ and $\log P^{N}{}_{oct}$ Thus, this method offers, when the composition of the mobile phase is strictly controlled, a new and innovative way of obtaining octanol/water partition coefficients data for basic compounds.

The recent advances in chromatographic sciences and in particular the emergence of Ultra Performance Liquid Chromatography (UPLC) also give promising perspectives for further improvement to increase the throughput of lipophilicity measurements without compromising chromatographic performance. Therefore, to speed up lipophilicity measurements, both HILIC methods developed in this work should be adapted and transferred to UPLC using HILIC UPLC columns that just begin to reach the market.

Structures of tested compounds



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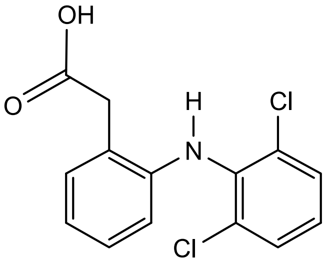
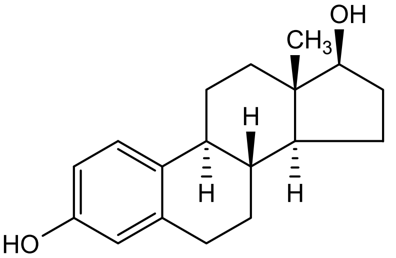
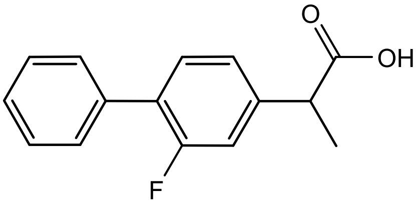


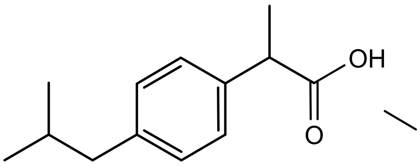
Diclofenac



Estradiol



Flurbiprofen



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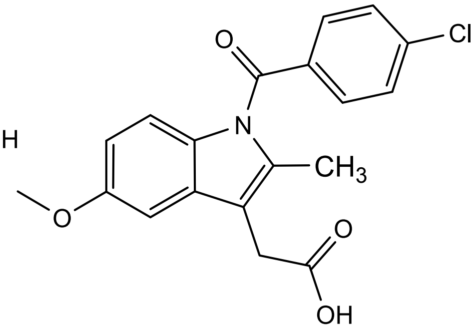
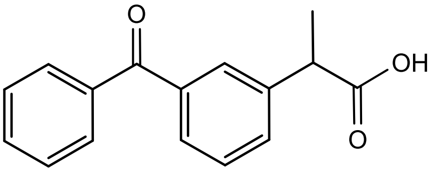
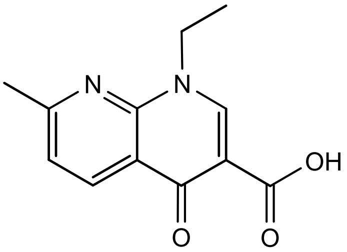


