Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 722–729

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

A rapid vesicle electrokinetic chromatography method for the *in vitro* prediction of non-specific binding for potential PET ligands

Zhengjin Jiang[a], John Reilly[a,*], Brian Everatt[a], Emmanuelle Briard[b]

[a] Novartis Institutes for Biomedical Research, Global Discovery Chemistry, Wimblehurst Road, Horsham, West Sussex RH12 5AB, UK
[b] Novartis Institutes for Biomedical Research, Global Discovery Chemistry, 4002 Basel, Switzerland

ARTICLE INFO

Article history:
Received 27 August 2010
Received in revised form 3 November 2010
Accepted 4 November 2010
Available online 11 November 2010

Keywords:
Vesicle electrokinetic chromatography
Positron emission tomography
Non-specific binding

ABSTRACT

High non-specific binding (NSB) is one of the most common reasons for candidate failure in potential positron emission tomography (PET) radiotracer development. It is of interest to develop high throughput *in vitro* methods for predicting non-specific binding prior to radiolabeling, which would help guide radiotracer candidate selection and assist decision making in new radiotracer discovery. We evaluated several electrokinetic chromatographic (EKC) systems to help identify PET ligands with low non-specific binding characteristics by mimicking the ligand–brain tissue interaction. The measured retention factors of tracers in clinical use or terminated candidates within AOT vesicle EKC systems were compared with literature *in vitro* or *in vivo* NSB data. We conclude that there is a statistical correlation between the chromatographic retention parameters of tested drugs and their NSB. The AOT vesicle EKC method can provide NSB *in vitro* trend analysis for a large number of drug candidates early in the novel radiotracer discovery process with minimal resources.

1. Introduction

In vivo dynamic molecular imaging of the brain with positron emission tomography (PET) is a rapidly expanding research area in preclinical drug development and clinical investigations [1]. It has been shown that the imaging quality is closely related to the properties of the radiotracers as well as the advancement of instrumentation and image analysis software [2]. However developing suitable and adequate PET radiotracers for matching the growing numbers of receptors, transporters, enzymes, and other targets is still a challenge. Conventional methods for developing a novel PET radiotracer are time-consuming, expensive, low throughput and even raise ethical issues in respect to reduce and refine animal testing. One common reason for failure is the high non-specific binding (NSB) of candidate PET radiotracers. It is often observed that a candidate PET radiotracer with higher affinity for target receptor exhibited worse suitability than one with lower affinity for target receptor, due to its higher NSB, which will sacrifice the signal-to-background ratio of the imaging [2]. Increased affinity is often linked with increased lipophilicity. Therefore, it is of interest to develop high throughput *in vitro* methods for predicting the non-specific binding prior to radiolabeling, which would help guide radiotracer candidate selection and assist decision making in new radiotracer discovery. Few *in vitro* methods have been reported for measuring and predicting the NSB of potential PET radiotracers. The most popular method is autoradiography, in which radiotracers are incubated on rodent and rhesus brain tissue sections. However, such a method requires either long exposure time for autoradiographic imaging or long analysis time for quantifying imaging results and manifestly radiolabeled material. A rapid assay was developed by removing the tissue sections from the slides and counting the activity bound to the section directly and the non-specific binding can be determined in presence of excess of suitable blocking drug for a 20 min period [3]. A useful tool for estimating the non-specific binding of potential PET ligands has been developed by the evaluation of the efflux of test compounds from whole rat brain homogenate over time [4]. Single compounds at a concentration of 1 mM were added to whole rat brain homogenate contained within a dialysis membrane compartment that was dialysed against phosphate buffered saline. A control beaker without brain homogenate was run in parallel. Samples of extra-compartmental buffer were taken at 5–10 min intervals over 1 h and analysed for drug content using a mass spectrometer [4]. However, the throughput of these methods is still low and is not

Abbreviations: **AOT**, docusate sodium salt; **BBB**, blood brain barrier; **CHI IAM$_{7.4}$**, chromatographic hydrophobicity index values referring to immobilized artificial membrane chromatography at pH 7.4; **EKC**, electrokinetic chromatography; **IAM**, immobilized artificial membrane; **LEKC**, liposome electrokinetic chromatography; **log $D_{7.4}$**, logarithm of the octanol–water partition coefficient at pH 7.4; **NSB**, non-specific binding; **PET**, positron emission tomography; **PS**, 3-*sn*-phosphatidyl-L-serine from bovine brain; **POPC**, 2-oleoyl-1-palmitoyl-*sn*-glycerol-phosphocholine.

* Corresponding author. Tel.: +44 1403323370; fax: +44 1403323837.
E-mail address: john.reilly@novartis.com (J. Reilly).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.11.004

Table 1
List of selected potential PET ligands with NSB ratio data.

Compound	NSB ratio [4]	log $k_{PC_{80}PS_{20}}$	log $k_{PC_{65}PS_{35}}$	log $k_{PS_{100}}$	log k_{AOT}	log $D_{7.4}$	CHI $IAM_{7.4}$
Sertraline	0.03	2.30	–	–	2.18	2.70	68.55
Paroxetine	0.07	1.89	2.15	–	2.01	1.40	56.98
Fluoxetine	0.10	2.21	2.15	–	2.16	1.88	56.36
GBR12909	0.10	2.15	2.34	–	2.03	4.76	53.95
Haloperidol	0.22	1.71	1.93	–	1.79	1.97	51.24
Imipramine	0.25	1.45	1.62	–	1.77	2.10	54.21
Spiperone	0.25	1.50	1.87	–	1.69	1.78	48.75
Zimelidine	0.34	1.29	1.55	–	1.75	1.52	51.08
Reboxetine	0.37	1.45	1.73	1.86	1.38	1.66	45.24
ICS205-930	0.42	1.03	1.00	1.79	1.30	0.20	46.18
SCH23390	0.56	1.10	1.06	1.49	1.25	2.10	47.42
Citalopram	0.56	1.37	1.42	–	1.62	1.44	48.01
N-Methyl-Spiperone	0.58	1.13	1.60	–	1.68	2.23	48.49
Nisoxetine	0.59	1.13	1.35	1.73	1.51	1.02	46.78
Nomifensine	0.63	1.01	1.18	1.90	1.24	1.98	40.32
Bupropion	0.73	0.17	0.40	1.10	1.04	2.81	40.32
MK801	0.75	0.57	1.03	1.54	0.99	1.45	41.19
WAY100635	0.75	−0.01	0.52	1.12	0.91	3.44	38.90
MDL100907	0.77	0.51	0.88	1.62	1.33	1.66	44.26
SKF10047	0.78	0.17	0.63	0.91	0.75	0.47	42.47
Raclopride	0.86	0.34	0.91	1.52	0.79	1.96	35.35

–: Data not measurable.

suitable for compound screening in the early phases of potential PET ligand discovery.

