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Highlights

- Chromatographic retention of 26 molecules was studied by IAM LC and LEKC
- LEKC data could not be achieved for hydrophilic neutral or anionic compounds
- IAM related better with partition than with distribution coefficients in *n*-octanol
- For LEKC, the relationships were linear to partition coefficients in *n*-octanol
- LEKC outperformed IAM in modelling the pulmonary permeability of drugs

Immobilised artificial membrane liquid chromatography *vs* liposome electrokinetic capillary chromatography: Suitability in drug/bio membrane partitioning studies and effectiveness in the assessment of the passage of drugs through the respiratory mucosa

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Abstract

This study pioneers a comparison of the application of biomimetic techniques, immobilised artificial membrane liquid chromatography (IAM LC) and liposome electrokinetic capillary chromatography (LEKC), for the prediction of pulmonary drug permeability. The pulmonary absorption profiles of 26 structurally unrelated drug-like molecules were evaluated using their IAM hydrophobicity index (CHI IAM) measured in IAM LC, and the logarithm of distribution constants (log K^{LEKC}) derived from the LEKC experiments. Lipophilicity (phospholipids) parameters obtained from IAM LC and most LEKC analyses were linearly related to the n-octanol/water partitioning coefficients of the neutral forms (*i.e.*, log $P_{o/w}$ values) to a moderate extent. However, the relationship with distribution coefficients at the experimental pH (7.40) (*i.e.,* log $D_{7.4}$) were weaker overall for IAM LC data and sigmoidal for some liposome compositions (phosphatidyl choline (PC): phosphatidyl inositol (PI) 85:15 mol% and 90:10 mol%) and concentrations (4 mM) in LEKC. This suggests that phospholipid partitioning supports both hydrophobic and electrostatic interactions occurring between ionised drugs and charged phospholipid moieties. The latter interactions are original when compared to those taking place in the more established *n*-octanol/water partitioning systems.

A stronger correlation (R^2 > 0.65) was identified between the LEKC retention parameters, and the experimental apparent lung permeability (*i.e.*, log P_{app} values) as opposed to the values obtained by IAM LC. Therefore, LEKC offers unprecedented advantages over IAM LC in simulating cell membrane partitioning processes in the pulmonary delivery of drugs. Although LEKC has the advantage of more effectively simulating the electrostatic and hydrophobic forces in drug/pulmonary membrane interactions *in vitro*, the technique is unsuitable for analysing highly hydrophilic neutral or anionic compounds at the experimental pH. Conversely, IAM LC is useful for analysing compounds spanning a wider range of lipophilicity. Its simpler and more robust implementation, and propensity for high-throughput automation make it a favourable choice for researchers in drug development and pharmacological studies.

Keywords: *biomimetic chromatography, drug/phospholipid interactions, immobilised artificial membrane chromatography, liposome electrokinetic capillary chromatography, pulmonary drug permeability*

1. Introduction

During the initial phases of drug development, the evaluation of the physicochemical properties of drug candidates extends beyond the assessment of biological activity [1]. Lipophilicity and solubility are also essential parameters which play a role in the absorption of drugs administered via parenteral and enteral routes, including the pulmonary route [2]. Therefore, there is a need for the development of reliable, reproducible and high-throughput screening methods to characterise these critical parameters. The lipophilicity of drugs, particularly when they exist in their non-ionised form, is commonly assessed through the determination of their partition coefficient (log $P_{o/w}$) between immiscible phases. Typically, log $P_{o/w}$ is assessed using an isotropic *n*-octanol/water system, with *n*-octanol representing the body's lipophilic environment and water depicting the characteristics of extracellular fluids. Log $P_{o/w}$ determination is often employed as an indicator of transmembrane permeability [3]. However, most commercially available drugs are weak acids or bases and exist as a mixture of charged and neutral species depending on the pH value. Thus, for ionisable drugs, the apparent distribution coefficient (log D_{pH}) at a specific pH is typically used to assess lipophilicity. The aqueous phase is buffered to a defined pH value, and *n*-octanol serves as the organic phase [4]. However, even with the consideration of the ionisation state, the *n*-octanol/water system lacks the precision to evaluate lipophilicity as it is too simplistic to capture the complexity of interactions between ionised molecules and phospholipids found in biological membranes. This makes it an inadequate model for drug absorption and transport through biological membranes [5]. Grumetto *et al.* [6] studied the intestinal permeability of 47 compounds, thereby revealing the fundamental role of polar/electrostatic forces in the absorption of drugs through biological membranes. One of the alternatives is IAM chromatography, which presents superior biomimetic properties compared to traditional lipophilicity measurements. In this method, phosphatidylcholine molecules, the primary phospholipid in cell membranes, are covalently bonded to silica, effectively replicating the phospholipid monolayer. Chromatographic analysis considers intermolecular and electrostatic interactions that affect the partitioning

pattern, according to the so-called 'pH-Piston Hypothesis' [7, 8].In recent years, several studies have been published focusing on the validation of chromatographically obtained phospholipid lipophilicity (phospholipophilicity) parameters in the prediction of intestinal drug absorption and blood-brain barrier passage [9, 10]. Phospholipophilicity elucidates the preferential partitioning of a drug molecule between a phospholipid membrane and an aqueous environment [11]. However, there is a sparsity of published works investigating the permeability of the alveolar epithelium or the mechanisms that affect the rate of pulmonary permeability. Existing studies highlight the role of phospholipophilicity as the most important physicochemical parameter.

In recent years, capillary electromigration (CE) techniques, which retain the advantages of conventional LC methods, have gained notoriety in drug absorption and permeability studies [12]. Various CE-based techniques, including microemulsion electrokinetic capillary chromatography (MEEKC) [13], bio partitioning micellar electrokinetic chromatography (BMEKC) [14], and liposome electrokinetic capillary chromatography (LEKC) [15-17], have been developed. CE methods are recognised for their high separation efficiency, cost-effective solvent usage, and other environmentally friendly features [12].

The current study focuses on the potential of application, usefulness, and limitations of LEKC and immobilised artificial membrane (IAM) HPLC methods to estimate the membrane permeability of 26 structurally unrelated small molecule drugs (Figure S1). The results are compared with *in vitro* permeability data (P_{app}) obtained from Calu-3 cell lines to evaluate whether these techniques are a predictor for human pulmonary drug absorption.

In IAM HPLC applications, the stationary phase is made by covalent attachment of phosphatidylcholine (PC) monolayers to amino-propyl silica particles which are intended to mimic the *in vivo* interactions of drug molecules with phospholipids [18-22]. However, as the model comprises of a single-layer phospholipid structure, the analytes only bind to the surface and do not pass through the membrane. In this regard, the model is unlike physiological conditions where a bilayer (or multiple bilayers) exists. Thus, the complexity and diverse lipid composition of natural biological membranes may not be fully captured [23]. Conversely, LEKC offers substantial advantages for evaluating drug-membrane interactions compared to other existing models like *n*-octanol/water or IAM HPLC.

In LEKC, liposomes serve as a pseudo-stationary phase where each lipid vesicle possesses a lipid bilayer microstructure and contains carefully controlled proportions of phospholipids that are prevalent in biological membranes, such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) or phosphatidylglycerol (PG) [23, 24]. The liposome bilayer structure (similar to the cell membrane), along with the ability to adjust the type and mole fraction of each phospholipid, provides a closer similitude to natural membranes, thereby enhancing the model of complex drug-lipid interactions [15]. The main differences of these analytical approaches are depicted in Figure 1.

Lipidomic research has elucidated that different tissues comprise different lipid compositions [25]. While zwitterionic PC is the most common, lipid extracts from the brain and lung are also rich in anionic phospholipids such as PI. Consistently, LEKC represents a much more versatile platform than IAM HPLC, as the composition of the liposomes used as a pseudo stationary phase can be finely adjusted to mirror a specific body tissue where the permeation is occurring. In contrast, while considerable synthetic work has been done to couple phospholipids other than PC to amino-propyl silica for biomimetic LC analysis [26], such phases are not commercially available and/or are not broadly implemented due to high batch-to-batch variability [27].