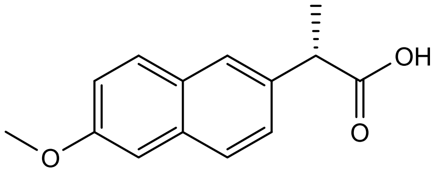
Indomethacine



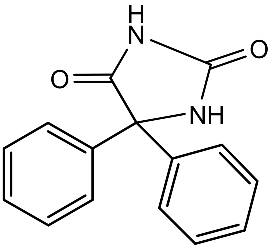
Ketoprofen



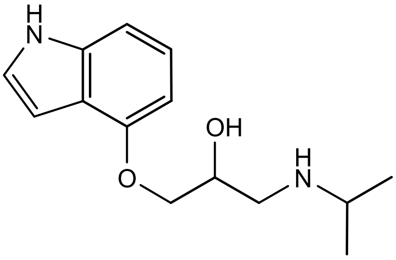
Nalidixic acid



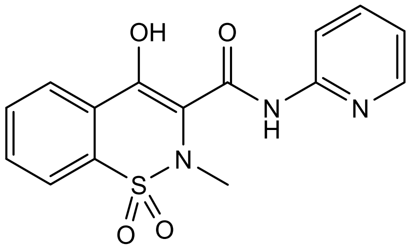
Naproxen



Phenytoin



Pindolol



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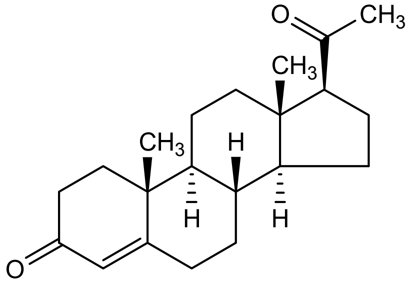
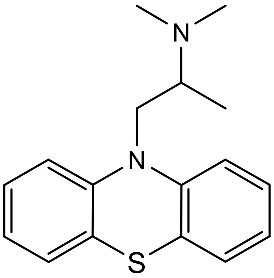


Progesterone



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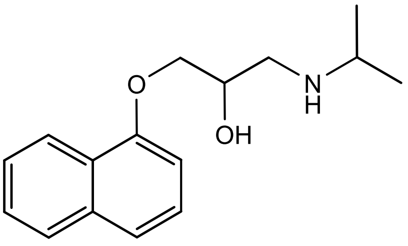

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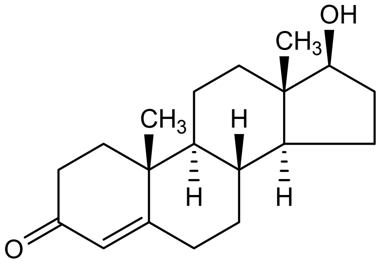
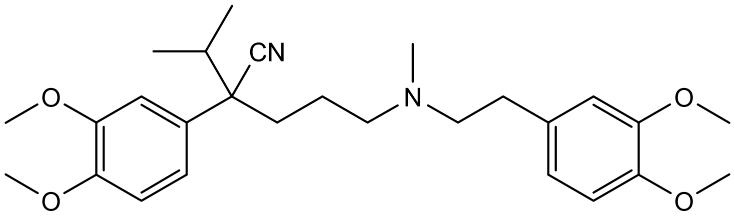
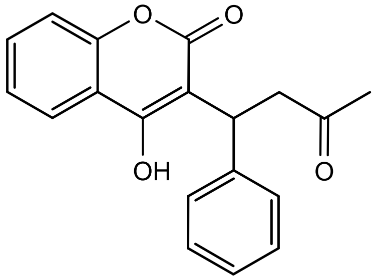