The three compartment model (also known as the two tissue-compartment model) is currently the most widely used configuration to derive the PET tracer binding potential to neuroreceptors [2,5,6]. The three compartments are the arterial plasma compartment, the intracerebral free and non-specifically bound compartment and the specifically bound compartment. The non-specific binding is related to the drug tissue affinity to the non-specifically bound compartment as well as the un-metabolized free fraction in the same compartment which is assumed to be the same as the free fraction in plasma at equilibrium. A computational model has been developed using quantum chemical calculations to predict the non-specific binding of a particular drug molecule [5,7]. Their results showed a correlation between the estimate of the energy of interaction between single drug molecule and lipid molecule and the *in vivo* NSB as estimated by PET data analysis.

However such models are built with limited data set consisting of only 10 well-studied central nervous system PET radiotracers acting on a variety of molecular targets. A biomathematical modeling approach has been used to predict the *in vivo* performance of radioligands directly from *in silico*/*in vitro* data [8]. This model incorporates components accounting for blood brain barrier (BBB) penetration, specific binding, nonspecific binding and kinetic interactions.

Chromatographic techniques are widespread within the pharmaceutical industry and hold great promise for studying NSB since they can effectively mimic drug–membrane interactions [9–14]. For example, immobilized artificial membrane (IAM) chromatography has been evaluated as a permeability screen method [9,10,12]. Electrokinetic chromatography (EKC) with either liposome or surfactant unilamellar vesicles as a pseudostationary phase has also been used to estimate membrane affinity related properties of drug candidates, such as intestinal absorption, volume of distribution and blood brain barrier permeability [11,13,14].

In this work, three liposome and one surfactant vesicle system were evaluated as high throughput, cost-effective, and predictive screening models for non-specific binding of PET radiotracer candidates. Chromatographic characteristics and statistical correlations were applied for comparison purposes. The retention factors (k) of a series of commercial drugs in different separation systems were determined and correlated to *in vitro* NSB ratio data and *in vivo* qualitative NSB data in literature.

2. Materials and methods

2.1. Chemicals and reagents

The ammonium acetate, ammonium formate, 3-*sn*-phosphatidyl-L-serine from bovine brain (PS), 2-oleoyl-1-palmitoyl-*sn*-glycerol-phosphocholine (POPC), docusate sodium salt (AOT), 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) and all *n*-alkylphenones were purchased from Sigma–Aldrich (Gillingham, UK). All validated and candidate PET ligands listed in Tables 1 and 2 were obtained from various pharmaceutical companies or provided by Novartis GDC. Sodium hydroxide (1 M and 0.1 M), HCl solution (1 M), dimethyl sulfoxide (DMSO), HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Leicestershire, UK). The water used throughout all experiments was purified using an Elga water purifier (Bucks, UK).

2.2. Instruments

All CE experiments were carried out on the Beckman P/ACE MDQ system (Munich, Germany) equipped with diode array UV detector and a temperature control system (25 °C for all experiments). A 32 Karat™ software version 5.0 was used for instrument control, data acquisition and data handling. The fused-silica capillaries with dimensions of 50 μm I.D. (375 μm O.D.) and 37.5 cm total length (29 cm effective length) were purchased from Beckman Instruments (Hallow, Worcestershire, UK). All HPLC experiments were performed on the Waters Alliance 2695 HPLC system (Milford, MA, USA) equipped with 2487 dual λ absorbance detector. Regis IAM PC DD2 column (100 mm × 4.6 mm I.D., 10 μm particle size) was obtained from Hichrom Limited (Reading, Berkshire, UK), while Gemini C_{18} column (50 mm × 3.0 mm I.D., 3 μm particle size) was purchased from Phenomenex (Macclesfield, Cheshire, UK). The extrusion of liposome was performed through polycarbonate membrane using a Northern Lipids Lipex extruder (Vancouver, BC, Canada).

2.3. Surfactant vesicle and liposome preparation

Stock solutions of 50 mM ammonium acetate and 25 mM HEPES were prepared in advance and stored in the refrigerator. The buffer pH of both solutions was adjusted to 7.4 with NaOH solution (1 M)

Table 2
List of selected PET ligands with qualitative non-specific binding data.

Name	Species	Target	Affinity (nM)	log $D_{7.4}$	*In vivo* specificity	Maximum brain uptake[b]	log k_{AOT}	Outcome
ABP688	Human	mGlu5	3.5	2.4[a]	Specific	7 kBq/mL	0.45	Clinically used
AC-5216	Human	TSPO	0.3	3.40	Specific	8 kBq/mL	0.94	Promising
AL438	Rat	Glucocorticoid	8.1	5.60	Unspecific	284%SUV	1.99	Terminated
Altanserin	Human	5-HT_{2A}	0.1	3.08	Specific	3%ID/L	1.42	Clinically used
Heteroarylalkynyl-benzylpiperidine	Rat	NMDA	5.3	2.52	Unspecific	21%SUV	1.63	Terminated
Citalopram	Human	5-HTT	5.0	1.44	Unspecific	100%SUV	1.62	Terminated
CPEB	Mice	ORL1	2.3	4.15	Unspecific	50%SUV	1.65	Terminated
Deprenyl	Human	MAO-B	7	3.17	Specific	35 kBq/mL	0.47	Clinically used
Diprenorphine	Human	Opiate	0.2	2.51	Specific	400%SUV	−0.21	Clinically used
Donepezil	Human	AChE	5.7	2.19	Specific	300%SUV	1.24	Clinically used
Fallypride	Human	D2/D3	0.03	2.38	Specific	29 kBq/mL	0.70	Clinically used
Flumazenil	Human	Benzodiazepine	2	0.86	Specific	25 kBq/mL	−0.51	Clinically used
GR103545	Monkey	k-Opioid	0.5	1.89	Specific	240%SUV	1.49	Promising
Imipramine	Mice	Norepinephrine	4.8	2.10	Unspecific	114%SUV	1.77	Terminated
MPPF	Human	5HT1A	0.34	3.01	Specific	3 kBq/mL	0.85	Clinically used
NMeSpiperone	Human	D2	0.25	2.23	Specific	26 kBq/mL	1.61	Clinically used
Nisoxetin	Monkey	NET	1	1.02	Unspecific	130%SUV	1.50	Terminated
Nomifensine	Human	Monoamine reuptake inhibitor	56	1.98	Specific	600%SUV	1.24	Clinically used
NPA	Human	D_{2high}	0.27	0.91	Specific	400% SUV	0.62	Clinically used
Physostigmine	Human	AChE	0.7	0.35	Specific	5%ID/L	0.37	Promising
PK11195	Human	TSPO	4.5	4.11	Specific	6 kBq/mL	0.97	Clinically used
Raclopride	Human	D2	1.8	1.96	Specific	20 kBq/mL	0.79	Clinically used
Rolipram	Human	PDE4	2.5	1.77	Specific	5%ID/L	−0.07	Clinically used
SCH23390	Human	D1	0.1	2.10	Specific	9 kBq/mL	1.25	Clinically used
Setoperone	Human	5HT2A	0.4	1.26	Specific	9 kBq/mL	0.96	Terminated
Spiperone	Mice	D2	0.07	1.78	Unspecific	130%SUV	1.69	Terminated
WAY100635	Human	5HT1A	0.4	3.44	Specific	7 kBq/mL	0.91	Clinically used

