Talanta 84 (2011) 809–813

Contents lists available at ScienceDirect

Talanta

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

Frozen water phase method for log *D* measurement using a 96-well plate

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ARTICLE INFO

Article history:
Received 24 November 2010
Received in revised form 8 February 2011
Accepted 9 February 2011
Available online 19 February 2011

Keywords:
Physicochemical screening
1-Octanol
log *D*
Physical property
Frozen water phase method
Liquid nitrogen

ABSTRACT

In this study, a frozen water phase method for log *D* measurement using a 96-well plate was developed. In the case of log *D* measurement of compounds, the problem of octanol contamination often occurs; in lipophilic compounds, the concentration of the octanol phase is much higher than that of the water phase. When the water phase is separated from the octanol phase, a small amount of octanol phase contamination could strongly influence the concentration of the water phase. To avoid this problem, the frozen water phase method was developed. The water phase was frozen in liquid nitrogen and then the unfrozen octanol phase was removed. To remove the portion of the octanol remaining on the frozen water phase, the surface of the frozen water phase was washed with octanol and water/ethanol (50/50, v/v). The validity of the method was confirmed by results of commercially available drugs at the log *D* range from 0 to 4. Further, it was found that this method had the ability to evaluate the pH–log *D* profile of compounds in the range from pH 2 to pH 12. As a result, we developed the convenient and accurate method that is effective in preventing contamination with a wide dynamic range.

1. Introduction

During the early stages of drug discovery and development, lead compounds are optimized by synthesis of a large numbers of derivatives. However, many of the lead compounds identified by high throughput screening are highly lipophilic and poorly soluble. log *D* is the negative logarithm of the distribution coefficient between octanol and water. log $D_{7.4}$ (the log *D* value at pH 7.4) is often used as a parameter of lipophilicity for drug design and deriving quantitative structure activity relationship (QSAR) [1]. Optimization of lipophilicity is very important in the drug discovery process because lipophilicity is closely associated with absorption [2], distribution, metabolism [3–7] and excretion (ADME) properties of the compound, as well as other important pharmacological and pharmaceutical characteristics. As such, a screening method for lipophilicity evaluation in the early stages of drug discovery would be a valuable tool for pharmaceutical research and drug discovery [8–10].

Several methods have been developed to overcome these difficulties and increase throughput in obtaining the experimental log *D* measurement. For example, the conventional method is the shake-flask method [11]. There have also been other methods based on titration method [11,12], pH depending UV spectra change [11], chromatographic retention times [11,13–15] and liquid–liquid extraction method with PTFE membrane [16]. It has been suggested that each method has distinct advantages and disadvantages.

The shake-flask method is a simple and commonly used method to determine log *D* [11]; however, it is time-intensive and requires significant amounts of the active pharmaceutical ingredient. Recently miniaturized shake-flask methods using a 96-well plate-based automated injection technique have been reported with the advantages of requiring a small amount of active pharmaceutical ingredient and automated high-throughput systems in the early stage of drug discovery [17–20]. In the case of the miniaturized method, the problem of octanol contamination often occurs due to the decreased sample volume. In lipophilic compounds, the concentration of the octanol phase is much higher than that of the water phase. When the water phase is separated from the octanol phase, a small amount of octanol phase contamination could strongly influence the concentration of the water phase. In addition, it is difficult to separate the water phase from the octanol phase because of the high viscosity of octanol. Several reports of an automated injection technique have been reported to prevent contamination of the octanol [18,19], while disadvantages of the automated injection technique (such as clogging of the needle by insoluble matter) were also reported [21]. Thus, several approaches will be necessary to develop the miniaturized log *D* method without contamination of the octanol.

The liquid–liquid extraction method, utilizing the difference in melting points of each phase, was an often used technique for sample preparation of biological samples (such as therapeutic dose monitoring in clinical trials) [22]. For example, one phase was frozen in liquid nitrogen, and then the other unfrozen phase

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doi:10.1016/j.talanta.2011.02.015

was extracted. In this study, we investigated the applicability of this method for log *D* measurement to avoid contamination of the octanol during water phase sampling.