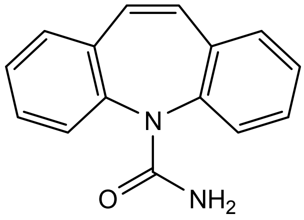
Testosterone



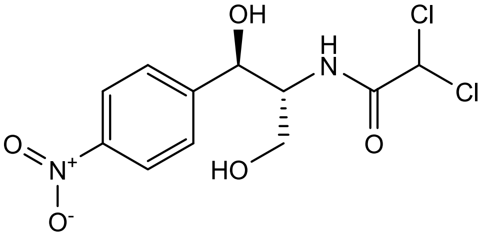
Verapamil



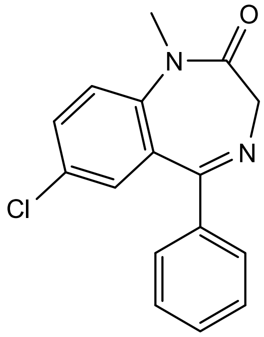
Warfarin



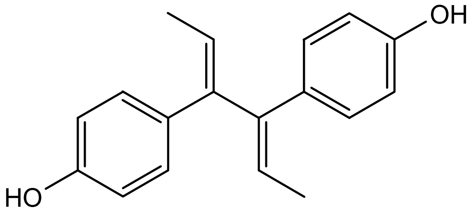
Carbamazepine



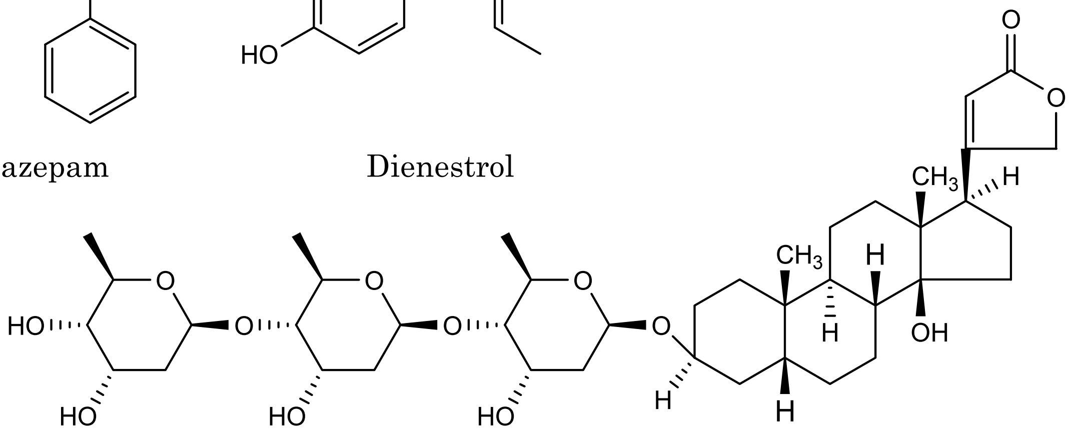
Chloramphenicol



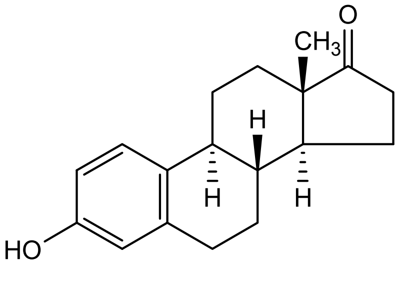
Diazepam



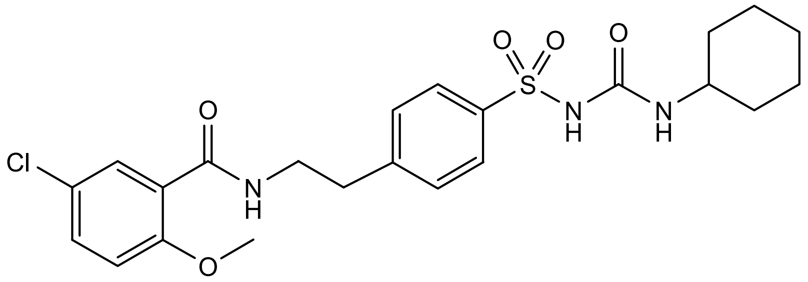
Dienestrol



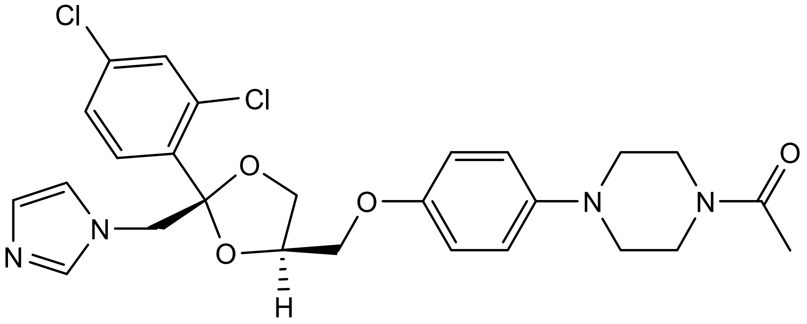
Digitoxin



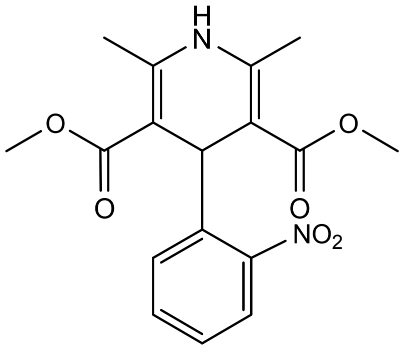
Estrone



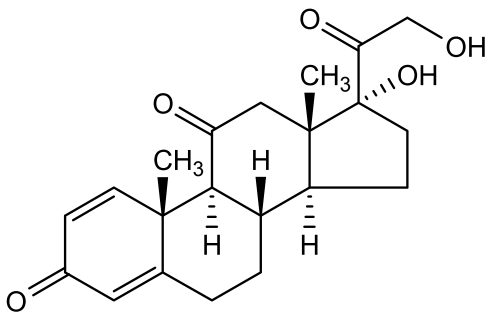
Glybenclamide



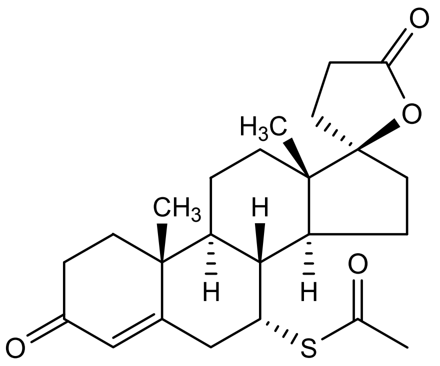
Ketoconazole



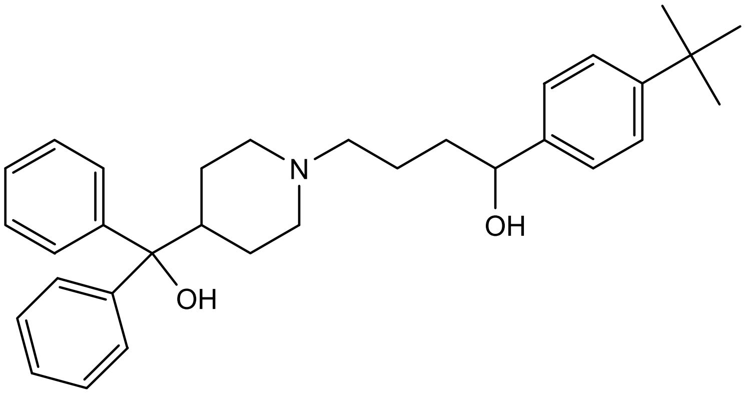
Nifedipine



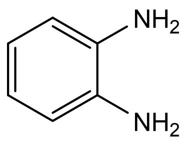
Prednisone



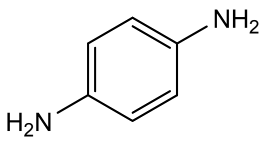
Spironolactone



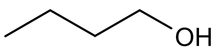
Terfenadine



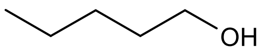
1,2-phenylenediamine



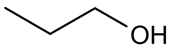
1,4-phenylenediamine



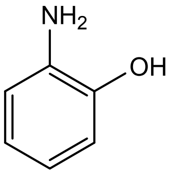
1-butanol



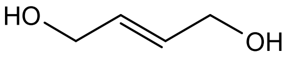
1-pentanol



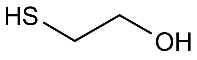
1-propranol



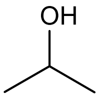
2-aminophenol



2-butene-1,4-diol



2-mercaptoethanol



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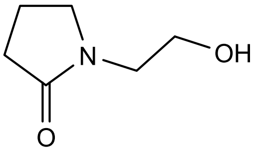
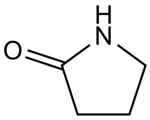
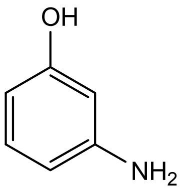