[a] log $D_{7.4}$ was calculated with Novartis in-house software.
[b] Specific tracers are tracers that show either reduced uptake in the presence of a blocker or low binding in region devoid of receptors and vice versa for unspecific tracers.

and HCl solution (1 M). Sample solutions were made by dilution of the solid sample with methanol to provide a final concentration around 1 mg mL^{-1}. 5–25% (v/v) of DMSO was added into the sample solution in order to aid dissolution if the sample was not readily soluble.

The 1% (w/v) double-tailed anionic surfactant AOT vesicle solution was prepared by following the previous method [13]. The appropriate amount of AOT was dissolved in a mixture of 25 mM pH 7.4 HEPES stock solution and methanol (85/15, v/v) due to its lack of solubility in purely aqueous solutions. After the vesicle solutions were brought up to volume with the same mixture solution, they were vortex mixed for a further 30 min in order to control the size polydispersity and to ensure a relatively equilibrated vesicle size and size distribution.

All three liposome solutions were prepared by stepwise extrusion through polycarbonate membranes with different pore sizes [14]. In brief, the appropriate amounts of POPC and PS were dissolved in a 9:1 (v/v) mixture of chloroform and methanol. The organic solvent was stirred at 40 °C for 30 min and then removed under reduced pressure using a rotary evaporator in a water bath maintained at 60 °C. After evaporation, the vessel was connected to a vacuum pump overnight. The thin lipid film was hydrated with the appropriate amount of 25 mM pH 7.4 HEPES buffer. The hydrated liposomes were subjected to a freeze–thaw procedure five times. This procedure involved freezing the vesicles by immersion in dry ice (with acetone), followed by thawing (reconnect the flask to rotary evaporator in 60 °C water bath). The obtained multilamellar vesicles were then processed to unilamellar vesicles by stepwise extrusion through polycarbonate membranes while the extruder was maintained at a temperature of 65 °C by a circulating water bath. The vesicles were extruded through the 400, 200 and 100 nm filters three times each and through the 50 nm filters 20 times. The finally obtained liposome solutions appeared with a light blue translucent hue.

2.4. CE and HPLC conditions

New capillaries were flushed first with NaOH solution (1.0 M) for 1 h, followed by acetonitrile, water, and running buffer for 30 min each. Between runs, the capillaries were flushed for 0.5 min at 50 psi with NaOH (0.1 M), water, ACN and vesicle running buffer, respectively. The capillaries were thermostated at 25 °C. All separations were detected at λ = 254 nm (230 nm or 214 nm) depending on the UV absorbance of analyte. Sample introduction was performed hydrodynamically at 0.2 psi for 3 s, and the applied voltage of the separations was 20 kV. In order to measure the electroosmotic flow and the migration time of analytes in CZE system (buffer in the absence of the liposome or AOT vesicle), all samples were first run with either 25 mM pH 7.4 HEPES buffer or 25 mM pH 7.4 HEPES/methanol (85/15, v/v) buffer.

All HPLC experiments were carried out in gradient mode using 100% 50 mM ammonium acetate (pH 7.4) buffer as mobile phase A and 100% acetonitrile as mobile phase B. log $D_{7.4}$ was measured on Gemini C_{18} column following a methodology originally described by Kerns et al. [15]. The gradient was: 0 min/0% B, 2.5 min/95% B, 4.0 min/95% B, 4.1 min/0% B, 5.5 min/0% B. The mobile phase flow rate was 1.0 mL/min. A set of standards (atenolol, metoprolol, labetolol, diltiazem and triphenylene) was selected and a calibration graph of retention time *versus* log $D_{7.4}$ (literature) was constructed. A method described by Valko et al. [16] was used to measure the CHI $IAM_{7.4}$ values of all test drugs on Regis IAM PC DD2 column where the stationary phase support is immobilised phosphatidylcholine. The gradient was: 0 min/0% B, 6.0 min/100% B, 6.5 min/100% B, 7.0 min/0% B, 9.0 min/0% B. The mobile phase flow rate was 1.0 mL/min. With a set of alkylphenone standards, the gradient retention times can be converted to CHI $IAM_{7.4}$, which approximates to an acetonitrile concentration at which an equal distribution of compound can be achieved between the mobile phase and IAM.

2.5. Calculations

Retention factors of the compounds in EKC were measured as previously reported [13]:

$$k_{\text{neutral}} = \frac{(t_R - t_{eof})}{t_{eof}(1 - (t_R/t_{ves}))} \quad (1)$$

$$k_{\text{charged}} = \frac{(t_R - (t_0 t_{eof}/t'_{eof}))}{t_0 t_{eof}/t'_{eof}(1 - (t_R/t_{ves}))} \quad (2)$$

t'_{eof}, t_{eof}, t_0 and t_R is the electroosmotic flow and the migration time of analytes in CZE system (in the absence of vesicles) and EKC system (in the presence of vesicles), respectively. Methanol was used as the electroosmotic flow marker. The elution time of AOT vesicles or liposome (t_{ves}) was determined using a co-injected vesicle marker decanophenone. All samples were run in triplicate and the mean value was used for further correlation.

3. Results and discussions

3.1. Chromatographic characteristics

Recently, EKC with either liposome or surfactant vesicles has been successfully used to study and predict the drug–cell membrane interaction or the drug transport mechanisms through different biological systems [11,13,14]. One of the most noticeable advantages of EKC over HPLC based methodologies is that the composition of vesicle is easily controlled for effectively mimicking the properties of the physiological membrane.