2. Experimental

2.1. Materials and reagents

Propranolol, ketoconazole, imipramine, carbamazepine, metoprolol, dexamethasone, desipramine, alprenolol and chlorpromazine were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Antipyrine was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). Flufenamic acid, ketoprofen and indomethacin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Compounds were dissolved in dimethylsulfoxide (DMSO; Wako Pure Chemical Industries) at 10 mM. 1-Octanol (OctOH) was obtained from Tokyo Chemical Industries. Phosphoric acid, ethanol (EtOH), acetonitrile (MeCN), acetic acid, boric acid, sodium hydroxide, potassium chloride, NaH_2PO_4 and Na_2HPO_4 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Water was purified using a MilliQ Gradient A10 system (Millipore Co., Billerica, MA, USA). 50% EtOH (water/ethanol (50/50, v/v)) was prepared by mixing the same volume of water and ethanol. 10 mM phosphate buffer (pH 7.4) was prepared by mixing 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4. Universal buffer (ionic strength 0.3, from pH 2 to pH 12) was prepared according to the previous report [23]. Octanol and buffer were mutually presaturated. As for the liquid nitrogen, in house liquid nitrogen was used.

2.2. HPLC conditions

HPLC/UV analyses were performed using a 1100 series HPLC system fitted with a binary pump, plate auto sampler, thermostat in the column compartment, and diode array detector controlled by Chemstation, version 9.01 (Agilent Technologies, Palo Alto, CA). Chromatography was conducted using a YMC Pro C18 column (35 mm × 4.6 mm; particle size 3 μm; YMC, Kyoto, Japan). The mobile phase was composed of a mixture of 0.1% phosphoric acid in water (solvent A) and MeCN (solvent B). The gradient was delivered at 2 mL/min as follows: 0 min, 5% solvent B; 3.0 min, 90% solvent B; 3.5 min, 90% solvent B; 3.51 min, 5% solvent B; and 5 min, 5% solvent B; loop time = 5.0 min. The column was maintained at 40 °C. The diode array detector was set at 210 nm.

2.3. Sample preparation of log $D_{7.4}$ measurement

Compounds were dissolved in DMSO to a concentration of 10 mM. A volume of 10 μL of each 10 mM DMSO solution was placed in a 96-well plate (31 mm, deep well plate, polypropyrene, Agilent Technologies, parts no. 5042-6454). Next, 300 μL of octanol was added to each well using an eight-channel electric Biohit eLINE pipette (Biohit OYJ, Helsinki, Finland), and the plate was sealed with a silicone pre-slit well cap. The plate was then agitated on a plate shaker (Taitec Micro mixer E-36, Taitec Co., Saitama, Japan) for 5 min. A volume of 600 μL of 10 mM phosphate buffer (pH 7.4) was added to each well using an 8-channel electronic pipette. After sealing, the plate was vigorously mixed on the plate shaker for 1 h at room temperature. The plate was centrifuged at 2000 rpm for 5 min in a swinging-bucket centrifuge (KUBOTA 7780, KUBOTA Manufacturing Co., Gunma, Japan), and the seal was removed. A volume of 10 μL of the octanol phase was transferred into a new plate and 490 μL of 50% EtOH was added using an 8-channel electronic pipette (50-fold dilution of octanol phase). The 50-fold diluted octanol phase plate was sealed and placed in the injector of HPLC for analysis of the octanol phase. The bottom of the plate that was initially shaken for 1 h was put into a dewar flask filled with liquid nitrogen for approximately 60 s to freeze the water phase. The unfrozen octanol phase was removed by using an 8-channel electronic pipette. 400 μL of octanol was added on the frozen water phase and removed by using an 8-channel electronic pipette to wash out the compound that was partitioned in the octanol phase. The wash-out process using octanol was carried out twice. 400 μL of 50% EtOH was added on the frozen water phase and removed using an 8-channel electronic pipette to wash out the remaining octanol. The plate was put into a water bath to melt the frozen water phase. The water phase plate was sealed and placed in the injector of HPLC for analysis of the water phase. A volume of 5 μL of 50-fold diluted octanol phase and a volume of 50 μL of water phase were injected onto the column by an auto liquid sampler.

2.4. Sample preparation for the pH–log *D* profile

To obtain the pH–log *D* profiles, the universal buffer (ionic strength 0.3, from pH 2 to 12) was used for the aqueous phase. log *D* measurement was carried out according to the same procedure in Section 2.3.