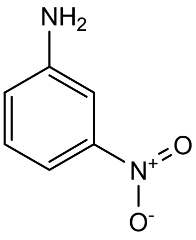
2-pyrrolidinone,
1-(2-hydroxyethyl)-



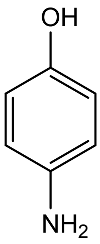
2-pyrrolidinone



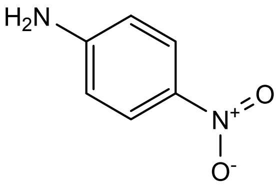
3-aminophenol



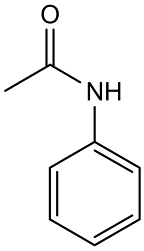
3-nitroaniline



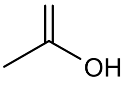
4-aminophenol



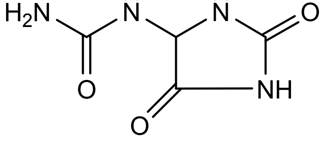
4-nitroaniline



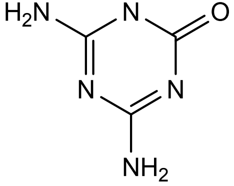
Acetanilide



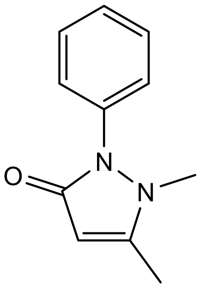
Acetic acid



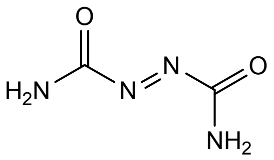
Allantoin



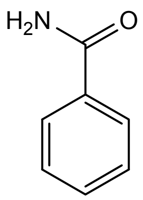
Ammeline



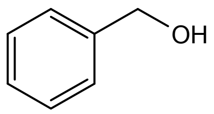
Antipyrine



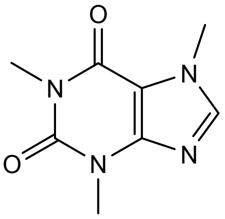
Azodicarbonamide



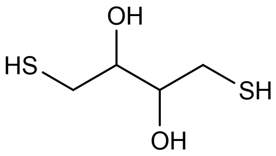
Benzamide



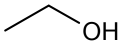
Benzyl alcohol



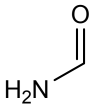
Caffeine



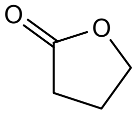
Dithiothreitol



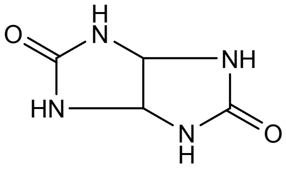
Ethanol



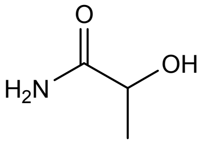