It has been extensively accepted that electrostatic interaction between the charged drugs and the acidic phospholipids plays an important role in charged drug–tissue affinity [17–20]. In brain, reported tissue levels of the acidic phospholipids vary by a factor of *ca* 25 between researchers [17,21,22]. More recently, the phospholipid molecular species was analysed in the brain by ^{31}P NMR spectroscopy [21]. It was also observed that brain membrane of both human and rat contains an abundance of phosphatidylserine (PS). In this work, three liposome systems with different PS molar concentrations (POPC/PS, 80/20, 65/35 or 0/100) were evaluated for predicting the non-specific binding of PET radiotracer candidates. Our previous work [13] also showed that a surfactant EKC method with negatively charged AOT vesicles can effectively mimic both the hydrophobicity/lipophilicity interactions and electrostatic interactions between the compound and cell membrane. As this method has been successfully used to predict the lung tissue affinity of inhaled drugs, it was also evaluated in this work.

Firstly, we compared the robustness, reproducibility and the method run times of four EKC methodologies. A set of seven alkylphenones was selected as test compounds. As can be seen in Fig. 1, the $POPC_{80}PS_{20}$ liposome system observed the fastest elution for all seven alkylphenones, but co-elution was observed for the longer retained compounds octanophenone and decanophenone. By increasing the PS molar fraction in the liposome composition, the resolution between the octanophenone and decanophenone slightly improved and the migration time of liposome (measured with marker decanophenone) also increased, while the EOF remained almost constant. These observations can be rationalised by the properties of liposomes. The neutral POPC and the negatively charged PS have a very similar molecular weight. Therefore, the increase of PS molar fraction in the POPC/PS liposome composition will significantly increase the ratio of the charge to mass of the liposome and then the absolute value of its mobility (opposite direction to EOF), which appeared in the increasing migration time of decanophenone. This will also cause the increase in migration of analyte if it adsorbed on the liposome. The stronger the interaction between the analyte and the liposome, gives a longer migration

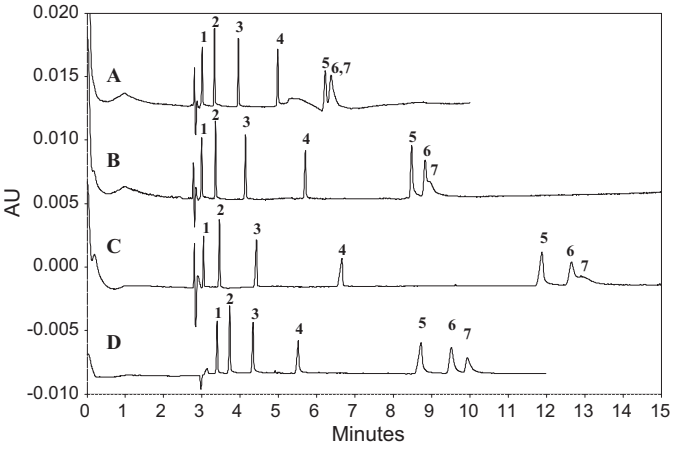

Fig. 1. Separation of seven alkylphenones with different electrokinetic chromatographic methods. Conditions: fused-silica capillary, 50 μm I.D. (375 μm O.D.) × 37.5 cm (29 cm effective length); injection by a pressure of 0.2 psi for 3 s; column was thermostated at 25 °C; applied voltage, 20 kV; detection at λ = 254 nm. Compounds: 1 = acetophenone; 2 = propiophenone; 3 = butyrophenone; 4 = valerophenone; 5 = heptanophenone; 6 = octanophenone and 7 = decanophenone. A: 4 mM $POPC_{80}PS_{20}$ liposome system; B: 4 mM $POPC_{65}PS_{35}$ liposome system; C: 4 mM PS_{100} liposome system; D: 1% w/v AOT vesicle system.

time. Compared to the three liposome EKC systems, the 1% w/v AOT vesicle system exhibited baseline separation for all seven compounds. Additionally, the preparation and stability of vesicles is a major concern in EKC in order to develop a robust method. As described above in the experimental section, the preparation procedure for liposomes is more complex than that for AOT vesicle preparation. After preparation, no deterioration of separation with the AOT vesicles was observed for at least 3 weeks, while a clear change in separation with the liposome vesicles, such as increase in analysis time and baseline noise, was observed after one week. It was also noticed that a better baseline was observed with the AOT vesicle EKC system rather than with the liposome EKC system. Therefore, the AOT vesicle EKC system has an advantage over liposome EKC systems as long as it provides a similar accuracy of prediction.

In Fig. 2, the separation of p-MPPF, Bupropion, PK11195, SCH23390, Spiperone and Sertraline with the AOT vesicle EKC method is observed. All six compounds are clinically used or terminated PET tracers and they have shown different *in vivo* non-specific bindings. The six compounds can be separated rapidly

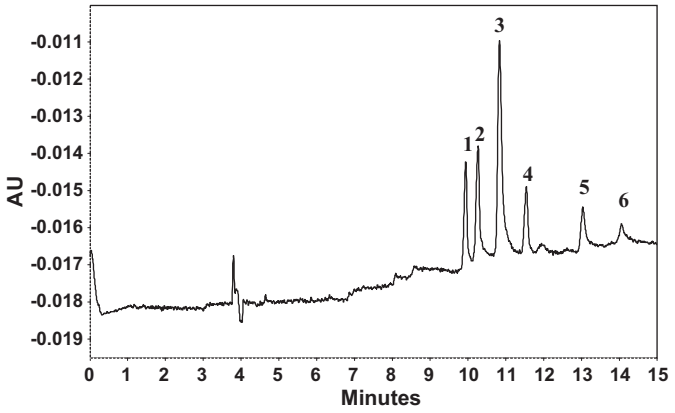

Fig. 2. Separation of several PET candidates with AOT vesicle EKC. Conditions: fused-silica capillary, 50 μm I.D. (375 μm O.D.) × 37.5 cm (29 cm effective length); injection by a pressure of 0.2 psi for 3 s; column was thermostated at 25 °C; applied voltage, 15 kV; current, 23 μA; detection at λ = 254 nm. Compounds: 1 = p-MPPF; 2 = bupropion; 3 = PK11195; 4 = SCH23390; 5 = Spiperone; 6 = Sertaline.