The present study aimed to compare the selectivity of both analytical platforms to investigate the relationship between phospholipophilicity measurements using each technique, and their intrinsic capability to assist in evaluating the permeation of model drugs through bronchial epithelial cell membranes, which are particularly rich in PI (up to ~15% (v/v)) [28]. To the best of our knowledge, this is the first time two biomimetic techniques, exploited in CE and in LC, are systematically compared both in terms of analytical retention characterisation and inherent suitability in profiling the absorption of therapeutics *in vitro* using a dataset of structurally unrelated compounds. Our results can inform pharmaceutical/separation scientists on the best analytical approach to undertake depending on a number of factors including throughput required, polarity range and ionisation behaviour of the intended dataset, and environmental impact.

2. Materials and methods

2.1 Chemicals

All samples were purchased from commercially available sources. Acyclovir, chlorothiazide. cromolyn, enalaprilat, imipramine, levofloxacin, oxacillin, quinidine, ranitidine, salbutamol, sulfanilic acid, sulfaguanidine, sulfapyridine, theophylline and uridine were purchased from Thermo Scientific (Renfrew, United Kingdom). Famotidine, flunisolide, labetalol, pefloxacin, rosiglitazone and rosuvastatin were sourced from Cayman Chemical Company (Ann Harbor, Michigan, USA). Budesonide, methotrexate and saquinavir were purchased from APExBIO (Houston, Texas, USA). Nadolol and norfloxacin were purchased from Selleck Chemicals (Cologne, Germany) and Sigma-Aldrich (St. Louis, Missouri, United States), respectively. Each sample had a purity ≥ 98% and were used without further purification.

For the LEKC study, the phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), liver phosphatidylcholine (liver PC) and liver phosphatidyl inositol (liver PI) were sourced from Avanti Polar Lipids (Alabaster, AL). Ethyl benzoate was purchased from Merck (Hohenbrunn, Germany), propyl benzoate from Aldrich (St. Louis, USA), butyl benzoate and pentyl benzoate from Fluka (Steinheim, Germany) and hexylbenzoate from Sigma (St. Louis, USA).

The buffer reagents disodium hydrogen phosphate dodecahydrate (Na_2HPO_4*12 H_2O) and sodium dihydrogen phosphate monohydrate (NaH_2PO_4*H_2O) were purchased from J.T. Baker (Deventer, the Netherlands) and Merck (Darmstadt, Germany), respectively. HPLC grade 99.5% dimethyl sulfoxide (DMSO) was sourced from Labscan (Dublin, Ireland). The standard pH solutions (4.0, 7.0 and 10.0) used for pH meter calibration were purchased from Merck (Darmstadt, Germany).

For the IAM-HPLC study, HPLC grade acetonitrile and methanol were obtained from Rathburn (Walkerburn, United Kingdom) and used without further filtration. Crystalline ammonium acetate (CH_3COONH_4), obtained from Alfa Aesar (India) with a purity of ≥ 97%, was used for buffer preparation. Sodium hydroxide (NaOH) for pH adjustment was purchased from VWR International (Rosny-sous-Bois, France). Standard pH solutions (4.0 and 7.0) employed for pH meter calibration, were procured from Thermo Scientific (Oxford, United Kingdom). Stock solutions were prepared by dissolving analytes in HPLC grade methanol (budesonide, chlorothiazide, enalaprilat, ganciclovir, famotidine, imipramine, labetalol, levofloxacin, moxifloxacin, nadolol, oxacillin, quinidine, ranitidine, rosiglitazone, rosuvastatin, salbutamol, saquinavir, sulfaguanidine, sulfapyridine, theophylline) or pure

water (cromolyn, pefloxacin, sulfanilic acid, uridine) to a concentration of 2.0 mg/mL, except for acyclovir and methotrexate, which required the addition of sodium hydroxide, and norfloxacin where diluted acetic acid was added to aid solubility. The analytical balance used was Sartorius RC210D, 21000106. Working solutions were freshly prepared each day by diluting the stock solution with the mobile phase to a concentration of 50 µg/mL.

All samples underwent filtration using 0.45 µm PTFE Membrane syringe filters from VWR International (Radnor, USA) before LC analysis.

2.2 Buffer preparation

To facilitate the LEKC work, a sodium phosphate buffer (PHB, I = 50 mM, pH 7.4) was prepared from Na_2HPO_4*12 H_2O and NaH_2PO_4*H_2O. Purified water was obtained from a water purification system Milli-Q Direct-Q 3 UV. The centrifuge used was a Micromax Thermo IEC with a Thermo IEC 851 rotor. The pH of the buffer solutions was measured with a WTW inoLab pH 7110 pH meter and adjusted to pH 7.4. Each solution was filtered through 0.45 µm membrane filters (Millipore) before CE analysis. The lipid stock solutions were prepared in hexane:2-propanol (3:2, by volume) or in chloroform (as supplied). The lipid stock solutions were stored in darkness at -20°C. The concentration of each phospholipid stock solution prepared was 4 mM. All studied compounds were prepared in water at a concentration of 50 µg/mL. An analytical balance Sartorius BP301S (Goettingen, Germany) was used for weighing the analytes. Dimethyl sulfoxide (DMSO) was used as the electroosmotic flow marker.

To accomplish the IAM-HPLC measurements, A 10 mM ammonium acetate buffer solution was prepared by dissolving crystalline ammonium acetate (AA) in pure water. Water with a resistivity of 18.2 MΩ cm^{-1} was purified in-house through a Suez water purification system (SUEZ Water Technologies and Solutions). The required pH of the buffer was verified using a Mettler Toledo pH meter (Peterborough, UK) and adjusted with sodium hydroxide to pH 7.4. Before each pH adjustment, the pH meter underwent calibration using a two-step calibration method, ensuring that the calibration slope remained within the 96 % to 104 % range. Prior to use, the mobile phase was filtered under vacuum through 0.45 µm nylon membrane filters from Cole-Parmer. HPLC grade acetonitrile served as the organic modifier phase without the need for further filtration.

2.2 LEKC study

2.2.1 Preparation and characterization of liposomes

Aliquots of the lipids were mixed in appropriate molar ratios (95:5, 90:10, or 85:15 mol% of liver PC/ liver PI or 80:20 or 85:15 mol% of POPC/POPS). Next, the phospholipids were placed under a stream of pressurised nitrogen to evaporate the organic solvent. Chloroform was added to the dried phospholipids and redried under nitrogen to form a lipid film, and the samples were placed under reduced pressure (8-100 mbar) overnight. The dry lipid films were hydrated with PBS solution to obtain a vesicle concentration of 1 or 4 mM for LEKC. The temperature of the buffer was maintained above the gel-to-liquid crystalline phase transition temperature of the lipid component with the highest melting temperature before adding to the lipid film. The dry lipid suspension was maintained at approximately 60°C during the hydration step of 60 min, with intermittent vortexing to yield multi lamellar vesicles (MLVs). For obtaining large unilamellar vesicles, the MLV dispersion was extruded a minimum of 11 times through Millipore 100 nm pore size polycarbonate filters (Bedford, MA, USA) using an Avanti® Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA). The size distribution of the prepared vesicle solutions was measured with a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, Worcestershire, U.K.). The average vesicle sizes (based on intensity distribution) were in the range of 100-130 nm.

2.2.2 Capillary electrophoresis (CE) analysis

An Agilent Technologies 7100 CE capillary electrophoresis system equipped with an UV/DAD detector was used. The capillary was a 48.5 cm fused coated silica capillary (Polymicro Technologies) with 40 cm length to the detector and inner/outer diameters of 50/360 µm. The software used was Agilent OpenLAB CDS ChemStation Edition c.01.05.

During each CE run the capillary was flushed prior to each sequence with 0.1 M NaOH for 3 min, with H_2O for 2 min, and with phosphate buffer (PHB) for 5 min in high pressure mode (ca 940 mbar). Before each injection, the capillary was flushed with NaOH, H_2O and the background electrolyte in use (PHB, 1 or 4 mM liposome dispersion) for 1 min each. The applied separation voltage was 30 kV, and the temperature was 25 °C. Hydrodynamic injection was applied at 50 mbar for 10 s and all samples were run with three replicate injections. UV spectra of the runs were recorded at wavelengths 190 - 400 nm with 2 nm

increments and the spectra of the analytes were saved in the UV spectrum library. Electropherograms were extracted at wavelengths 200 nm, 230 nm, 214 nm and 254 nm. Migration times were recorded for the distribution constant calculations. CE data processing details can be found in SI.

