Development of methods for determination of physico-chemical parameters (pK_a, log$P$) of water-insoluble compounds in early phase of drug discovery

Theses of doctoral (PhD) dissertation

Gergely Völgyi

Semmelweis University

Doctoral School of Pharmaceutical and Pharmacological Sciences

Supervisor:	Prof. Dr. Krisztina Takács-Novák, D. Sc.
Opponents:	Dr. Éva Szökő, D. Sc.
	Prof. Dr. György Miklós Keserű, D. Sc.
Head of Examination Committee:	Prof. Dr. Imre Klebovich, D. Sc.
Members of Examination Committee:	Dr. Pál Perjési, C. Sc.
	Dr. Miklós Józan, Ph. D.

Semmelweis University, Department of Pharmaceutical Chemistry

Budapest, 2007

1 Introduction

The physico-chemical profiling (solubility, ionization, lipophilicity, permeability) of the newly synthetised compounds provide useful information for the forecast of pharmacokinetic parameters and biological effects.

The new paradigm in drug research introduced in the early 90-ies has increased the rate of finding of biologically active molecules. It is due to the new technologies such as combinatoric chemistry and high throughput screening technologies. These technologies have been proved to be effective in lead discovery. However the bottleneck of drug research has shifted from hit and lead discovery to lead optimization and even more to the selection of potentially drug-like molecules. This required the physico-chemical profiling in early stage of drug development. New, high capacity methods have been developed.

However, modern techniques of drug discovery often produce molecules that are very poorly soluble in water, and the assessment of physico-chemical property such as ionization and lipophilicity in aqueous solution can be difficult and problematic, because many assays require the drugs to be in solution during measurement.

Therefore the previously applied procedures can only be used restrictedly. Every method which makes the high throughput physico-chemical profiling of many very sparingly soluble compounds possible, it has considerable benefits in early drug discovery.

2 Objectives

2.1 Development of methods for pK_a determination of water-insoluble compounds

The cosolvent method (mixed solvent procedure) is the most widely used procedure for the pK_a determination of water-insoluble compounds. The solubility of some unionized molecules can be enhanced by mixing solvents such as methanol, dioxane or acetonitrile with water, but experience shows that not all compounds dissolve in any single solvent-water mixture.

The main objectives were the followings:

(1) Investigation of solubility of structurally diverse substances in different cosolvent systems in order to find the most universal multicomponent cosolvent system.

(2) Physico-chemical characterization of the new cosolvent system (determination of relative density and relative permittivity).

(3) Study of the solvation by quantum chemical calculation.

(4) Investigation of the applicability of the mixed-solvent procedure for pK_a determination in the new cosolvent system.

(5) Validation.

2.2 Development of standardized reversed-phase thin-layer chromatographic method for log*P* determination

Determination of log*P* value of drug candidates is an everyday routine in drug research. Due to the well-known limitations of direct log*P* measurements (like shake-flask method or dual-phase potentiometry) reversed-phase chromatography (TLC or HPLC) is widely used for log*P* estimation of highly lipophilic or nonionizable compounds. These indirect methods provide reliable

log*P* values if the calibration equation has been set up using closely related compounds. Since in the early stage of drug research such calibration set is rarely available there is a need for general method.

The main objective of the this study was to develop an optimized and validated reversed-phased thin-layer chromatography (RP-TLC) method for estimation of log*P* values of chemically diverse, lipophilic, neutral compounds or weak acids and bases.

3 Methods

3.1 Preparation of MDM-water mixtures

For p_sK_a (cosolvent dissociation constant) determination a 60 %/v MDM stock solution was prepared which contained 20 %/v methanol (**M**eOH), 20 %/v 1,4-**d**ioxane, 20 %/v acetonitrile (**M**eCN), 40%/v distilled water and KCl in 0.15 M concentration, then diluted with 0.15 M ionic strength adjusted water until the desired MDM concentration was reached.

3.2 Determination of relative density

The densities of MDM-water mixtures were measured using a pycnometer of 25mL volume according to the specification of the Hungarian Pharmacopoeia. This method is also described in the European Pharmacopoeia.

3.3 Determination of relative permittivity

Relative permittivities of MDM-water mixtures were determined by a Universal Dielectrometer (Type: OH-301, Radelkis, Hungary) at constant temperature (25.0 ± 0.1°C). The capacity of the instrument's condenser (measured in vacuum between the plates of the condenser) increases when filled with dielectric medium according to the equation

$C = \varepsilon_r C_o$,

where ε_r is the relative permittivity of the dielectric medium, C_o is the electric capacity in vacuum and C is the electric capacity in the medium. The following relationship describes the dielectric constant of MDM-water mixtures:

$$\varepsilon_{MDM} = (\varepsilon_{water} - 1)\frac{C_{MDM} - C_0}{C_{water} - C_0} + 1$$

The relative permittivity of distilled water is well-known (ε_{water} = 78.3), so the relative permittivity of MDM-water mixtures can be calculated from the measured capacities.

3.4 Potentiometric pK_a determination

GLpKa automated pK_a analyser (Sirius Analytical Instruments Ltd., Forest Row, UK) fitted with combination Ag/AgCl pH electrode was used for determination of dissociation constants. The pK_a and p$_s K_a$ values were calculated by RefinementPro^{TM} software (Sirius Analytical Instruments Ltd., Forest Row, UK).

The four-parameter technique (Four Plus^{TM} method) was used for electrode calibration in both aqueous medium and MDM-mixtures.

For bases and ampholytes, in each experiment, 10.00 ml of a 1 mM aqueous solution of sample was preacidified to pH 1.8-2.0 with 0.5 M HCl, and then titrated with 0.5 M KOH to an appropriately high pH, usually 12. In the case of acids, the titration was performed in the opposite direction. The titrations were carried out at constant ionic strength (I = 0.15 M KCl) and temperature (t = 25.0 ± 0.5 ºC), and under nitrogen atmosphere. The pK_a values of samples were calculated by RefinementPro^{TM} software.

The cosolvent dissociation constants (p$_s K_a$ values) of the compounds were also determined in various MDM-water mixtures between 15-56 wt%. The same titration protocol was performed as above. Each sample was measured at least in four different MDM-water mixtures. To obtain the best aqueous pK_a value from p$_s K_a$ data three different extrapolation methods have been tried. First, the traditional plot of p$_s K_a$ versus R (wt% of organic solvents) was applied using

$$p_s K_a = a\, R_{wt\%} + b$$

equation.

The second extrapolation method is based on the linear relation between p_sK_a and the dielectric constant (ε) of cosolvent mixture:

$p_sK_a = a/\varepsilon + b$

The third method known as Yasuda-Shedlovsky extrapolation also establishes a correlation with the dielectric constant but uses a modified equation:

$p_sK_a + \log[H_2O] = a/\varepsilon + b$

where $\log[H_2O]$ is the molar water concentration of the given solvent mixture.

3.5 Spectrophotometric pK_a determination

The UV/pH titrations were performed using D-PAS technique (Sirius Analytical Instruments Ltd., Forest Row, UK) attached to a GLpKa. The pK_a and p_sK_a values were calculated by RefinementPro™ software. All measurements were performed in solutions of 0.15 M KCl under nitrogen atmosphere, at t = 25.0 ± 0.5 ºC. Sample concentrations of 5-100 μM were used for UV/pH titration.

3.6 Determination of log*P* values by the shake-flask method

The octanol-water partition coefficients of compounds of the calibration set were measured using the traditional shake-flask technique at 25.0 ± 0.1 ºC temperature. The organic and the aqueous phases were mutually saturated before the experiments. The samples were dissolved in aqueous Britton-Robinson buffer solution (stock solution: 1-6 mg/100 ml) and aliquots of the stock solution were equilibrated with n-octanol for 1 h in a thermostatted shaker (Lauda, M20S). The phase ratio was varied from 5 ml/10 ml to 0.1 ml/50 ml (n-octanol/water) depending on the expected log*P* value of the examined compound. After separation of the equilibrated phases (by centrifugation at 2000 g for 10 min) the concentration decrease of the solute was determined in the

aqueous phase by UV spectrophotometry (Hewlett-Packard 8452A, UV-Vis spectrophotometer) at the λ_{max} above 230 nm of each compound.

3.7 Determination of log*P* values by the RP-TLC method

The RPTLC experiments were performed on 20 cm x 20 cm chromatography plates pre-coated with 0.25 mm layers of silanized silica gel $60F_{254}$ (Merck, Germany, article 5747) or on RP-8 F_{254}s (Merck). The samples were dissolved in methanol or in 1:1 methanol-chloroform mixture (c = 0.5 or 1 mg/ml) and 0.5 µl of these solutions was spotted onto the plate. Methanol-water, ethanol-water, 1-propanol-water, 2-propanol-water, acetone-water, acetonitrile-water and 1,4-dioxane-water mixtures were used as mobile phases. The paper-lined chromatography chamber (Camag) was saturated with the actual mobile phase for at least 30 min before development. After development (150 mm) the plates were dried and the spots were detected by densitometry (λ = 200 and 254 nm; Shimadzu, CS-9301PC).

4 Results and conclusions

4.1 Development of methods for pK_a determination of water-insoluble compounds

The four-component cosolvent system, MDM dissolves sufficiently the lipophilic compounds and it can be applied for compounds which are not soluble in methanol-water or other single organic cosolvent mixtures (e.g. 2-propanol, DMF, DMSO, acetone, etc.). However, MDM also dissolves polar compounds so it can be considered a more universal cosolvent for pK_a determination in drug research (Figure 1).

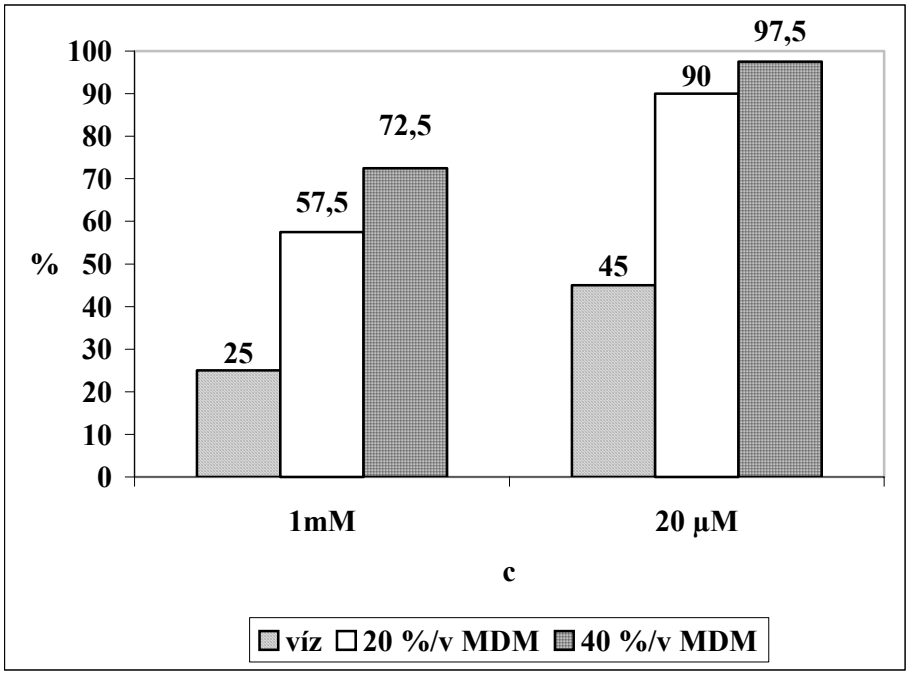

Figure 1. Improvement of the solubility of 40 samples in MDM-water mixtures (expressed as % of molecules dissolved in two analytical quantities: 1 mM and 20 µM concentrations).

The physicochemical characteristics of MDM-water mixtures (relative density, relative permittivity) were determined.

The solvation properties of MDM-water mixture have been evaluated using computer simulations based on molecular modeling via Monte Carlo and molecular dynamics simulations, which found for a series of fluoroquinolones that water mainly solvated the polar sites of the solutes, while dioxane was the most important organic component.

The pK_a determination in MDM-water mixtures was validated:

(1) The application of MDM-water mixture improves the solubility of poorly water soluble drugs and therefore their p$_s K_a$ values could be measured at lower proportion of organic solvent in MDM-water mixture than in methanol-water mixture (Table 1). This makes it possible to avoid the long-distance extrapolations from organic solvent-rich regions (R > 40 wt%) thereby resulting in more reliable extrapolated pK_a values to zero organic solvent content for lots of poorly soluble compounds.

Compound	**Methanol (wt%)**	**MDM (wt%)**
Chlorpromazine HCl	34	16
Diphenoxylate HCl	44	38
Haloperidol	40	34
Hydrochlorotiazide	23	16
Sertraline HCl	43	26

Table 1. The lowest methanol and MDM content, in which the compounds do not precipitate.

(2) The cosolvent dissociation constants (p$_s K_a$) of 50 compounds were determined in 15-56 wt% MDM-water mixtures by potentiometric or spectrophotometric methods.

(3) The MDM-water mixtures did not cause large shifts in p$_s K_a$ values and the Yasuda-Shedlovsky extrapolation procedure was proposed to obtain the aqueous pK_a values. The extrapolated data are in good agreement with pK_a values measured in water (Figure 2). The average deviation is ΔpK_a = 0.13.

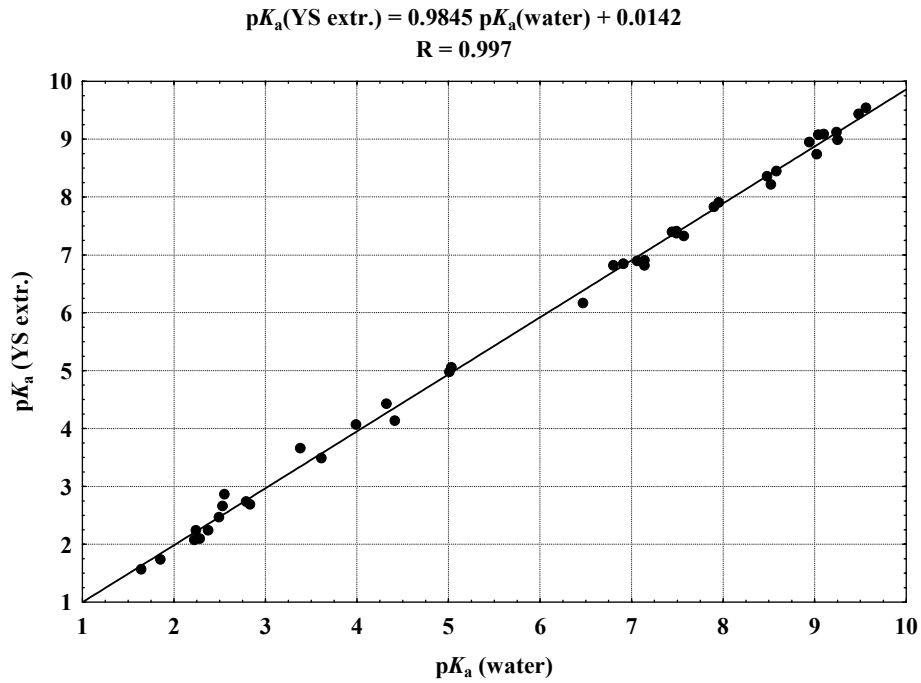

Figure 2. Relationship between aqueous pK_a values and the pK_a values extrapolated from MDM-water mixture.

(4) The linearity of the Yasuda-Shedlovsky equations is valid up to 55 wt% MDM-mixture (ε = 48).

(5) The recently developed SGA method for high throughput pK_a determination can be extended with measurements in 20 %/v MDM. Our results provide general calibration equations for acids and bases:

for acids: $pK_{a(aqueous)} = 1.016\ p_sK_{a\ (20\ \%/v\ MDM)} - 0.382$

for bases: $pK_{a(aqueous)} = 0.992\ p_sK_{a\ (20\ \%/v\ MDM)} + 0.256$

Thus the single point estimation procedure may provide rapid aqueous pK_a values for water-insoluble compounds in the early phase of drug research.

4.2 Development of standardized reversed-phase thin-layer chromatographic method for log*P* determination

A validated reversed-phase thin-layer chromatographic (RPTLC) method was developed for parallel estimation of lipophilicity of chemically diverse,

neutral compounds or weak acids and bases. To cover a wide range of lipophilicity two, optimized chromatographic systems were developed: one for the log*P* determination of less or moderately lipophilic (log*P*: 0-3) and one for highly lipophilic (log*P*: 3 – 6) compounds. The method uses general calibration equations obtained with chemically non-related compounds. RP-diC_1 silanized silica gel plates were applied as stationary phase. Several organic solvent-water systems have been tried as mobile phase, and the acetone-water mixture was found to be optimal in both systems with respect of the correlation of R_M values to the octanol/water partition coefficients.

TLC/logP$_{0-3}$ system

The acetone-water mixture 45 + 55 (V/V) was found to be optimal for the estimation of log*P* values in 0-3 range. The calibration set contains seven compounds, namely caffeine, acetaminophen, acetanilide, hydrocortisone, propyphenazone, nitrazepam, and diazepam.

TLC/logP$_{3-6}$ system

The optimized chromatographic system consists of RP-diC_1 silanized silica gel as stationary phase and acetone-water 60 + 40 (V/V) mixture as mobile phase. The calibration set contains five compounds, namely diazepam, benzophenone, biphenyl, simvastatin, and tolnaftate. The diazepam acts as connecting link between the two calibration sets.

The universal applicability of the optimized chromatographic systems was then tested using 20 randomly selected structurally diverse compounds. Mainly, there was good agreement between the log*P* values obtained by shake-flask method and by RPTLC technique.

The following prescription is suggested for the determination of logP_{TLC} value:

(1) for compounds of unknown lipophilicity first the appropriate system has to be selected based on predicted log*P* value;

(2) the compounds have to be run together with the appropriate calibration set either using 45 % or 60 % acetone/water system;

(3) then R_M values from three parallel chromatographic runs must be determined;

(4) finally with the help of calibration equation set up in the same experiments the log*P* values can be obtained.

On one plate 15-20 compounds can be simultaneously investigated. The proposed two TLC experiments with the automation of the sample application and imaging detection of the compounds can be considered as a possible alternative for fast and acceptable accurate estimation of lipophilicity of drug candidates in the early phase of drug research.

5 References

Papers of the thesis work

1. **Völgyi G.,** Ruiz R., Box K., Comer J., Bosch E., Takács-Novák K. Potentiometric and spectrophotometric pK_a determination of water-insoluble compounds: validation study in a new cosolvent system. *Anal. Chim. Acta* **2007,** *583,* 418-428. IF: 2,894

2. Box K.J., **Völgyi G.,** Ruiz R., Comer J.E., Takács-Novák K., Bosch E., Ráfols C., Rosés M. Physicochemical properties of a new multicomponent cosolvent system for the pK_a determination of poorly soluble pharmaceutical compounds. *Helv. Chim. Acta* **2007,** *90,* 1538-1553. IF: 1,550

3. **Völgyi G.,** Deák K., Vámos J., Valkó K., Takács-Novák K. Study on log*P* determination of structurally diverse neutral compounds by RPTLC method. *JPC-J. Planar Chromatogr.-Modern TLC* (submitted for publication)

4. Nagy P.I., **Völgyi G.,** Box K., Takács-Novák K. Computer modeling for the solution structure of a prototype polar organic molecule in pure and multi-component solvents. *Phys. Chem. Chem. Phys.* (submitted for publication)

Papers related to the thesis

1. Nagy P.I., **Völgyi G.,** Takács-Novák K. Monte Carlo structure simulations for aqueous 1,4-dioxane solutions. *J. Phys. Chem. B* (accepted for publication) IF: 4,115

Further publications

1. **Völgyi G.,** Takácsné Novák K. Alkalimetry in alcohol/water mixture with potentiometric end-point detection. Critical remarks on a new method of the European Pharmacopoeia. *Acta Pharm. Hung.* **2003,** *73,* 179-183.

2. Takács-Novák K., **Völgyi G.** Alkalimetry in alcohol-water mixtures with potentiometric end-point detection. Critical remarks on a newer method of European Pharmacopoeia. *Anal. Chim. Acta* **2004,** *507,* 275-280. IF: 2,558

3. Nagy P.I., **Völgyi G.,** Takács-Novák K. Tautomeric and conformational equilibria of tyramine and dopamine in aqueous solution. *Mol. Phys.* **2005,** *103,* 1589-1601. IF: 1,351

4. Takácsné Novák K., **Völgyi G.** Physico-chemical profiling in drug research. *Magy. Kém. Foly.* **2005,** *111,* 169-176.

5. Box K.J., **Völgyi G.,** Baka E., Stuart M., Takács-Novák K., Comer J.E.A. Equilibrium versus kinetic measurements of aqueous solubility, and the ability of compounds to supersaturate in solution – a validation study. *J. Pharm. Sci.* **2006,** *95,* 1298-1307. IF: 2,228

6. Sinkó B., **Völgyi G.,** Horváth P., Takácsné Novák K. Current problems in the quality control of pharmaceutical preparations manufactured in pharmacies II. Paracetamol containing preparations. *Acta Pharm. Hung.* **2006,** *76,* 173-180.

7. Kóczián K., **Völgyi G.,** Kökösi J., Noszál B. Site-specific acid-base properties of tenoxicam. *Helv. Chim. Acta* **2007,** *90,* 1681-1690. IF: 1,550

Citable abstract

1. **Völgyi G.,** Vámos J., Takács-Novák K.: A new RP-TLC method for log*P* determination of neutral compounds. *Eur. J. Pharm. Sci.* 2007, *32, Supplement 1*. 25-26.