(within 15 min) and the results are reproducible. More importantly, their resulting calculated retention factors, which represent the interaction between drug and AOT vesicle, are quantitatively different and appear to reflect the degree of NSB of six compounds tested. Sertraline, a selective serotonin re-uptake inhibitor, has showed extensive non-specific binding to tissue [23] and Cella et al., predicted it to have a high NSB ratio in brain tissue [4]. It was deemed significant that Sertraline exhibited the highest retention ($\log k_{AOT} = 2.18$) in the current AOT vesicle EKC system. Spiperone, which has been found to have medium NSB in mice brain tissue [24] and is a terminated PET tracer, exhibited relatively lower retention ($\log k_{AOT} = 1.69$). SCH23390, a good tracer with a reported medium *in vitro* NSB ratio [4,25], exhibited lower retention ($\log k_{AOT} = 1.25$). The retention factors of other three test drugs (p-MPPF, Bupropion and PK11195) are significantly lower than for sertraline ($\log k_{AOT} = 0.85$, 1.10 and 0.97, respectively). These compounds are not considered to have high NSB issue *in vivo* and have been assigned as good PET tracers. Noticeably, the retention $\log k$ value with all three liposome EKC systems also exhibited the similar order (Sertraline $\gg$ Spiperone > SCH23390 > PK11195 $\approx$ Bupropion $\approx$ p-MPPF).

3.2. Correlations of EKC retentions to literature in vitro NSB ratio data

In order to evaluate the prediction of non-specific binding based on several EKC systems, 21 clinically used as well as unsuccessful PET radiotracer candidates, whose NSB ratio have been evaluated using equilibrium dialysis method from whole rat brain homogenate [4], were collected for this research. Their quantitative NSB ratio values were correlated respectively to the logarithm of the measured retention factors within different EKC systems ($\log k_{POPC_{80}PS_{20}}$, , $\log k_{PS_{100}}$ and) as well as their $\log D_{7.4}$ and CHI $IAM_{7.4}$ values. All physicochemical properties ($\log k_{POPC_{80}PS_{20}}$, $\log k_{POPC_{65}PS_{35}}$, $\log k_{PS_{100}}$, $\log k_{AOT}$, $\log D_{7.4}$ and CHI $IAM_{7.4}$) were measured using chromatographic approaches and summarised in Table 1. It was noticed that by increasing the PS molar fraction in the liposome composition, the retention factor of analytes also increased. This could be attributed due to the increasing negative charge density of liposomes, which further increases the electrostatic interaction between positively charged analytes and liposomes. However for strongly interacting compounds (such as sertraline and paroxetine), their retention factors are too high to be measured precisely at high PS molar fraction as their elution time is too close to that of the vesicle marker. Such a limitation is not the case within the AOT vesicle EKC system because good resolution was achieved between highly retained compounds.

The *in vitro* NSB ratio data using the equilibrium dialysis method was successfully used for evaluating the non-specific binding of PET candidates [4]. In brief, compounds that are currently in clinical use as PET ligands exhibited a high NSB ratio value in their assay, whereas compounds known to exhibit high non-specific binding have a low NSB ratio value.

We first compared our $\log D_{7.4}$ measured data by HPLC trend analysis in this work with the literature *in vitro* NSB ratio values. Lipophilicity is an empiric parameter use to predict non-specific binding. Current practice suggests that successful ligands have an octanol–water partition coefficient ($\log P$, $\log D_{7.4}$) of around 1.5–3.0 with an appropriate affinity for the receptor or enzyme of interest. A high $\log D_{7.4}$ value greater than 3.0 indicates that the candidate is most likely to have high NSB. However, many exceptions to this rule exist, suggesting that other factors, such as its ion charge state, size and shape, contribute to the non-specific binding phenomenon [5]. Indeed no statistical correlation ($r^2 = 0.04$) was found between the *in vitro* NSB ratio and $\log D_{7.4}$ (Fig. 3A), which was expected since the $\log D_{7.4}$ does not involve the contribution of electrostatic interactions.

Similarly, CHI $IAM_{7.4}$ data of all 21 test compounds were correlated to their *in vitro* NSB ratio (Fig. 3B). Significant correlation ($r^2 = 0.79$) was observed between CHI $IAM_{7.4}$ and the *in vitro* NSB ratio value. The CHI $IAM_{7.4}$ data was measured on a Regis IAM PC DD2 column, where a single layer of phosphatidylcholine (PC) is covalently bonded onto the silica gel. Such zwitterionic functional groups can provide binding sites for either hydrophobic/hydrophilic interaction or weak electrostatic interaction. Due to the fact that both the AOT vesicles and the POPC/PS liposome contain net negative charge at pH 7.4, electrostatic interaction is expected to be a significant factor in the retention of charged compounds. As seen in Fig. 3C, a clearly improved correlation ($r^2 = 0.88$) was observed between $\log k_{POPC_{80}PS_{20}}$ and the *in vitro* NSB ratio. However the correlation decreased with the increase of PS molar fraction to 35% and 100% ($r^2 = 0.82$ or 0.37, respectively) (Fig. 3D and E). It is difficult to explain this observation since the interaction between some analytes and the liposome become too strong to be measured precisely, which sacrificed the accuracy of data. However, several recently reported PC/PS ratio in rat brain tissue using TLC [26] or ^{31}P NMR [21] methods are approximate 80/20–73/27 (m/m). The POPC/PS liposome EKC system with higher PS molar fraction might overemphasize the contribution of electrostatic interaction.

A significant correlation ($r^2 = 0.85$) was also observed in Fig. 3F between $\log k_{AOT}$, and the *in vitro* NSB ratio even though AOT vesicles bear negative charge. The reason is still not clear. Since the AOT vesicle EKC system provides a more stable baseline, better peak shape for strongly interacting compounds and better robustness compared to the POPC/PS liposome EKC system as well as the comparable accuracy of prediction to that of $POPC_{80}PS_{20}$ liposome EKC system, it was selected for further research.