2.3 IAM-HPLC study

2.3.1 HPLC analysis

Agilent 1260 Infinity II high performance liquid chromatograph was used with 1260 Infinity Diode Array Detector (1290 DAD). Both the IAM DD2 column (150 mm x 4.5 mm) and IAM P.C. DD2 guard cartridge system (1 cm x 3.0 mm) were purchased from Regis Technologies (Morton Grove, IL, USA). Chromatographic parameters and the sequenced analysis were configured using the *Microsoft® Windows* 10 Professional operating PC with Agilent 1290 Infinity 2D-LC Chemstation software, version 1.4.36.

The mobile phases used for these studies were 50 mM ammonium acetate buffer (AA) at pH 7.4 and acetonitrile. The chromatographic separation was carried out under gradient conditions (Table S2) at 25 °C, with a flow rate of 1 mL/min and an injection volume of 10 µL. This method was developed and validated by Valko, where the isocratic and fast-gradient separations were compared based on the log k_{IAM} values and the CHI_{IAM} values of 48 analytes [29]. Each sample was run in triplicate with a blank run every 4-5 samples. The determination of time zero was accomplished using an injection of citric acid. Before each LC analysis, the column underwent an 18 min equilibration with pure ammonium acetate buffer, maintaining a flow rate of 1 mL/min. UV spectra of each run was recorded at 214 nm, 230 nm, 254 nm, 260 nm and 290 nm wavelengths.

2.3.2 Data processing

Chromatographic hydrophobicity index (CHI_{IAM}) links the isocratic and gradient retention factors by the organic phase concentration parameter (ϕ_0) [29]. Further information is available in SI.

2.4 Lipophilicity and biological activity parameters

Log $P_{o/w}$ values (partition coefficients *n*-octanol/aqueous phase of neutral form of analytes) were taken from the indicated literature sources. The *n*-octanol/aqueous buffer at pH 7.4

partition coefficients (log $D_{7.4}$) were also obtained from the literature except for sulfapyridine and budesonide, whose values were calculated according to Equation 1,

$$logD_{7.4} = \log P_{o/w} - \log(1 + 10^{7.4-pK_a}) \quad (1)$$

and pefloxacin, chlorothiazide and sulphaguanidine for which, the experimental log D values measured at pH 6.9, 6.5 and 7.6, respectively, were used as reasonable estimates for their log $D_{7.4}$ values. The ionisation percentage of monofunctional electrolytes was calculated by equations S8a and S8b as detailed in SI.

For ampholytes, calculations were performed using Marvin Sketch software v. 24.1.2 (ChemAxon (http://www.chemaxon.com)). These also informed their ranking according to their prevalent electric charge at the experimental pH value.

The experimental apparent lung permeability (P_{app}) values of 26 investigated compounds were acquired from 8 published studies in Calu-3 cell cultures [30-37]and were used for comparison with data generated during IAM-HPLC and LEKC experiments. P_{app} values were further log-transformed and collected in Table 2.

3. Results and discussions

In IAM-HPLC, partition coefficients were determined for the compounds of interest and the measurements were performed at pH 7.4 adapting a standardised fast-gradient method, developed by Valkó *et al.* [29]. This choice was made due to the high lipophilicity of certain compounds within the dataset (saquinavir and imipramine), which would require the use of elevated percentages of organic modifier under isocratic conditions for effective elution.

3.1 Relationships of IAM indices with *n*-octanol/water lipophilicity values

Analytical retention in both IAM-HPLC and LEKC is strongly affected by both hydrophobic and electrostatic interactions between the analytes and the membranes, thus the dataset was split into subsets according to the prevalent charge supported at the experimental pH. The physico-chemical properties of the dataset are reported in Table 2, whereas Table 3 summarises all the biomimetic measurements. The relationships between IAM indices and the *n*-octanol/water lipophilicity of the neutral forms of the analytes (A) and that of the

mixture at pH 7.4 (B) are shown in Figure 2. A clear ascending trend is visible in Figure 2A with anions evidently less retained – on average – than neutrals, cations and zwitterions. This is fully consistent with the 'pH piston hypothesis' [38] according to which bases engage more intensely with the negatively charged phosphate group of PC than neutral isolipophilic, *i.e.* of identical log $P_{o/w}$, molecules. Indeed, in the IAM.PC network, the phosphate groups are located at a deeper site than the positively charged choline moieties which supports a more superficial – and therefore weaker – interaction with anions. This behaviour was observed – albeit with some small differences – also for the 'new' IAM selectivity [39]. Both the acids enalaprilat and sulfanilic acid deviated from an imaginary trend line with a CHI IAM lower than expected. A weaker linear relationship can be observed when considering CHI IAM *vs* log $D_{7.4}$ instead of log $P_{o/w}$ (Figure 2B). Specifically, acidic compounds appear more scattered than in Figure 2A, with methotrexate and theophylline deviating from the distribution of the datapoints, indicating that for both these compounds analytical retention on the IAM phase seems to be log P^N rather than log $D_{7.4}$ driven. This is consistent with our previous works [9, 10, 40-42] according to which IAM data related more with log $P_{o/w}$ than with log D_{pH}, offering substantial orthogonality for ionisable compounds when compared with the "classic" selectivities exploited in reversed phase LC such as silica based C18 or C8 phases.

3.2 Relationships of LEKC indices with *n*-octanol/water partitioning of the neutral forms

Figure 4 proffers an overview of how the distribution constants in LEKC are related to the *n*-octanol/water partitioning of the neutral forms for three liposomal compositions implemented *ad hoc* to mimic the characteristics of the respiratory epithelium bilayers [28]. The rationale was to compare IAM-HPLC with LEKC in modelling the passage of therapeutics through a barrier containing a remarkable concentration of anionic phospholipids such as PI. In these regards, this study and its findings are original as the only tangentially related research is that by Masucci and co-workers [43] who assessed the retention behaviour of a set of β-blockers using both IAM-HPLC and electrokinetic capillary chromatography. However, the main differences are that (1) there were only eight solutes investigated which were structurally related, (2) these possessed similar ionisation characteristics making the electrostatic interactions of the retention much more predictable and less distinct, and (3) the authors used lysophospholipid micellar electrokinetic (LMEKC) capillary chromatography

which feature a completely different setup as compared to LEKC. Aside from the aforementioned study, partitioning experiments in the scientific literature are usually performed separately, on a smaller dataset or by using other techniques such as MEEKC that are not, due to their nature, relatable to LEKC.

The first noteworthy aspect in Figure 3 is that twelve compounds, mostly acidic and anionic at the pH 7.4, were experimentally inaccessible by LEKC but could be measured by IAM-HPLC. While the neutrals that exhibited no retention at all have negative log $P_{o/w}$ values, some of the unretained acidic compounds have remarkable *n*-octanol/water lipophilicity (*e.g.* rosuvastatin (log $P_{o/w}$ = 2.40)). It is expected that the interaction with a negatively (5-15 mol% PI) charged membrane would favour cations, as in this sense, the retention of the positively charged analytes could be measured except famotidine and sulphaguanidine which are the least lipophilic of the subset with highly negative lipophilicity values (log $P_{o/w}$ < -0.8). It is much more difficult to discount the behaviour of zwitterions as their chromatographic retention is certainly affected by complex mechanisms of charge overlapping. This is heavily dependent on features such as charge distribution, pK_a values of the ionisable moieties involved, the molecular flexibility of the compounds among others [44]. However, certainly charge seems to play a pivotal role, particularly if we look at the structures of norfloxacin and pefloxacin, two fluoroquinolone antibiotics whose chemical structures differ only by the substitution of the nitrogen in position 4 of the piperazine ring. In fact, LEKC retention of pefloxacin, which supports a methyl group covalently bound to the nitrogen in position 4, is not measurable, counter to what would be expected. Indeed, the addition of an alkyl group should in principle increase – albeit only marginally – the hydrophobicity of the molecule. However, it is known that tertiary amines are less basic than secondary amines due to the reduced accessibility of the lone pair of electrons of the nitrogen. In this it is likely that this instance leads to the negative charge becoming dominant, thereby hindering the retention in LEKC due to electrostatic repulsion with the negatively charged membranes of the pseudo stationary phase. This certainly represents a drawback of LEKC, as the contemporary druggable range for small molecules (MW < 1,000 Da) typically spans 0-3 log $P_{o/w}$ and an analysis of the World Drug Index suggests over one fifth of the marketed active ingredients supports a weak acid moiety [45]. However, Figure 3 shows that there is again a positive, ascending trend between log $P_{o/w}$ and distribution