Formamide



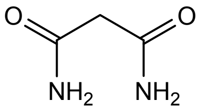
gamma-butyrolactone



Glycoluril



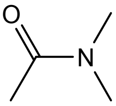
Lactamide



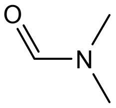
Malonamide

CH_3OH

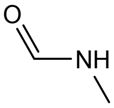
Methanol



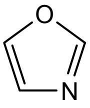
N,N-dimethylacetamide



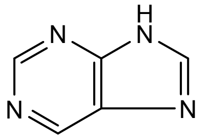
N,N-dimethylformamide



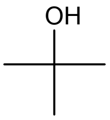
N-methylformamide



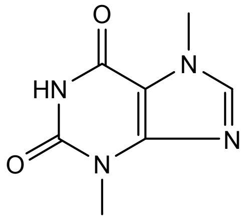
Oxazole



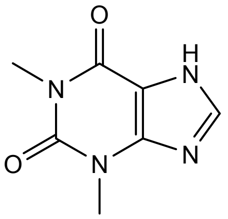
Purine



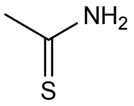
tert-butanol



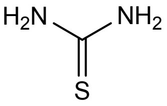
Theobromine



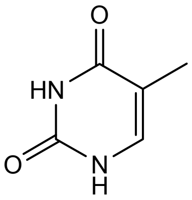
Theophylline



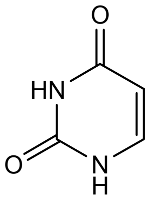
Thioacetamide



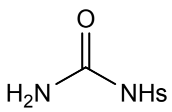
Thiourea



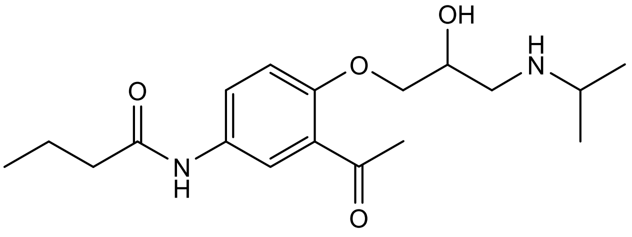
Thymine



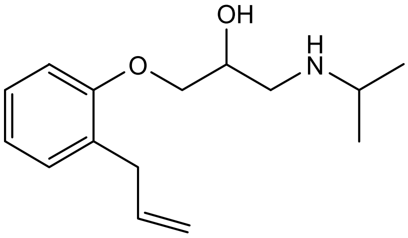
Uracil



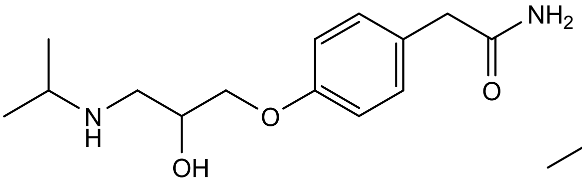
Urea