3.3. Correlation of surfactant vesicle EKC retention to in vivo NSB of potential PET tracer

In order to further evaluate the predictability of non-specific binding based upon the AOT vesicle EKC system, we required accurate *in vivo* NSB data of PET radiotracers. Unfortunately, although the ratio between specific and non-specific binding of most of the common PET radiotracers has been evaluated *in vivo*, the data concerning the absolute non-specific binding of PET tracers are often not quoted in the literature or assessed using different models and so not comparable. Therefore, we compared the $\log k_{AOT}$, with some literature reported qualitative non-specific binding data from different resources. Twenty-seven compounds, whose non-specific binding has been qualitatively evaluated *in vivo*, were selected for this comparison (see Table 2). Specific tracers are tracers that show either reduced uptake in the presence of a blocker or low binding in region devoid of receptors and vice versa for unspecific tracers. As can be seen in Fig. 4, there is a correlation between $\log k_{AOT}$ and the qualitative non-specific binding of selected compounds. In brief, it appears that the higher the $\log k_{AOT}$, the higher the qualitative non-specific binding. Practically, NSB properties of PET tracer N-methyl Spiperone was consider as critical point [27]. If we defined the $\log k_{AOT}$ value of N-methyl Spiperone (1.61) as a threshold, most qualitative high NSB compounds, except Nisoxetine ($\log k_{AOT} = 1.51$) will appear above it, whereas all qualitative low NSB compounds will appear below it. Nisoxetin is a NET tracer. The expression of NET in the human brain is very low and localized within small brain regions that make the development of NET tracers challenging. Spiperone, which displayed medium *in vivo* qualitative non-specific binding, also appeared above this threshold line. Interestingly, all compounds above this threshold line appeared to be failed or terminated PET candidates. There-

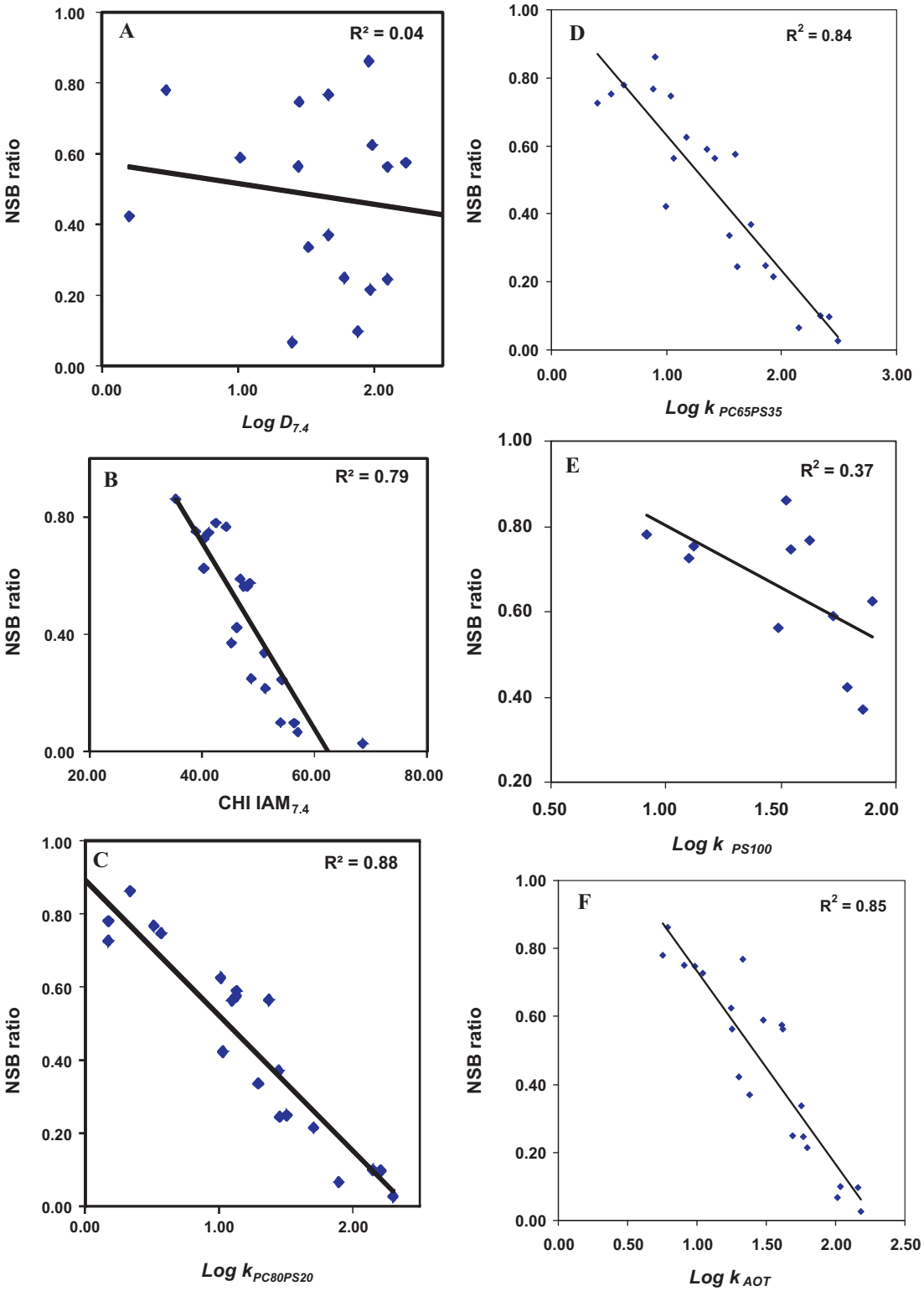

Fig. 3. Correlations between equilibrium dialysis NSB ratio and different chromatographic parameters.

fore, the log k_{AOT} information could help us to identify promising candidates from novel series that may be suitable for use as PET tracers.

To confirm this, we expanded this test set to further clinically used and failed candidates with an appropriate specificity and selectivity for the desired receptor or enzyme under investigation. As can be seen in Fig. 5, if we defined critical range of the log k_{AOT} values between 1.50 and 1.60, 36 compounds can be classified into three groups. All compounds in the group with log $k_{AOT} < 1.5$ have been proved to be good or promising tracers, whereas the compounds in the group with log $k_{AOT} > 1.7$ have been proved to be failed or terminated tracers. Only one compound in the critical zone, i.e. N-methyl Spiperone, appears to be good tracer, whereas the others in the critical zone failed.