constant using LEKC suggesting that there is involvement of a lipophilic component in the overall interaction with the lipid vesicles. However, when compared with Figure 2A, the datapoints appear in general much more scattered suggesting an original partitioning behaviour of LEKC when compared to IAM-HPLC. In general, when moving from left to right (from 3A to 3C, and from 3D to 3F), the membranes are progressively becoming less negative as the percentage of PI is decreasing. The retention of neutral compounds overall is largely unaffected by the liposome composition. Indeed, this variable impacts the electrostatics of the interactions, which in this case is negligible given that these analytes have prevalently no charge at the experimental pH. Nevertheless, an involvement of interactions of a polar nature between the investigated neutral compounds and the lipids headgroups is likely and these may be responsible for the slight fluctuations that are observed. An effect of charge is more visible for the higher concentration of lipids (4 mM), as the datapoints become more scattered from 15 mol% to 5 mol% of PI with most basic compounds shifting downwards from the data point distribution, suggesting a comparatively inferior retention which is consistent with a less negative electrostatic potential of the liposome bilayers. This means that cations would be less firmly held to the lipid bilayers when these contain less PI and are therefore expected to be less negatively charged. It is more difficult, however, to draw conclusions for acidic compounds given that unfortunately only two could be measured by LEKC. However, we can certainly compare these acids at the varying liposome compositions (15 mol% to mol 5% of PI) at both the chosen concentrations. The retention is higher at 5 mol% of PI, suggesting a specular behaviour for acids.

3.3 Relationships of LEKC indices with *n*-octanol/water partitioning of the distribution coefficients at the experimental pH

In Figure 4, relationships between LEKC data at three liposome compositions and varying concentration levels were studied against the logarithms of the distribution coefficients at the experimental pH (log $D_{7.4}$). Similarly, the retention of neutral compounds was minorly affected by the lipid composition of the vesicles and by their concentration, where for neutrals, log $P_{o/w}$ = log D. At 1 mM liposome concentration, an ascending trend can be seen for all the compounds, except the acids which seem to deviate from the data distribution line slope. However, no linearity is observed at log $D_{7.4}$ values < 0. This is expected as for

such highly hydrophilic analytes, an extra interaction of a polar/electrostatic nature is likely to become established and predominate at such low log $D_{7.4}$ values and the trend appears to be unrelated to this. At 4 mM liposome concentration, instead a sigmoidal trend can be seen, particularly for the 85:15 mol% and 90:10 mol% PC:PI liposome compositions. This implies that log K does not change much in LEKC for compounds with log $D_{7.4}$ < 0 or > 2, suggesting that a linear relationship exists between LEKC indices and log $D_{7.4}$ for moderately lipophilic analytes only. In terms of the liposome concentration effect, this seems to modulate a different chromatographic retention, mainly for the analytes with lower (< 0) log $D_{7.4}$ values, for which the interactions are reasonably driven by polar and electrostatic forces, being the hydrophobic interactions underrepresented

3.4 Relationship between IAM and LEKC data

CHI IAM and LEKC data were compared, and the outcome is depicted in Figure 5. The datapoints appear in general very scattered and the relationships observed, particularly at 1 mM, are rather weak. In both cases, the decrease in PI % does not correspond to a specular increase in the R^2. This would have been expected as the pseudo stationary phase in LEKC would, – in theory, increasingly resemble the lipid composition of the IAM phase. Slightly more pronounced relationships were observed by applying a 4 mM liposome concentration with a max R^2 as high at 0.75 for 10% (v/v) PI. Indeed, pioneer work by Pidgeon and co-workers [46] used ^{31}P NMR to study the interfacial motional properties of the IAM phases and they concluded that these were similar to the motional properties of the mobile lipids in fluid POPC liposomes.

For the original nature of the present work, it is challenging to place these findings in the context of the scientific literature underpinning the topic. The only study that can really assist – albeit with the limitation mentioned above – is that authored by Masucci *et al*. [43] who compared LMEKC values with IAM measurements and liposome partitioning data. Interestingly, while the relationships of LMEKC values with liposome partitioning data were very high (R^2 = 0.95), their relationships with IAM data were much poorer (R^2 = 0.60) and of the same order of magnitude as our own with the additional drawback of their dataset being composed of structural analogues, which is not the case of the present article.

However, it is noteworthy to mention that liver PC and liver PI contain mixtures of natural phospholipids with a specific distribution of fatty acids (displayed in Figure S2). These structures differ from that of the IAM.PC.DD2 phase used in this research, in which the fatty acid chain contains no unsaturation, and the lipids are covalently bound to amino propyl silica. Moreover, to maximise the stationary phase stability the unreacted amino propyl silica groups are end capped with propionic and decanoic anhydrides. This adds to the liposomes' noticeably superior density of the lipid network, distinctive lipid lateral mobility and inherent capability to effectively isolate two aqueous compartments, which all have no counterpart in IAM-HPLC. This may explain why analytical retention on LEKC although to some extent (R^2 = 0.57 - 0.75) related to IAM indices, encode for interaction forces that are original *per se* and not related solely to the phospholipid composition of the liposomes applied as a pseudo stationary phase. In fact, according to published research[9], endcapping of the IAM phase was demonstrated to play an only minor role in the analytical retention.

3.5 Influence of the headgroup chemistry in LEKC experiments

The influence of the headgroup was also studied by comparing the selectivities in LEKC by using pseudo stationary phases based on PI and on PS, which are both negatively charged phospholipids. The results are shown in Figure 6. Notably, the liposome concentration (1 mM vs 4 mM) appears to influence the analytical retention more than the chemistry of the head group. As, indeed, the datapoints appear more homogeneously distributed when we compare the LEKC distribution constants at an identical liposome concentration *i.e.* 1 mM, rather than when comparing the retention at 1 mM vs. 4 mM. This is interesting as while these residues are both negatively charged, certainly the chemistry of the headgroup is different with PI having a more rigid, cyclic structure supporting several hydrogen bond donor/acceptor groups, and the PS being instead less rigid and more flexible.

When comparing LEKC data achieved with 1 mM liposomes dispersions of 85:15 mol% PC:PI and 85:15 mol% POPC:POPS, a clear logarithmic trend is visible for all of the compounds, except for the basic compound imipramine, which behaves as a (weak) outlier. Notably, this was the most retained cationic molecule in our IAM-HPLC experiments.

Moreover, the significance of the headgroup chemistry was evaluated by comparing analytical retention indexes obtained on a PC-based IAM column with those from the LEKC

method, where varying proportions of POPC:POPS (80:20 mol% and 85:15 mol%) were used as the pseudo stationary phases in 1mM concentration (shown in Figure S4). A stronger correlation was observed when the molar percentage of POPS decreased from 20 mol% to 15 mol% (r^2 = 0.57 and 0.67, respectively), making the system more comparable to the IAM column. This improved relationship at a lower percentage of POPS may be attributed to the more homogeneous fatty acid composition compared to natural LiverPC (14% 16:0, 33% 18:0, 17% 18:1, and 12% 18:2) and LiverPI (46% 18:0 and 17% 20:4).