2.5. Calculation of log *D*, pK_a and log *P*

log *D* value was calculated using the following equation:

$$\log D = \log\left\{\frac{\text{concentration in octanol phase}}{\text{concentration in buffer phase}}\right\} \quad (1)$$

pK_a and log *P* were calculated from the pH–log *D* profiles by the fitting program of Microsoft Excel 2003:

$$\log D = \log P - \log\left[1 + 10^{(\mathrm{pH}-pK_a)}\right]$$

for monovalent acidic compound (2)

$$\log D = \log P - \log\left[1 + 10^{(pK_a-\mathrm{pH})}\right]$$

for monovalent basic compound (3)

3. Results and discussion

3.1. Development of the frozen water phase method

Fig. 1 shows the flow of the frozen water phase method. To develop the high throughput screening system, the liquid nitrogen was selected to freeze the water phase immediately. The water phase was frozen in liquid nitrogen at a depth of approximately 14 mm from the surface of the liquid nitrogen to the bottom of the plate, and then the unfrozen octanol phase was removed. To remove the portion of the octanol remaining on the frozen water phase, the surface of the frozen water phase was washed with octanol and 50% EtOH. It was also possible to measure log *D* of the poorly soluble compound, because it was dissolved in octanol prior to the measurement.

To remove the portion of the octanol remaining on the frozen water phase, several washing procedures were examined. 400 μL of the 50% EtOH or octanol was added on the surface of the frozen water phase and then removed by an 8-channel pipette. Table 1 shows the results of the washing procedure. To easily monitor contamination of the octanol, we used ketoconazole with a log $D_{7.4}$ of 3.8 (reference value [24]). Washing with 50% EtOH four times resulted in log $D_{7.4}$ of 2.9. It was insufficient to remove the remaining octanol by washing four times with 50% EtOH. On the other hand, it was possible to remove the residual octanol portion by the introduction of an octanol wash; the log $D_{7.4}$ value of our method measured almost the same as the reference

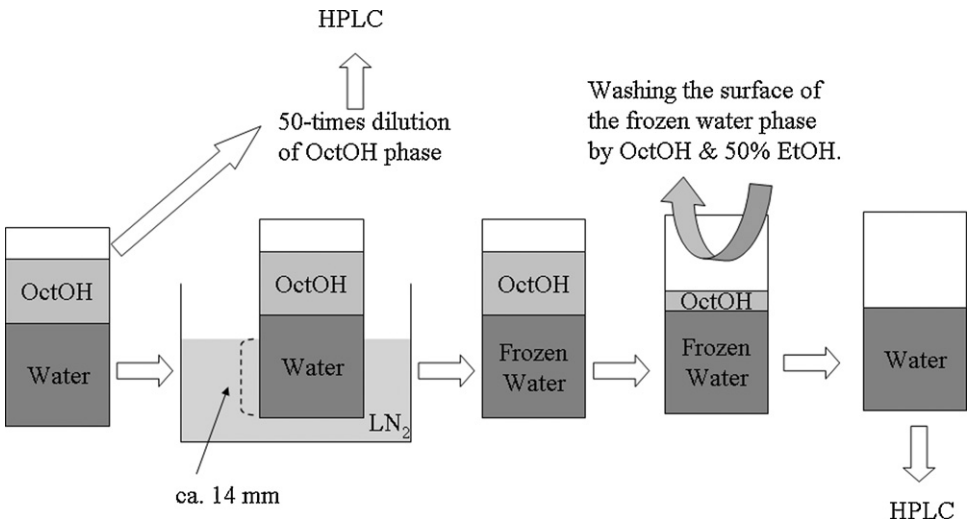

Fig. 1. Flow of the frozen water phase method using 96-well plate. OctOH and LN_2 refer to 1-octanol and liquid nitrogen, respectively.

Table 1
Effect of the washing procedure on log $D_{7.4}$ value of ketoconazole.

Washing procedure	log $D_{7.4}$ (n = 3)	
	Mean	SD
50% EtOH wash 4×	2.9	0.2
OctOH wash 1×, 50% EtOH wash 1×	3.5	0.1
OctOH wash 1×, 50% EtOH wash 2×	3.7	0.1
OctOH wash 2×, 50% EtOH wash 1×	3.8	0.1
Reference value [24]	3.8	–

value. It was important to select a high affinity washing solvent to remove the remaining octanol portion. As a result, washing two times with octanol and one time with 50% EtOH was selected. The duplicate wash with octanol successfully removed the residual octanol on the frozen water, and the single wash with 50% EtOH removed the washing solution of octanol remaining on the frozen water.