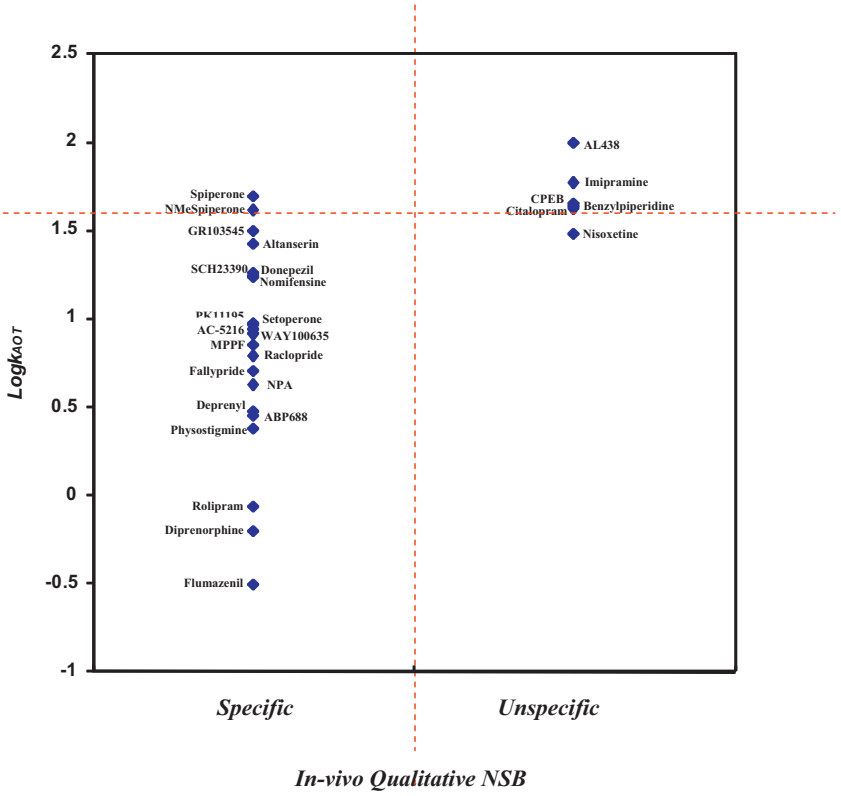

Fig. 4. Relationship between log k_{AOT} of selected PET ligands and their *in vivo* qualitative NSB.

For comparison we also regarded the predictability of non-specific binding using log $D_{7.4}$. As can be seen in Table 2, *in vivo* nonspecific binding of 11 out of 27 compounds cannot be correctly predicted qualitatively. For example, AC-5216 and PK11195, whose log $D_{7.4}$ values are 3.40 and 4.11, respectively, suppose to have high NSB, while their *in vivo* data did not show such properties. Conversely, citalopram and imipramine, whose log $D_{7.4}$ values are 1.44 and 2.10, respectively, exhibited high *in vivo* NSB.

There are, however, other parameters that need to be considered while trying to predict the success of a tracer *in vivo*. Currently the AOT vesicle EKC system does not take in account the brain exposure. After intravenous injection, the free PET radiotracer in

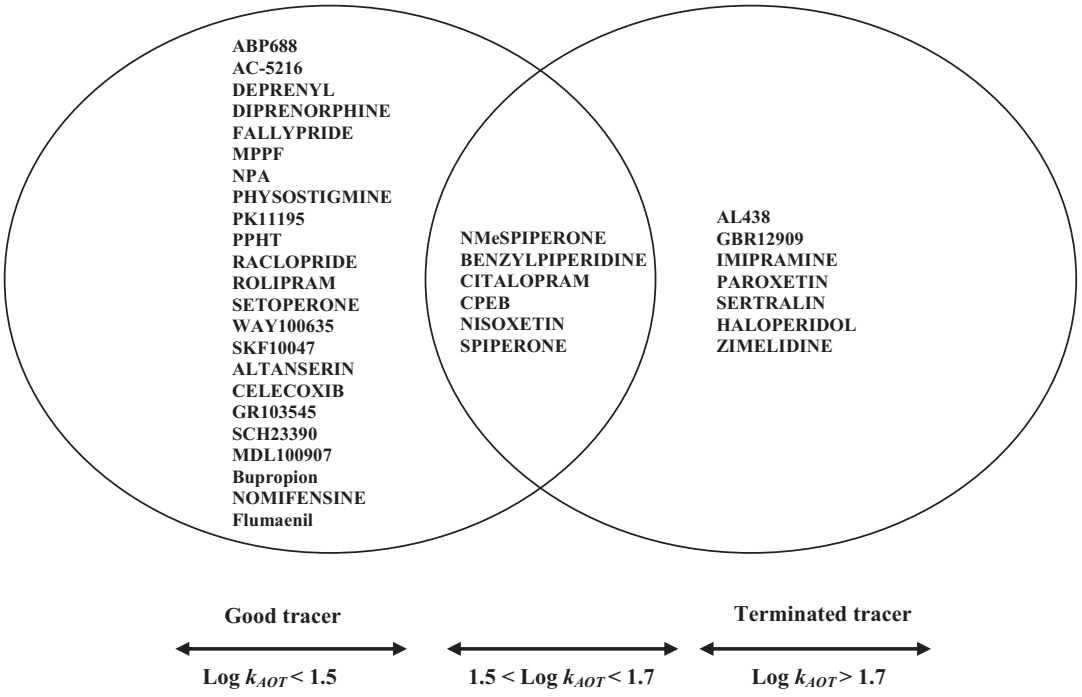

Fig. 5. Predicting PET potential of candidates with their log k_{AOT} values.

plasma needs to cross the blood–brain barrier (BBB) before arriving at the non-specific or specific binding sites. This implies a good flux and passive BBB permeability. However, this requirement might be in conflict with the requirement for low non-specific binding. For example, in general within a given structure family, lipophilicity promotes BBB permeability, which is good, but increases non-specific binding, which is detrimental. To sum up, the properties of candidates required for proper PET radiotracer quantification include many factors, such as affinity, selectivity, permeability, metabolism etc. The importance of low non-specific binding cannot be overemphasised. Limitations aside, there is still a significant correlation between retention in the AOT vesicle EKC system and NSB, suggesting AOT vesicle EKC can provide a primary profile screening for a large number of drug candidates early in the drug discovery process.

4. Conclusions

In this study, three POPC/PS liposome and one AOT vesicle EKC methods as well as HPLC $IAM_{7.4}$ and $\log D_{7.4}$ chromatographic methods were compared and evaluated as high throughput profile screening methods for NSB of potential PET radiotracer in respect of chromatographic characteristics and statistical correlations. The AOT vesicle EKC analysis offered the best high throughput approach owing to the short run times, high reproducibility, high selectivity and low sample and solvent cost. The retention within AOT vesicle EKC system of selected clinically used as well as unsuccessful candidates exhibited good correlation to their *in vitro* NSB ratio data, measured through a equilibrium dialysis method from whole rat brain homogenates, as well as to *in vivo* qualitative NSB data. The AOT vesicle EKC method provides a useful tool for the estimation of non-specific binding characteristics. Compounds that are currently in clinical use as PET ligands have achieved low retention in this system, whereas compounds known to be terminated tracer have shown high retention value. From this data we are able to identify promising candidates from a large number of novel series that may be suitable for use as PET tracers in the drug discovery process with minimal resources.

References

[1] J.K. Willmann, N. Van Bruggen, L.M. Dinkelborg, S.S. Gambhir, Molecular imaging in drug development, Nat. Rev. Drug Discov. 7 (2008) 591–607.