3.6 Biomimetic potential of IAM-HPLC and LEKC to mimic the interactions of active ingredients with the pulmonary epithelial barrier

The biomimetic potential of both LEKC and IAM-HPLC is studied in Figure 7. Evidently, for most of the LEKC related plots, a parabolic trend can be seen indicating that an intermediate retention promotes better apparent permeability through the Calu-3 cells. This is in agreement with a similar study by Avdeef *et al.* [47] in which the authors observed a parabolic dependency of apparent permeability through Caco-2 monolayers – a robust *in vitro* model to study the intestinal absorption of pharmaceuticals - and log $D_{7.4}$. This emphasizes that for optimal absorption log $D_{7.4}$ must range between 1 and 3, as molecules with log $D_{7.4}$ < 1 would rather stay in the aqueous biophase, whereas molecules with log $D_{7.4}$ > 3 may get entrapped in the phospholipid network without necessarily access to the cytoplasm. Overall, the best relationships with log P_{app} are 4 mM PC:PI 90:10 mol% and 1.0 mM PC:PS 85:15 mol% (both R^2 = 0.65). Moreover, this is very close to the composition of bronchial epithelial cells, whose characteristics were mirrored faithfully by LEKC. In addition, poorer relationships are established when the anionic lipids are scarcely (5 mol%) present, which indicate the need to incorporate these in suitable concentrations (> 5 mol%) in the separation medium. Overall LEKC indices were superior in the prediction of drugs' pulmonary delivery than IAM indices, as relationships of the latter with log P_{app} were unsatisfactory. This implies that IAM, which was effective in assessing the passage of barriers such as the small intestine and the BBB [19, 21, 22, 48], cannot necessarily capture the characteristics of a membrane with a higher negative to zwitterionic lipid ratio.

4. Conclusions

In this study, LEKC and IAM-HPLC have been systematically compared in terms of their suitability to study interactions between drugs and phospholipid membranes, and their capability to model the passage through membranes with higher composition of negatively charged phospholipids such as PI and PS. Our results show that LEKC encodes for original interaction forces that are not depicted in IAM-HPLC and are not solely related to the lipid composition of the liposomes used as a pseudo stationary phase. On the other hand, twelve compounds could not be measured in LEKC but, by could be eluted in IAM-HPLC. Most of these molecules are hydrophilic and/or anionic at the experimental pH and some of the latter possess moderate lipophilicity. At present, this makes LEKC, at least with the current setup, difficult to implement in drug development programmes for acidic compounds. However, LEKC outscored IAM-HPLC particularly when applying 10-15 mol% PI/PS liposome dispersions. This emphasises the requisite for the incorporation of anionic phospholipids in the pseudo stationary phases which are unfortunately lacking from the IAM phases on the market today. Equally significant is the inherent environmentally friendly nature that LEKC possesses over IAM LC; its speed, simplicity and notable lack of the need for organic solvents makes it a greener choice of method.

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Investigation, Data Curation; Susanne K. Wiedmer: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition; Giacomo Russo: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition

Declaration of generative AI and AI-assisted technologies in the writing process: No generative AI and AI-assisted technologies were used in the writing process.

Declaration of competing interest: The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

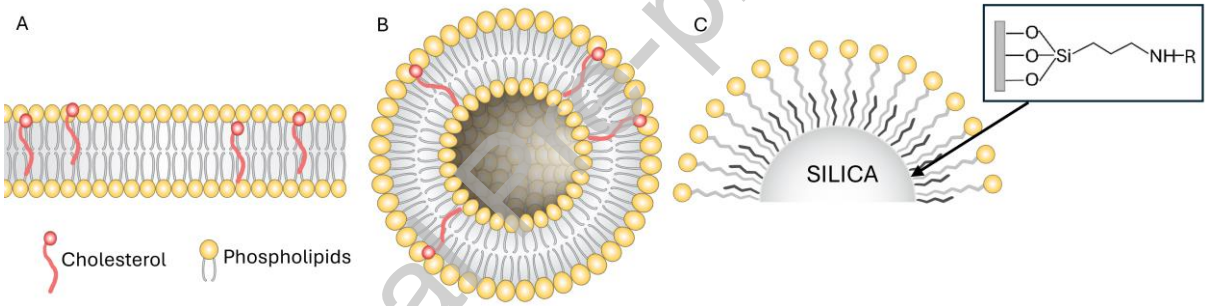

Figure 1. Schematic representations of: (a) the membrane lipid bilayer; (b) a liposome, a small lipid vesicle composed of natural or synthetic phospholipids, which serves as a pseudo-stationary phase in LEKC; and (c) the IAM stationary phase, where phospholipid monolayers are covalently attached to amino-propyl silica particles.

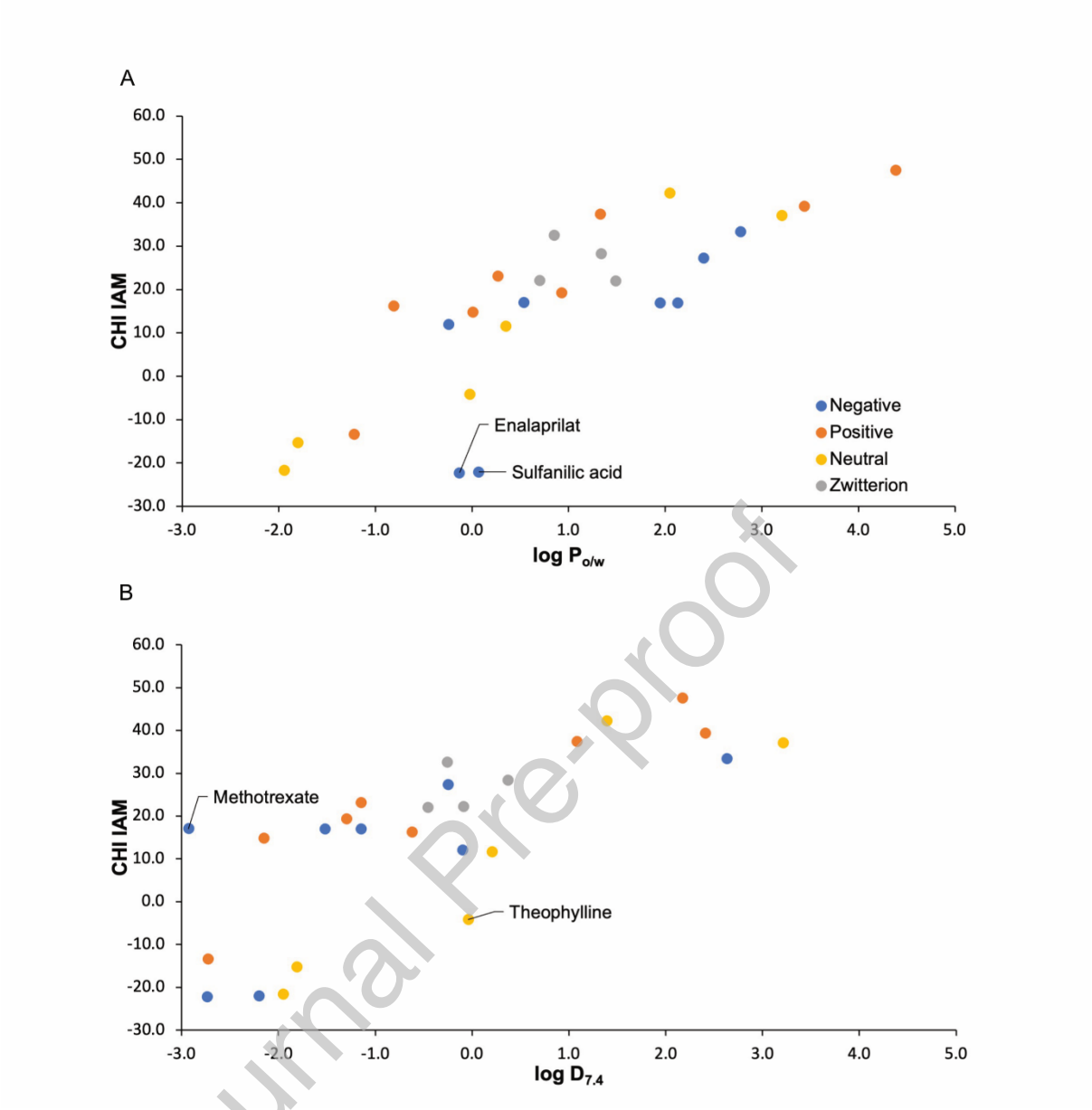

Figure 2. CHI IAM indexes plotted against log $P_{o/w}$ (A) and log $D_{7.4}$ (B) values.