Regarding the period for the freezing process, approximately 60 s was selected. The water phase was frozen from the bottom of the 96-well plate by liquid nitrogen. The freezing process of the water phase was controlled by visible check and time control. The melting point of octanol was previously reported as −16 to −17 °C [25]. When the bottom of the plate was in the liquid nitrogen more than 120 s, frozen octanol was visually observed. As a result, approximately 60 s was selected as the period for the freezing process.

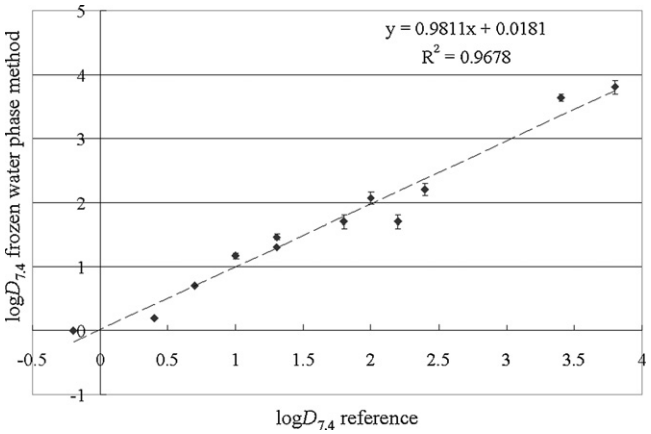

Fig. 2. Relationship between the log $D_{7.4}$ results of the frozen water phase method and the reference values.

3.2. Validation of the frozen water phase method

For the validation study of the frozen water phase method, log $D_{7.4}$ values of several commercial compounds were obtained using the frozen water phase method and were compared with reported log $D_{7.4}$ values [24,26–28] (Table 2 and Fig. 2). As a result, there was excellent positive correlation between the results of the frozen water phase method and that of the reported values over a wide dynamic range (log $D_{7.4}$ from 0 to 4). In the highly lipophilic compounds (log D > 4), it was difficult to quantify the concentra-

Table 2
log $D_{7.4}$ values of commercial medical supplies measured by 96-well plate frozen method.

Commercial medical supplies	log $D_{7.4}$ Mean n = 3	SD n = 3	log $D_{7.4}$ (reference)	Reference
Propranolol	1.3	0.0	1.3	[26]
Ketoconazole	3.8	0.1	3.8	[24]
Imipramine	2.2	0.1	2.4	[26]
Carbamazepine	1.7	0.1	2.2	[26]
Metoprolol	0.0	0.0	−0.2	[26]
Antipyrine	0.2	0.0	0.4	[26]
Desipramine	1.5	0.0	1.3	[26]
Flufenamic acid	2.1	0.1	2.0	[27]
Alprenolol	1.2	0.0	1.0	[26]
Chlorpromazine	3.6	0.1	3.4	[26]
Dexamethason	1.7	0.1	1.8	[26]
Indomethacin	0.7	0.0	0.7	[28]

Table 3
log *P* and pK_a values of ketoprofen and metoprolol.

Commercial medical supplies	pK_a[a]	pK_a (reference)	log *P*[a]	log *P* (reference)	Reference
Ketoprofen	4.3	4.5	2.9	2.7	[17]
Metoprolol	9.5	9.6	1.9	1.8	[17]

[a] log *P* and pK_a values were obtained from the results of the measured pH–log *D* profiles using the fitting program of Microsoft Excel 2003.

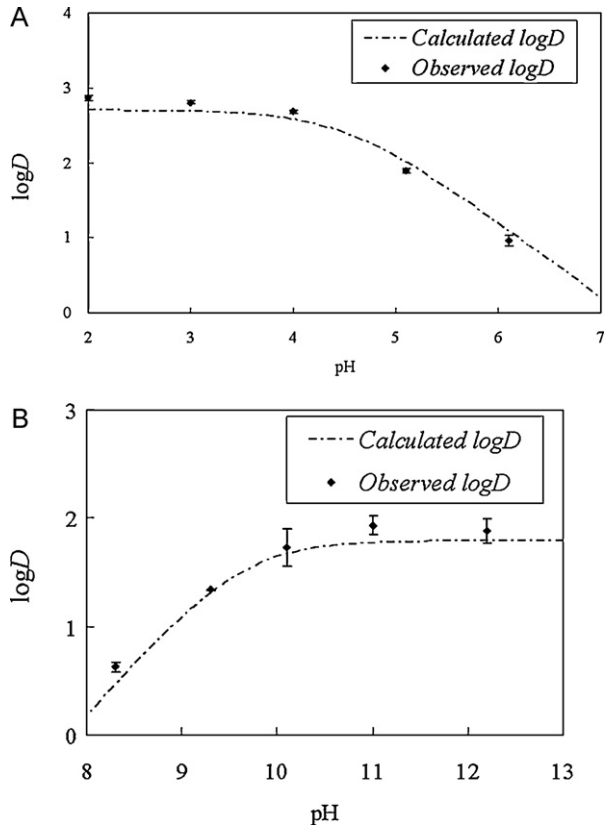

Fig. 3. pH–log *D* profiles of ketoprofen (A) and metoprolol (B) measured by the 96-well plate based frozen water phase method (SD, $n = 3$). Observed log *D* shows the result of the log *D* obtained by the frozen water phase method. Calculated log *D* curves were obtained as follows: $\log D = \log P - \log[1 + 10^{(\mathrm{pH}-\mathrm{p}K_a)}]$ for monovalent acidic compound, $\log D = \log P - \log[1 + 10^{(\mathrm{p}K_a-\mathrm{pH})}]$ for monovalent basic compound. pK_a and log *P* were obtained from the literature values [17].

tion of the water phase by UV detection, so the upper limit of log *D* was set as log *D* of 4. A general guide for optimal gastrointestinal absorption by passive diffusion permeability after oral dosing is to have a moderate log *D* (range 0–3) [29] and our method have the enough range to cover the range of the general guide.

1.1% (v/v) of DMSO was included in 300 μL octanol/600 μL water; however, it was supposed that there was little influence of DMSO on the log $D_{7.4}$ value from the previous report [18]. In fact, there was good correlation between the results of the frozen water phase method containing 1.1% (v/v) of DMSO and the reference values (Table 2 and Fig. 2).

3.3. Application for pH–log D profile

To investigate the applicability of the method for a wide pH range, pH–log *D* profiles of the model compounds were examined by this method. To obtain the pH–log *D* profiles, universal buffer (ionic strength 0.3, pH 2–12) was used for the aqueous phase. As for model compounds, ketoprofen and metoprolol were selected for the acidic and basic model compounds, respectively. Fig. 3 shows the results of the pH–log *D* profile. Observed log *D* shows the result of the log *D* obtained by the frozen water phase method. Calculated log *D* curves were obtained from Eqs. (2) and (3). As for the calculated log *D* curves, pK_a and log *P* were obtained from literature values, summarized in Table 3 [17]. In Fig. 3, the difference between the observed log *D* and the calculated log *D* curve were within 0.2 units. From the previous report [18], the difference of log *D* between the conventional shake-flask method and the miniaturized 96-well plate method was approximately 0.2–0.3 units, so excellent correlations were observed between the pH–log *D* profiles obtained by the frozen water phase method and those calculated from literature values (pK_a and log *P*).

pK_a and log *P* values were summarized in Table 3. The obtained values (log *P* and pK_a) from the measured pH–log *D* were almost the same as the reference values, and the agreements of log *P* and pK_a contributed to the agreement between the measured pH–log *D* profiles by the water phase frozen method and the calculated pH–log *D* profiles from the literature values. As a result, the 96-well plate-based water phase frozen method was applicable for a wide pH range from pH 2 to pH 12.

4. Conclusions

In this study, we developed the frozen water phase method for log *D* measurement. Validity of the method was confirmed by results of commercially available drugs from log *D* of 0–4. Further it was found that the method had an ability to evaluate the pH–log *D* profile of the compounds in the range from pH 2 to pH 12. As a result, we developed the convenient and accurate method that is effective in preventing contamination with a wide dynamic range.

Acknowledgements

We thank all staff members of the analytical research section at Tsukuba Research Laboratory (Eisai Co. Ltd.) for discussion regarding this work.

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