Journal of Pharmaceutical and Biomedical Analysis 94 (2014) 180–187

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

# The simultaneous determination of hydrophobicity and dissociation constant by liquid chromatography–mass spectrometry☆

P. Wiczling*, W. Struck-Lewicka, Ł. Kubik, D. Siluk, M.J. Markuszewski, R. Kaliszan

*Department of Biopharmaceutics and Pharmacodynamics, Medical University of Gdańsk, Gen. J. Hallera 107, 80-416 Gdańsk, Poland*

ARTICLE INFO

*Article history:*
Received 31 October 2013
Accepted 23 January 2014
Available online 31 January 2014

*Keywords:*
pH/organic modifier
Gradient RP HPLC
Hydrophobicity
Acidity
Throughput analysis

ABSTRACT

Convenient methods for testing drug candidates' lipophilicity and acidity are highly requested in modern pharmaceutical research and drug development strategies. Reversed-phase high-performance liquid chromatography (RP HPLC) might be particularly useful for the determination of both dissociation constant and the (pH-dependent) partition coefficient related parameters, applicable in high-throughput analysis of multi-component mixtures. The general theory of combined pH/organic modifier gradient has recently provided equations relating gradient retention time and pH of the mobile phase. The purpose of this work was to facilitate the identification of analytes in this technique by its transfer to RP HPLC coupled with time-of-flight mass spectrometry with electrospray ionization source (ESI-TOF-MS). The accuracy of the proposed methodology was assessed by analyzing a set of known drugs. The ammonium formate, ammonium acetate or ammonium bicarbonate buffers were used to control pH during chromatographic analysis. In result, the p$K_a$ and hydrophobicity parameters were determined and the accuracy of the estimated values was assessed by comparing them with literature data. The gradient RP HPLC coupled with ESI-TOF-MS methods allowed for the rapid determination of dissociation constant and hydrophobicity and was shown to be especially applicable for complex mixtures. The use of ESI-TOF-MS detection allowed to achieve the medium-throughput screening rate (100 compounds/day) and provided a simple approach to assess pharmacokinetically important physicochemical properties of drugs.



## 1. Introduction

A large number of compounds can be synthesized during the drug development process, which are then processed and screened for specific properties of interest to obtain the best possible drug candidates. Dissociation constant, p$K_a$, lipophilicity parameters, like log $P$ – logarithm of n-octanol–water partition coefficient and distribution coefficient, log $D$, of a drug (drug candidate) are of a primary concern in medicinal chemistry. Determination and/or calculation of those parameters at the early stage of studies can help in eliminating those molecules that are unlikely to become drugs due to their poor pharmacokinetic properties [1–5].

Lipophilicity is strictly connected with pharmacological properties of a substance, such as biological activity and toxicity. It describes an ability of a chemical compound to partition between two-immiscible liquids and is defined as an equilibrium reaction of this two-phase distribution. For ionizing compounds distribution coefficient $D$ is used to take into consideration the pH-dependence of partitioning [6]. log $P$ and log $D$ can be determined for different systems, however the n-octanol/water is believed to be the most reliable one, because it is congenial to the conditions that exist at the interface of the cell membrane [7]. The most common methods of log $P$ determination are direct methods (shake-flask, slow stirring method), filter probe method, pH metric (potentiometric) method, calculation methods (regressions with other properties, theoretical calculation with fragmental approach) and reversed-phase high performance liquid chromatographic methods (RP-HPLC) [8–10]. Since the partitioning of molecule depends on the charge-state of the molecule, the knowledge about the p$K_a$ is also useful in predicting pharmacokinetic properties [11]. The most common methods of p$K_a$ calculation are pH-metric (potentiometric) titration, spectrophotometric, capillary electrophoresis or RP-HPLC methods [12,13].

Currently, most desired are methods which allow for the simultaneous determination of log $P$, log $D$ and p$K_a$ with high accuracy and speed. The method that can be readily used for that purpose is RP-HPLC. The chromatographic retention depends mostly on the hydrophobic interactions between molecule and solvent that are the consequence of water "phobicity" to molecule (or its parts) [14,15]. Hydrophobicity is often measured as a retention factor of an

☆ Selected Paper presented at PBA 2013, Bologna, Italy, 30 June – 3 July 2013.

* Corresponding author at: Department of Biopharmaceutics and Pharmacodynamics, Medical University of Gdańsk, ul. Hallera 107, 80-416, Gdańsk, Poland. Tel.: +48 58 349 3260; fax: +48 58 349 3262.
*E-mail address:* wiczling@gumed.edu.pl (P. Wiczling).

0731-7085/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2014.01.038

analyte extrapolated to neat water as an eluent, $\log k_w$. Generally, it is well correlated with n-octanol/water lipophilicity although different nature of both partition systems leads to some unavoidable dissimilarities [7]. The hydrogen bond acidity of a molecule has been shown to be a major factor contributing to the differences in lipophilicity measures observed in different biphasic systems for example octanol/water and alkane/water or octanol/water and chromatographic one [16,17]

The hydrophobicity ($\log k_w$) and $pK_a$ values can be determined by studying the relationship between retention times obtained at different mobile phase compositions whenever an appropriate mathematical model is known. The numerous models and designs have already been proposed for the purpose of $pK_a$ and hydrophobicity determination using RP-HPLC. They mostly involve an isocratic and pH-gradient mode [18–28]. The high-throughput application of these modes is limited due to the fact that only few analytes can be analyzed simultaneously and a considerable amount of time is required to gather required data.