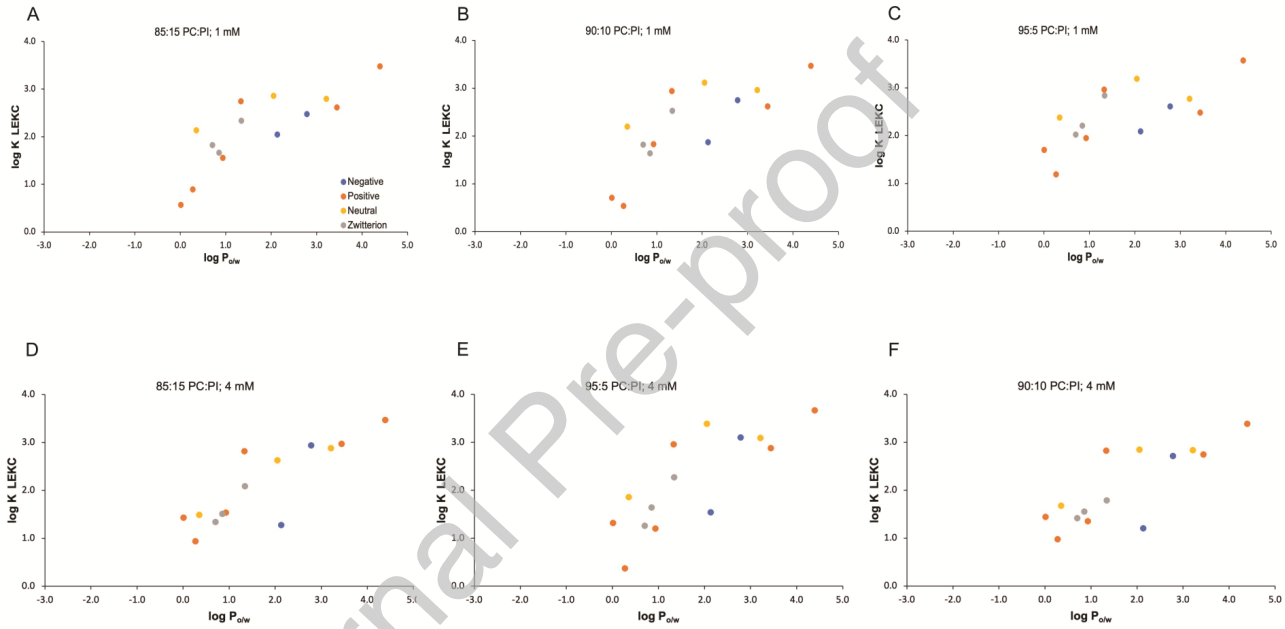

Figure 3. Logarithms of the distribution constants in LEKC studied vs the logarithm of the n-octanol/water partition values of the neutral form a three different compositions of the pseudo stationary phase.

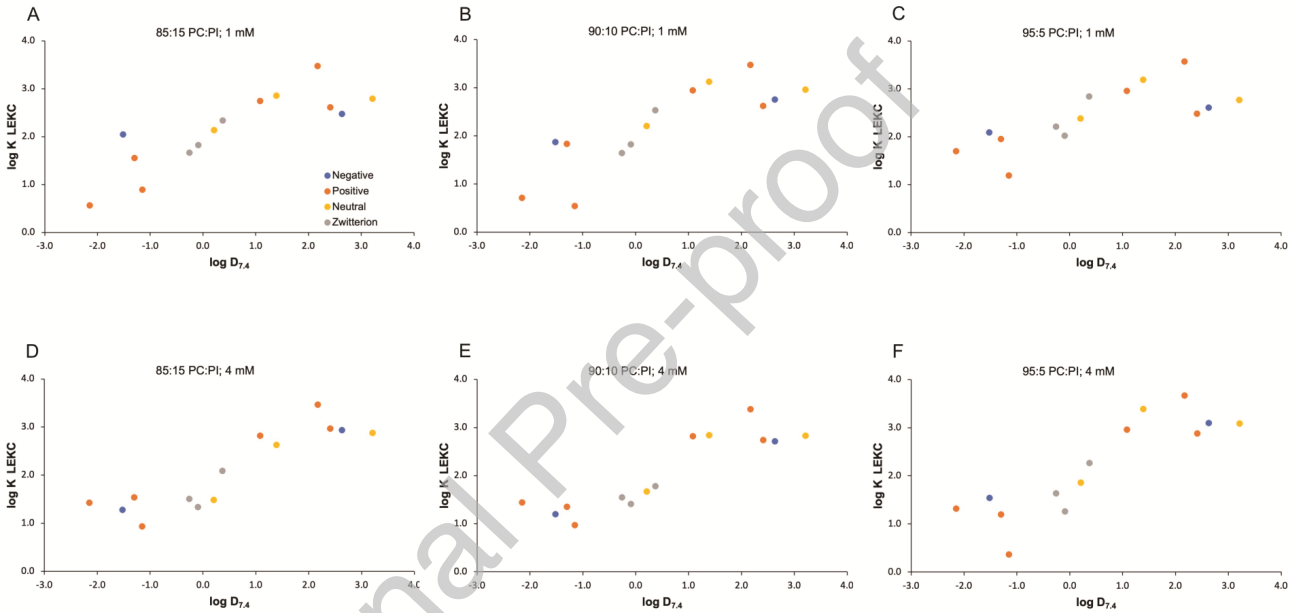

Figure 4. Logarithms of the distribution constants in LEKC studied vs the logarithm of the n-octanol/water partition values of the mixture undissociated/ionised at pH 7.4 a three different compositions of the pseudo stationary phase.