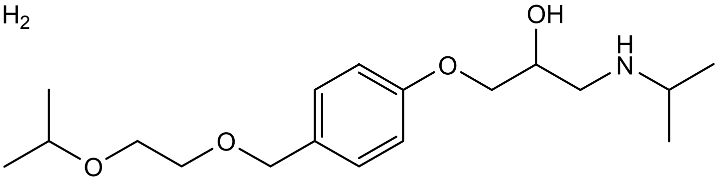
Acebutolol



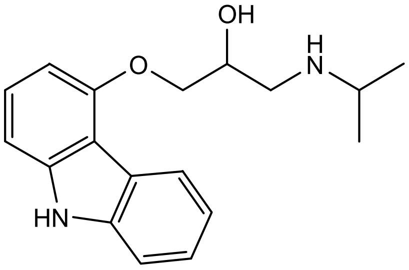
Alprenolol



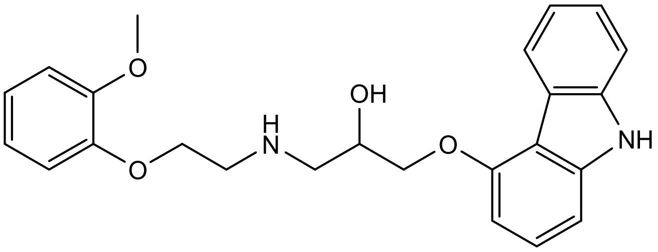
Atenolol



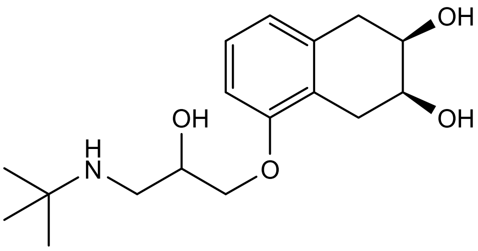
Bisoprolol



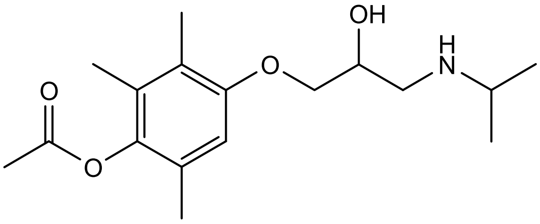
Carazolol



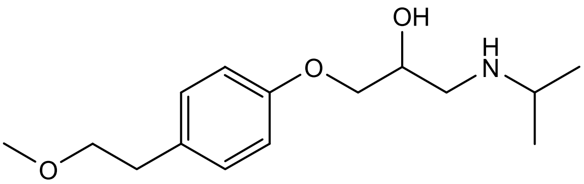
Carvedilol



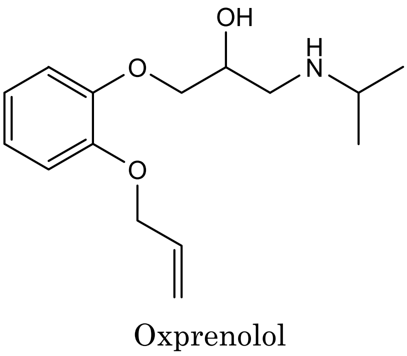
Nadolol



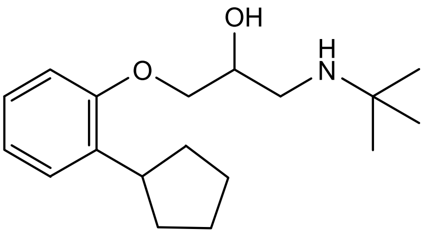
Metipranolol



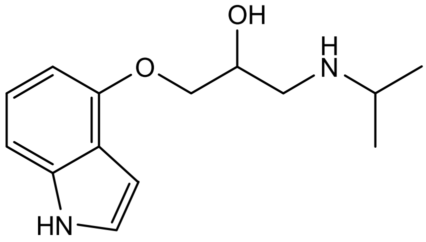
Metoprolol



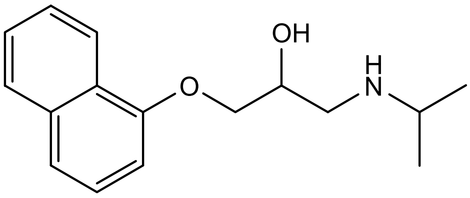
Oxprenolol



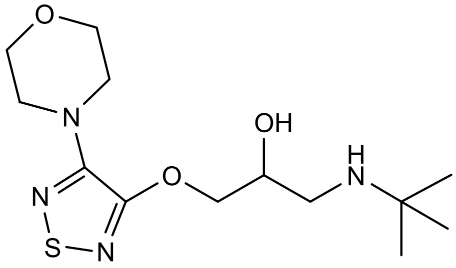
Penbutolol



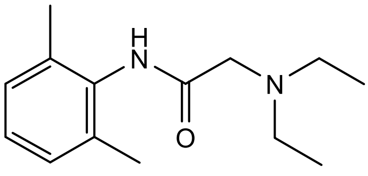
Pindolol



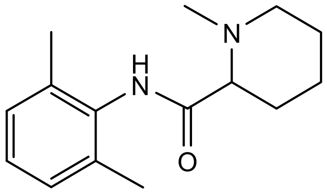
Propranolol



Timolol



Lidocaine



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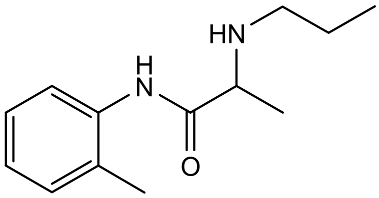

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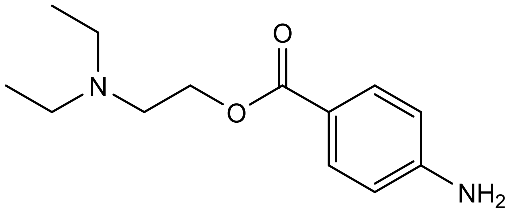
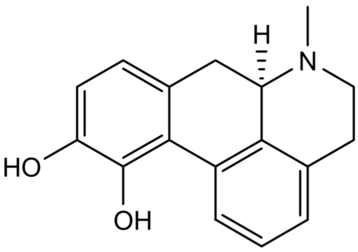
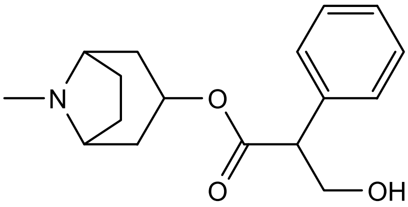


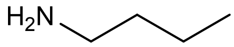
Procaine



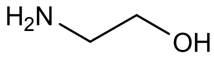
Apomorphine



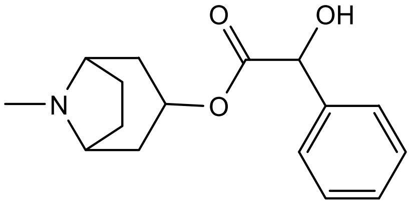
Atropine



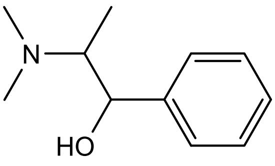
Butylamine



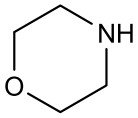
Ethanolamine



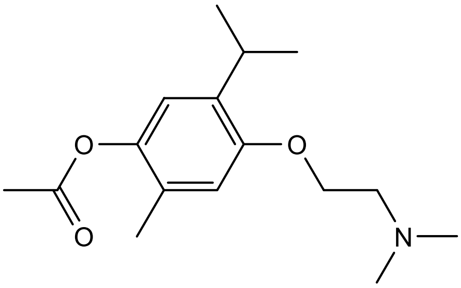
Homatropine



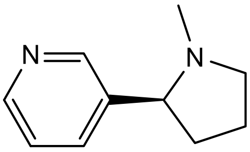
Methylephedrine



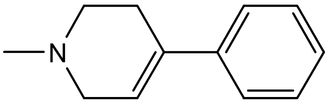
Morpholine



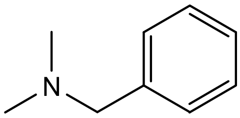
Moxisylyte



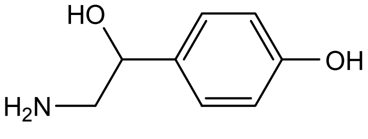
Nicotine



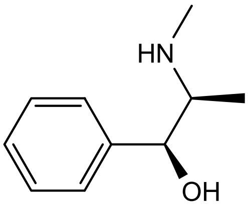
MPTP



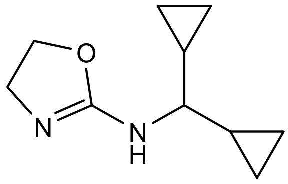
N,N-dimethylbenzylamine



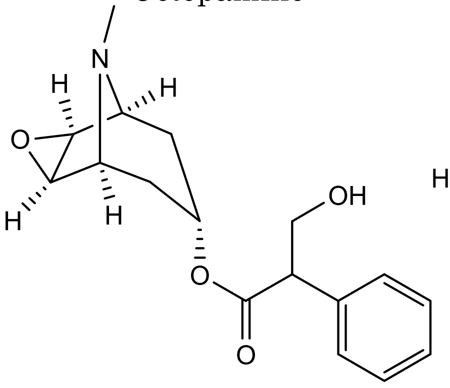
Octopamine



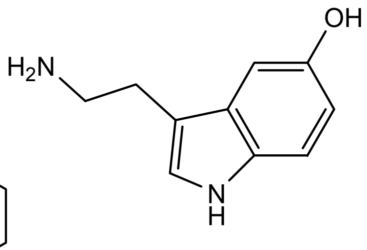
Pseudoephedrine



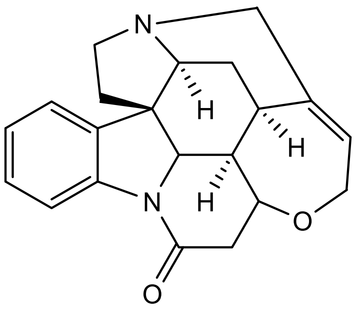
Rilmenidine



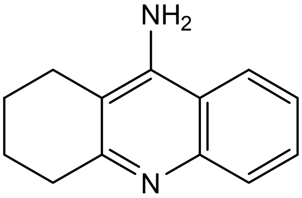
Scopolamine



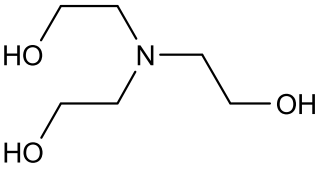
Serotonine



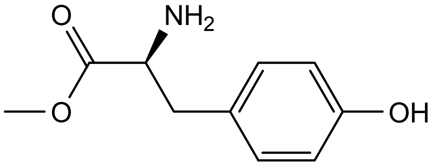
Strychnine



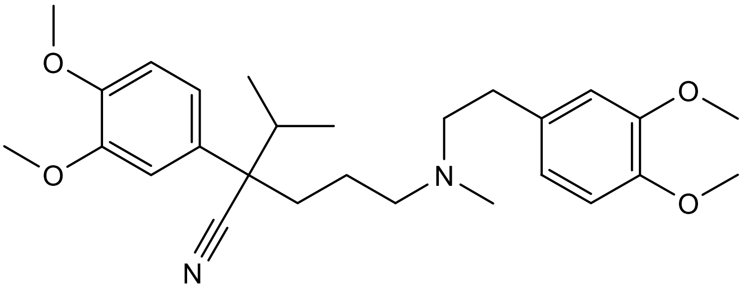
Tacrine



Triethanolamine



Tyrosine methyl ester



Verapamil