The RP-HPLC is particularly useful for multicomponent analysis especially when performed in an organic modifier gradient mode [11,29,30]. Typically it involves a series of wide range organic modifier gradients at varying gradient duration and pH of the mobile phase [11,29,31]. The Chromatographic Hydrophobicity Index (CHI) has been proposed in literature as a scale closely related to hydrophobicity [32,33]. CHI is obtained from the short-gradient retention time and scaled to lipophilicity, basing on a series of calibration compounds. A similar medium-to-high-throughput HPLC $pK_a$ assay was proposed and used in laboratory settings [34].

Generally when applying a classical UV detection it was not possible to analyze a complex mixture due to difficulties in obtaining a distinct separation of the components and proper identification of analytes peaks and retention times at different pH. The purpose of this work was to apply the previously proposed gradient RP HPLC method of $pK_a$ and $\log k_w$ determination and combine it with electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) detection. The use of ESI-TOF-MS detection technique allows for an easy peak tracking, analyte identification, and in consequence for the rapid determination of dissociation constant and hydrophobicity based on the one of the currently available models of chromatographic retentions.

## 2. Chemicals

The 40 reference substances (salicylic acid, paracetamol, nicotine, metronidazole, theophylline, clonidine, naproxene, aminophenazone, phenobarbital, lidocaine, procainamide, procaine, pindolol, cimetidine, diphenhydramine, nortriptyline, tetracaine, metoprolol, chlorpheniramine, amitriptyline, imipramine, promethazine, diclofenac, metoclopramide, cocaine, warfarine, phenylbutazone, chlorprotixene, chlorpromazine, quinidine, clozapine, papaverine, disopyramide, haloperidol, celiprolol, prazosine, diltiazem, bretylium tosylate, verapamil and ketoconazole) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol for LC–MS was obtained from J.T. Baker (Griesheim, Germany), formic acid 97% and acetic acid 99% were purchased from Alfa Aesar GMbH & Co KG (Karlsruhe, Germany), ammonium formate, ammonium bicarbonate, tetrahydrofuran were from Sigma–Aldrich (St. Louis, MO, USA) and ammonia was from POCH (Gliwice, Poland). Deionized water from a Milli-Q water system (Millipore Inc., Bedford, MA, USA) was used in the preparation of the samples and buffer solutions. All the reagents and the analytes employed were of a highest commercially available quality. The bretylium tosylate has been used to assess the repeatability of retention times for different pHs of the mobile phase. The ionization of either the bretylium cation or tosylate anion does not change with pH. Thus its retention should be constant at different pHs. The analytes were selected to have a "gold-standard" $pK_a$ values and when possible the "gold-standard" values of n-octanol/water $\log P$ for both ionized and nonionized form of analytes. They were extracted from Avdeef at al. [6] and Physical/Chemical Property database [35].

### 2.1. HPLC equipment

The experiments were carried out using liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) system from Agilent Technologies (Santa Clara, CA, USA) 1260 series, composed of a binary pump, an autosampler, a membrane degasser and a 6224 time of flight (TOF) mass spectrometer. The TOF mass spectrometer was equipped with a dual electrospray ionization source (Dual ESI). Analyses were performed in fast polarity switching mode. The nebulizer pressure and capillary voltage were set at 50 psi and 4000 V, respectively. Nitrogen was used as a drying gas (11 L/min, 350 °C). The fragmentor, skimmer and octopole voltage was set as 84 V, 65 V and 750 V, respectively.

For separation of the analytes an XBridge-C18 column (3 mm × 50 mm, 2.5 μm) from Wexford, Ireland) was used. The chromatographic separation was performed using 3 different buffers that were used to achieve the mobile phase pH spanning a wide range of pHs: from 2.50 to 10.50. Two series (I and II) of experiments were performed. The organic modifier gradients in series I was developed from 5% to 80% of methanol in buffer (v/v) from 0 till 10 min and composition of the eluent was held at 80% of methanol from 10 till 16 min. The series II comprised gradient program that started from 5% to 80% of B from 0 till 30 min and was held at 80% of methanol from 30 till 36 min. After each analysis, the 3.5 min post-run program was started, keeping the system at initial conditions in order to equilibrate the analytical column for the next analysis. Citric acid was injected to determine the column follow-up volume, $V_o$, which was 0.266 mL. The system dwell volume $V_d$ equaled 1.05 mL. The extra column volume equaled 0.020 mL. It served to find the extra column time that has been subtracted from all the retention times prior to any calculation. The injection volume was 2 μL, the flow rate ($F$) and the column temperature were set at 0.5 mL/min and 25 °C, respectively. The analyses were performed in a scan mode using 50–1200 $m/z$ range.

## 3. Samples and buffers preparation

Each reference substance was prepared at a concentration of 1 mg/mL by diluting the weighted substance in deionized water. Those samples that had limited solubility in water, were diluted in water with the addition of tetrahydrofuran. Subsequently, all substances were mixed together into one sample used for further analysis. After sample preparation, it was frozen immediately and stored at −20 °C. Directly before analysis, sample was thawed at room temperature. Three buffers: ammonium formate, ammonium acetate and ammonium bicarbonate were prepared at a concentration of 10 mM by diluting the weighed substances in deionized water. The ${}^{w}_{w}$pH (this symbol denotes pH measured in water without addition of any organic modifier) of those buffers was adjusted to span wide range of values (ammonium formate: 2.5, 3.3, 4.1, 8.9, 9.7; ammonium acetate: 4.9, 5.8; and ammonium bicarbonate: 6.8, 10.5) by appropriate addition of formic acid, acetic acid and ammonia, respectively. After buffers' preparation, they were filtered using 0.40 μm nylon filters and degassed. The RP-HPLC separation requires the use of organic modifier that is known to affect the pH values of the buffers [36,37]. The details of proper pH measurements in the organic/water mobile phase are discussed by Roses [38]. Briefly, the pH of the mobile phase was measured after