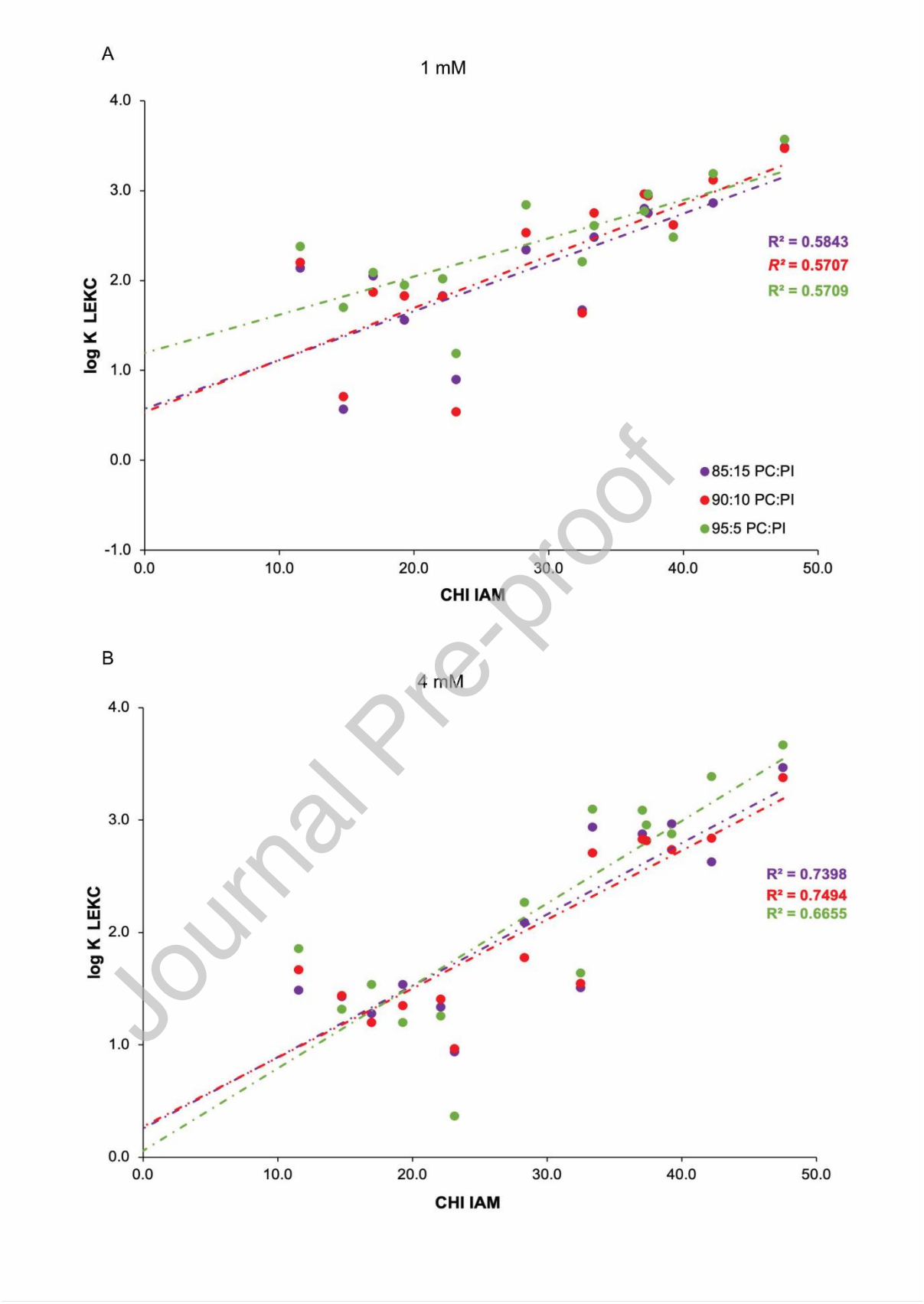

Figure 5. Comparisons between CHI IAM indexes and log K LEKC for three different liposome compositions (from 5 to 15 mol% of PI) at total concentrations of 1 mM (A) and 4 mM (B).

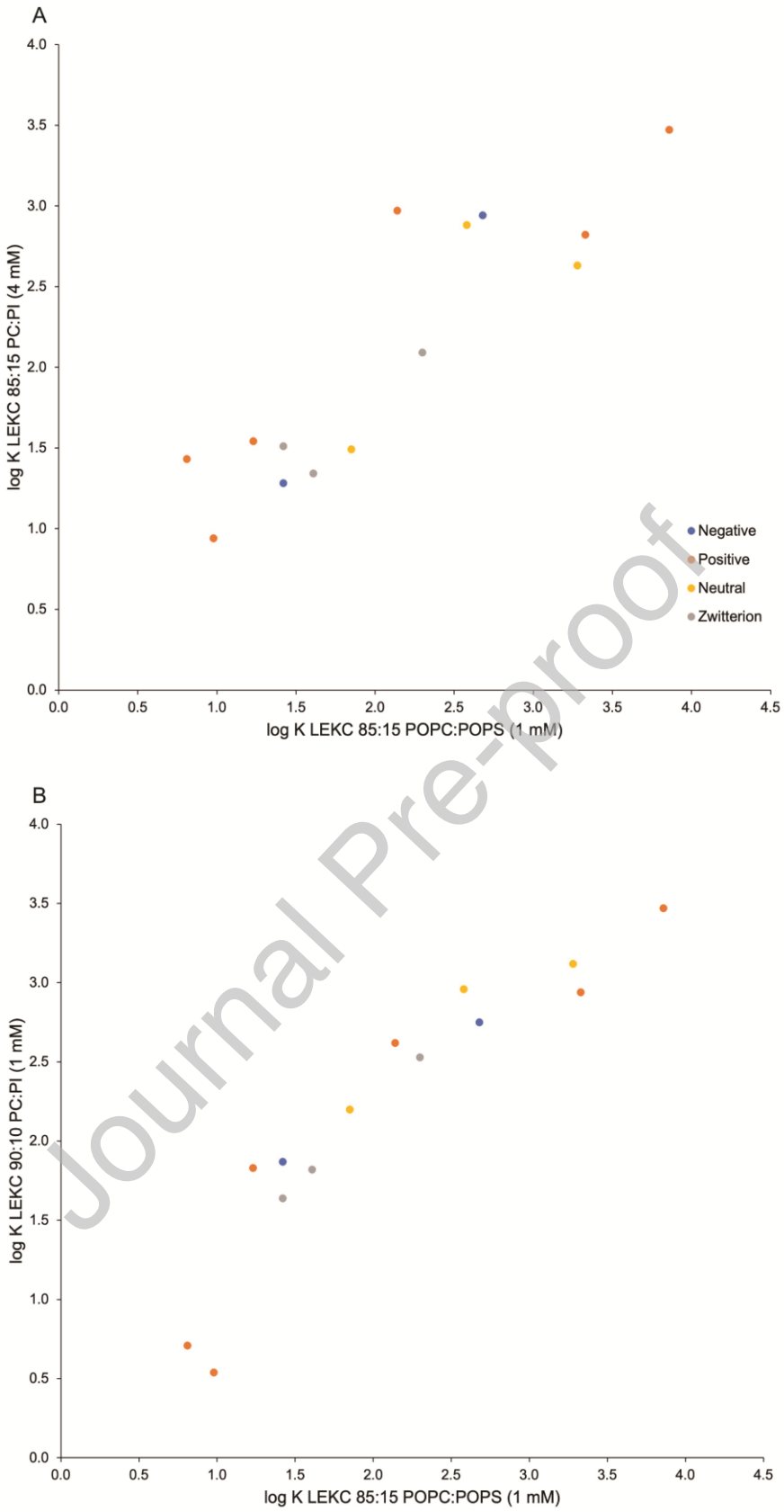

Figure 6. Log K LEKC PC:PI values vs log K LEKC POPC:POPS at two different concentrations of liposomes i.e., 1 and 4 mM.

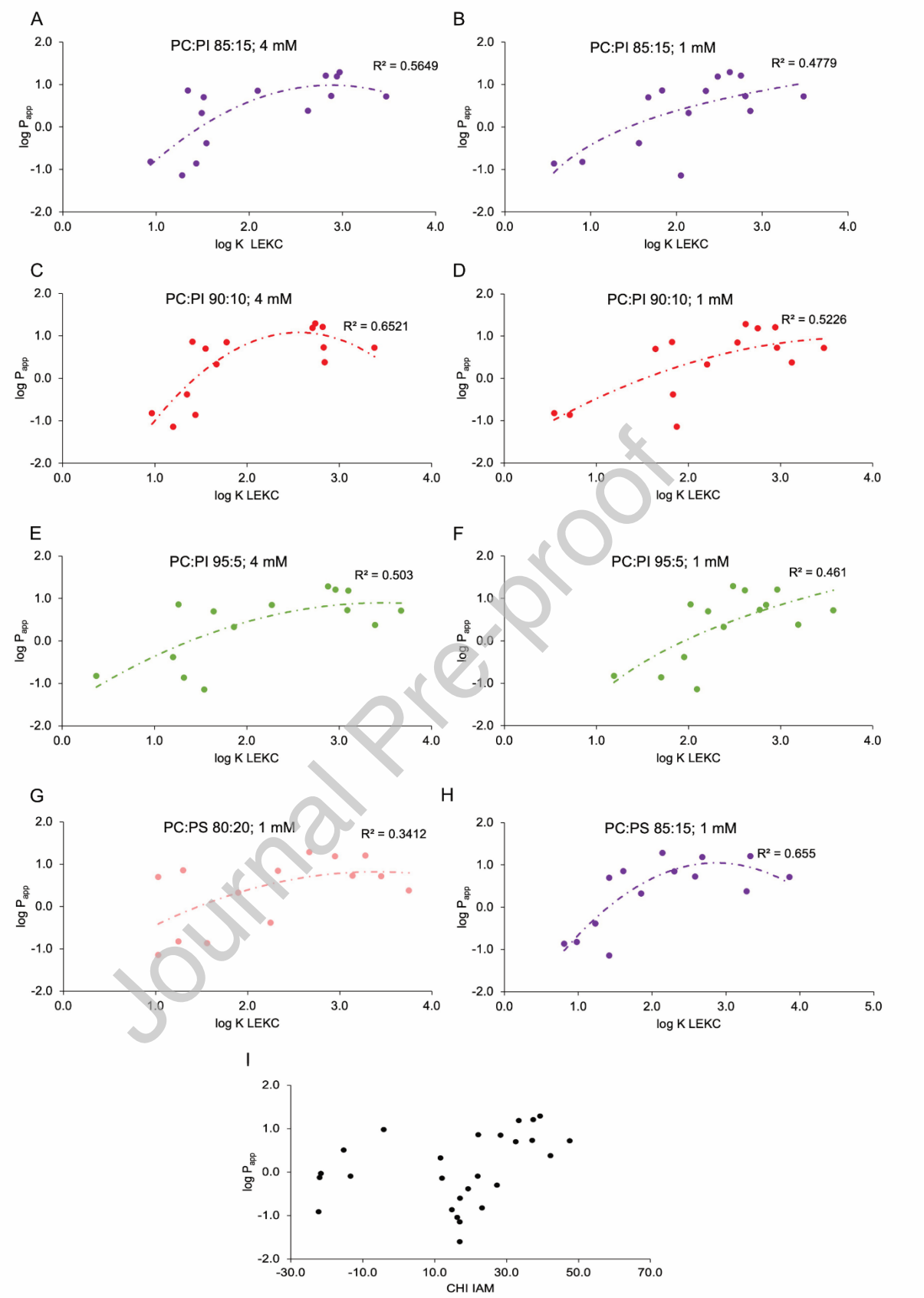

Figure 7. Relationships between the logarithm of the apparent permeability of the Calu-3 cell monolayer and the logarithms of the distribution constants in LEKC (A to H) and CHI IAM values (I).