[2] M. Laruelle, M. Slifstein, Y. Huang, Relationships between radiotracer properties and image quality in molecular imaging of the brain with positron emission tomography, Mol. Imaging Biol. 5 (2003) 363–375.

[3] S. Patel, T. Hamill, E. Hostetler, D. Burns, R.E. Gibson, An in vitro assay for predicting successful imaging radiotracers, Mol. Imaging Biol. 5 (2003) 65–71.

[4] C.V. Cella, S.M. Woodhouse, R.A. Lewis, A.E. Fray, A novel method for the assessment of potential PET ligands using an in vitro assay of non-specific binding, FENS A059 (2004) 4.

[5] L. Rosso, A.D. Gee, I.R. Gould, *Ab initio* computational study of positron emission tomography ligands interacting with lipid molecule for the prediction of nonspecific binding, J. Comput. Chem. 29 (2008) 2397–2405.

[6] M.A. Mintun, M.E. Raichle, M.R. Kilbourn, G.F. Wooten, M.J. Welch, A quantitative model for the in vivo assessment of drug-binding sites with positron emission tomography, Ann. Neurol. 15 (1984) 217–227.

[7] L. Rosso, A.D. Gee, I.R. Gould, Towards in silico descriptors of PET ligands non-specific binding, Neuroimage 41 (2008) T99.

[8] Q. Guo, M. Brady, R.N. Gunn, A biomathematical modeling approach to central nervous system radioligand discovery and development, J. Nucl. Med. 50 (2009) 1715–1723.

[9] A. Reichel, D.J. Begley, Potential of immobilized artificial membranes for predicting drug penetration across the blood–brain barrier, Pharm. Res. 15 (1998) 1270–1274.

[10] C. Pidgeon, S.W. Ong, H.L. Liu, X.X. Qiu, M. Pidgeon, A.H. Dantzig, J. Munroe, W.J. Hornback, J.S. Kasher, L. Glunz, T. Szczerba, IAM chromatography: an in vitro screen for predicting drug membrane permeability, J. Med. Chem. 38 (1995) 590–594.

[11] R.J. Pascoe, J.A. Masucci, J.P. Foley, Investigation of vesicle electrokinetic chromatography as an in vitro assay for the estimation of intestinal permeability of pharmaceutical drug candidates, Electrophoresis 27 (2006) 793–804.

[12] H.L. Liu, S.W. Ong, L. Glunz, C. Pidgeon, Predicting drug–membrane interactions by HPLC: structural requirements of chromatographic surfaces, Anal. Chem. 67 (1995) 3550–3557.

[13] Z. Jiang, J. Reilly, B. Everatt, A method for rapidly predicting drug tissue distribution using surfactant vesicle electrokinetic chromatography, Electrophoresis 29 (2008) 3674–3684.

[14] J.M. Carrozzino, M.G. Khaledi, Interaction of basic drugs with lipid bilayers using liposome electrokinetic chromatography, Pharm. Res. 21 (2004) 2327–2335.

[15] E.H. Kerns, L. Di, S. Petusky, T. Kleintop, D. Huryn, O. McConnell, G. Carter, Pharmaceutical profiling method for lipophilicity and integrity using liquid chromatography–mass spectrometry, J. Chromatogr. B 79 (2003) 381–388.

[16] K. Valko, C.M. Du, C.D. Bevan, D.P. Reynolds, M.H. Abraham, Rapid-gradient HPLC method for measuring drug interactions with immobilized artificial membrane: comparison with other lipophilicity measures, J. Pharm. Sci. 89 (2000) 1085–1096.

[17] N. Yata, T. Toyoda, T. Murakami, A. Nishiura, Y. Higashi, Phosphatidylserine as a determinant for the tissue distribution of weakly basic drugs in rats, Pharm. Res. 7 (1990) 1019–1025.

[18] T. Rodgers, D. Leahy, M. Rowland, Physiologically based pharmacokinetic modeling 1: predicting the tissue distribution of moderate-to-strong bases, J. Pharm. Sci. 94 (2005) 1259–1276.

[19] T. Rodgers, M. Rowland, Physiologically based pharmacokinetic modelling 2: predicting the tissue distribution of acids, very weak bases, neutrals and zwitterions, J. Pharm. Sci. 95 (2006) 1238–1257.

[20] J.P.H.T.M. Ploemen, J. Kelder, T. Hafmans, H. Van De Sandt, J.A. van Burgsteden, P.J.M. Salemink, E. Van Esch, Use of physicochemical calculation of pK_a and *C*log *P* to predict phospholipidosis-inducing potential—a case study with structurally related piperazines, Exp. Toxicol. Pathol. 55 (2004) 347–355.

[21] J.M. Pearce, R.A. Komoroski, Analysis of phospholipid molecular species in brain by ^{31}P NMR spectroscopy, Magn. Reson. Med. 44 (2000) 215–223.

[22] G.B. Ansell, J.N. Hawthorne, R.M.C. Dawson, Form and Function of Phsopholipids, Elsevier, Amsterdam, 1973.

[23] A.C. Schwartz, B.O. Rathbaum, Review of Sertraline in post-traumatic stress disorder, Expert Opin. Pharmacother. 3 (2002) 1489–1499.

[24] J.S. Fowler, C.D. Arnett, A.P. Wolf, R.R. MacGregor, E.F. Norton, A.M. Findley, [^{11}C] Spiroperidol: synthesis, specific activity determination, and biodistribution in mice, J. Nucl. Med. 23 (1982) 437–445.

[25] G.L.Y. Chan, J.E. Holden, A.J. Stoessl, D.J. Doudet, Y. Wang, T. Dobko, K.S. Morrison, J.M. Huser, C. English, B. Legg, M. Schulzer, D.B. Calne, T.J. Ruth, Reproducibility of the distribution of carbon-11-SCH 23390, a dopamine D_1 receptor tracer, in normal subjects, J. Nucl. Med. 39 (1998) 792–797.

[26] G.Y. Xu, H. Waki, K.Z. Kon, S. Ando, Thin-layer chromatography of phospholipids and their lyse forms: application to determination of extracts from rat hippocampal CA1 region, Microchem. J. 53 (1996) 29–33.

[27] K. Yanai, T. Ido, K. Ishiwata, J. Hatazawa, S. Watanuki, T. Takahashi, A. Ujiie, M. Ito, T. Matsuzawa, Characteristics of specific in vivo labeling of neuroleptic binding-sites with 3-*N*-[^{11}C]methylspiperone, Eur. J. Nucl. Med. 11 (1986) 438–443.