Table 1. The summary of considered parameters: pK_a, experimental n-octanol/water lipophilicity values (log $P_{o/w}$, log $D_{7.4}$), apparent permeability values (log P_{app}) obtained from Calu-3 cell lines, the chemical character of the molecules, prevalent species at pH 7.4 and molecular mass (g mol^{-1}).

Name	pK_a	log $P_{o/w}$	log $D_{7.4}$	log P_{app} [49]	Chemical character	Prevalent species at pH 7.4	Molecular mass (g mol^{-1})
Acyclovir	9.23/2.34[50]	-1.80[50]	-1.81[50]	0.51	B	N	225.21
Budesonide	13.75[a]	3.21[51]	3.21*	0.73	A	N	430.53
Chlorothiazide	6.85[52]	-0.24[53]	-0.10[53][b]	-0.14	A	(–)	295.72
Cromolyn	1.10[52]	1.95[50]	-1.15[50]	-1.60	A	(–)	466.35
Enalaprilat	1.25; 3.17/ 7.84[54]	-0.13[54]	-2.74[54]	-0.91	B/A	(–)	347.39
Famotidine	6.74[55]	-0.81[50, 56]	-0.62[50, 56]	-1.04	B	(+)	338.45
Imipramine	9.51[57]	4.39[50, 56]	2.17[50, 56]	0.72	B	(+)	281.42
Labetalol	9.42/7.48[55]	1.33[50, 56]	1.08[50]	1.21	B/A	(+)	329.41
Levofloxacin	5.83/8.75[58]	0.70[58]	-0.09[58]	0.86	B/A	Z	361.37
Methotrexate	4.70[52]	0.54[56]	-2.93[56]	-0.60	A	(–)	452.42
Moxifloxacin	10.32/6.28 [59]	0.85[60]	-0.26[59]	0.70	B/A	Z	401.43
Nadolol	9.69[57]	0.93[61]	-1.30[61]	-0.38	B	(+)	310.41
Norfloxacin	6.23/8.51[50]	1.49[62]	-0.46[62]	-0.09	B/A	Z	319.33
Oxacillin	2.72[52]	2.13[63]	-1.52[63]	-1.14	A	(–)	400.43
Pefloxacin	5.26/7.51[59]	1.34 [60]	0.37[59][c]	0.85	B/A	Z	333.36
Quinidine	8.55[57]	3.44[62]	2.41[62]	1.29	B	(+)	325.43
Ranitidine	8.20[64]	0.27[61]	-1.15[61]	-0.82	B	(+)	315.41
Rosiglitazone	6.84/6.23[a]	2.78[65]	2.63[65]	1.19	B/A	(+)	356.42
Rosuvastatin	4.00[a]	2.40[66]	-0.25[67]	-0.30	A	(–)	480.53
Salbutamol	9.30[68]	0.01[69]	-2.15[69]	-0.86	B	(+)	240.32

Saquinavir	7.15[70]	2.05[70]	1.39[70]	0.38	B	N	670.84
Sulfaguanidine	11.3[71]	-1.22[72]	-2.73[71][d]	-0.09	B	(+)	215.25
Sulfanilic acid	3.23[a]	0.07[a]	-2.20[a]	-0.12	A	(–)	172.18
Sulfapyridine	7.81[73]	0.35[74]	0.21*	0.33	A	N	248.28
Theophylline	8.81[52]	-0.02[52]	-0.04*	0.98	A	N	179.16
Uridine	9.25[75]	-1.94[76]	-1.95*	-0.03	A	N	244.20

[a] Values obtained from DrugBank and calculated using MarvinSketch.

[b] Log D of chlorothiazide measured at pH= 6.5.

[c] Log D of pefloxacin measured at pH= 6.9.

[d] Log D of sulfaguanidine measured at pH= 7.6.

* Log $D_{7.4}$ values calculated based on the log $P_{o/w}$ and pK_a values of the compounds.

A = acid; B = base; N = neutral; B/A = ampholyte; Z= zwitterion.

Table 2. CHI_{IAM} values determined by Immobilised Artificial Membrane High Performance Liquid Chromatography (IAM-HPLC) and distribution constants obtained by Liposome Electrokinetic Capillary Chromatography (LEKC) using varied liposome compositions and concentrations. Phospholipid composition for IAM-HPLC and LEKC detailed below.

Name	CHI_{IAM}	log K PC:PI ; 1 mM			log K PC:PI ; 4 mM			log K POPC:POPS ; 1 mM	
		85/15	90/10	95/5	85/15	90/10	95/5	80/20	85/15
Acyclovir	-15.32	NR	NR	NR	NR	NR	NR	NR	NR
Budesonide	37.09	2.80	2.96	2.77	2.88	2.83	3.09	3.14	2.58
Chlorothiazide	11.98	NR	NR	NR	NR	NR	NR	NR	NR
Cromolyn	16.91	NR	NR	NR	NR	NR	NR	NR	NR
Enalaprilat	-22.29	NR	NR	NR	NR	NR	NR	NR	NR
Famotidine	16.21	NR	NR	NR	NR	NR	NR	NR	NR
Imipramine	47.52	3.48	3.47	3.57	3.47	3.38	3.67	3.45	3.86
Labetalol	37.38	2.75	2.94	2.96	2.82	2.82	2.96	3.28	3.33
Levofloxacin	22.12	1.83	1.82	2.02	1.34	1.41	1.26	1.30	1.61
Methotrexate	17.03	NR	NR	NR	NR	NR	NR	NR	NR
Moxifloxacin	32.49	1.67	1.64	2.21	1.51	1.55	1.64	1.03	1.42
Nadolol	19.29	1.56	1.83	1.95	1.54	1.35	1.20	2.25	1.23
Norfloxacin	21.94	NR	NR	NR	NR	NR	NR	NR	NR
Oxacillin	16.96	2.05	1.87	2.09	1.28	1.20	1.54	1.03	1.42
Pefloxacin	28.31	2.34	2.53	2.84	2.09	1.78	2.27	2.33	2.30

Quinidine	39.25	2.62	2.62	2.48	2.97	2.74	2.88	2.67	2.14
Ranitidine	23.12	0.90	0.54	1.19	0.94	0.97	0.37	1.25	0.98
Rosiglitazone	33.36	2.48	2.75	2.61	2.94	2.71	3.10	2.95	2.68
Rosuvastatin	27.28	NR	NR	NR	NR	NR	NR	NR	NR
Salbutamol	14.76	0.57	0.71	1.70	1.43	1.44	1.32	1.56	0.81
Saquinavir	42.20	2.86	3.12	3.19	2.63	2.84	3.39	3.75	3.28
Sulfaguanidine	-13.40	NR	NR	NR	NR	NR	NR	NR	NR
Sulfanilic acid	-22.05	NR	NR	NR	NR	NR	NR	NR	NR
Sulfapyridine	11.56	2.14	2.20	2.38	1.49	1.67	1.86	1.90	1.85
Theophylline	-4.20	NR	NR	NR	NR	NR	NR	NR	NR
Uridine	-21.69	NR	NR	NR	NR	NR	NR	NR	NR

NR = unretained

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: