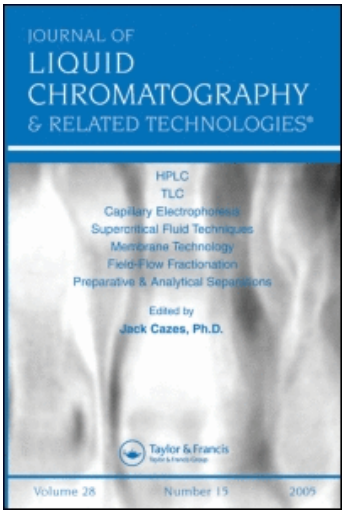

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Chromatography *In Silico*: Retention of Acidic Drugs on a Guanidino Ion-Exchanger

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Chromatography *In Silico*: Retention of Acidic Drugs on a Guanidino Ion-Exchanger

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Abstract: The chromatographic behavior of acidic drugs in ion-exchange liquid chromatography using a guanidino phase was analyzed *in silico* for developing a quantitative structure retention relationship in anion-exchange liquid chromatography. The directly calculated molecular interaction energy values between an analyte and a model phase using molecular mechanics calculations, correlated with high precision with the capacity ratios of acidic drugs measured in ion-exchange liquid chromatography. Retention order could be predicted based on the molecular interaction energy values using a model phase, even in ion-exchange liquid chromatography with a pH controlled eluent. The measured capacity ratio at pH 7.4 correlated well with the human serum albumin binding affinity measured using a modified Hummer-Dreyer method with purified human serum albumin.

Keywords: Chromatography *in silico*, Acidic drugs, Ion-exchange liquid chromatography, Guanidino-phase, HSA-acidic drug binding affinity

INTRODUCTION

Optimization of the quantitative structure retention relationship (QSRR) is required to enhance the analytic capabilities of chromatography. The octanol-water partition coefficient (log *P*) was first demonstrated to be a quantitative molecular property of analytes in reversed-phase liquid chromatography,[1] and later log *P* values were used for the quantitative analysis of

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chromatographic optimization.[2] A similar approach was commercialized by ACD.[3] Log *P*, however, is a property of the molecular form of compounds and not of the ionized form of compounds. Therefore, errors are introduced when using log *P* values to predict retention time for ionized compounds. A new approach was proposed based on the direct calculation of the molecular interaction energy value between a model phase and an analyte *in silico*. This approach was examined for use in reversed-phase liquid chromatography of various compounds: phenolic compounds,[4,5] benzoic acid derivatives,[6] acidic drugs,[7] and basic drugs.[8] Furthermore, the chromatographic behavior of basic compounds in ion-exchange liquid chromatography was analyzed successfully *in silico*.[9] The correlation coefficient between measured and predicted capacity ratios was equivalent, or better than, that obtained using the log *P* system in reversed-phase liquid chromatography. The new method can be applied to estimate the relative capacity ratio of newly designed compounds, because the new system does not require experimental data for predicting the elution order. This new approach is currently less precise than the approach using Dry-Lab (computer-assisted method),[10] but the theoretical approach provides a new dimension in which to study molecular interactions quantitatively, and for designing a new phase. On the other hand, the measurement of human serum albumin (HSA)-drug binding affinity to determine bioavailability, is necessary in the drug discovery process. The original methods (equilibrium dialysis, Hummer-Dreyer method, frontal analysis method) are time consuming and the HSA purity is poor (containing more than 30% glycated albumin). Previously, a new approach that eliminates the use of HSA was proposed. A combination of ion-exchange and reversed-phase liquid chromatography allows for the reproducible and fast measurement of HSA-drug binding affinity using a newly developed bonded phase.[11] In this report, a one column system is proposed for the development of a fast liquid chromatographic screening of acidic drugs in the drug discovery process.

EXPERIMENTAL

Preparation of Hexylguanidino Bonded Silica Gel

A hexylguanidino-bonded silica gel was synthesized from a hexenyl-bonded silica gel instead of binding 5-bromo-1-pentene with trichlorosilane, due to the availability of the silyl-reagent.[10] A mixture of hexenyltrichlorosilane (1.1 mL, Nacalai Tesque, Kyoto, Japan) and 5 μm silica gel (2 g; MS Gel EP-DF 100, 100 Å pore size, Dokai-Kagaku, Kitakyushu, Japan) in toluene, was refluxed. The hexenyl-bonded silica gel was bromated using 30% hydrogen bromide (HBr) in acetic acid (Sigma-Aldrich, St. Louis, MO). The bromate phase was converted to an azido phase using sodiumazide in aqueous solution. The conversion to azido groups was monitored using

infrared spectrophotometry where the azide group indicated strong absorption at 2110 cm^{-1}. The azido groups were converted to amino groups. Finally, the amino groups were converted to guanidine groups using a previously described method.[12] The total organic content of the bonded-silica gel was 15.5 wt%, and the ion-exchange capacity was 16.8 μeq/g by titration.

Capacity Ratio Measurement

Drugs and chemicals were obtained from Sigma-Aldrich and Wako Pure Chemicals (Osaka, Japan). Their properties are summarized in Table 1. Acetic acid, sodium acetate, sodium hydroxide, phosphoric acid, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Wako Pure Chemicals. HPLC grade methanol was obtained from Kanto-Kagaku (Tokyo). Milli-Q grade water was used.

The automated liquid chromatograph comprised a LC-10AD pump, a SIL-10AXL auto-injector, a SPD-10AV UV detector from Shimadzu (Kyoto, Japan), equipped with a UZ-SH-MIC microflow cell from LC Packing (The Netherlands), and an ERC-3522 degasser from ERC (Tokyo). The aluminum block column heater was made to specifications and controlled with a 965 Temperature & Process Controller from Sakaguchi E.H. Voc Co.

Table 1. Molecular properties of acidic drugs

Drug	log n*K*	log n*K**	p*K*a ref	p*K*a mes	MIm	MIi
Furosemide	5.54	5.70	4.2	5.661	23.592	31.184
Ibuprofen	—	6.61	5.2	6.032	24.515	32.909
Indomethacin	—	7.32	4.5	5.729	36.478	47.386
Iopanoic acid	—	7.84	—	6.051	34.234	39.888
Mefenamic acid	—	7.84	4.2	5.776	31.606	42.211
Nalidixic acid	—	4.18	6.0	6.481	23.849	28.415
Naproxen	5.81	5.75	4.2	5.942	22.827	32.190
Nicotinic acid	—	3.20	4.95	5.262	17.022	24.845
Phenylbutazone	5.95	6.05	4.4	5.665	28.226	42.237
Probenecid	—	5.29	3.4	5.537	24.142	37.505
Salicylic acid	4.92	4.81	3.0	5.079	21.661	30.691
Tolazamide	—	5.16	5.7	6.118	22.514	34.746
Tolbutamide	5.26	5.29	5.3	6.329	18.776	35.167
Warfarin	5.63	5.38	5.1	6.136	26.052	32.272

log n*K*: HSA-drug binding affinity from ref. [11]; log n*K**: HSA-drug binding affinity measured by 2 column system from ref. [11]; p*K*a ref from reference [11]; p*K*a mes: p*K*a measured by ion-exchange liquid chromatography; MIm: molecular interaction energy value for molecular form drugs (kcal/mol); MIi: molecular interaction energy value calculated for ionized acidic drugs.

(Tokyo). The operation and chromatographic data analysis were performed with a CLASS-LC10 workstation from Shimadzu.

The guanidino-bonded silica gel column (50 mm × 2.1 mm i.d.) was used for ion-exchange liquid chromatography of acidic compounds. The eluent was 50 mM sodium acetate solution, pH 3.20 to 9.50, containing 50% methanol; and 50 mM sodium phosphate solution containing 50% methanol. The column temperature was 37°C. The void volume marker was fructose. The flow rate was 0.2 mL/min.

Quantitative Analysis of the Retention *In Silico*

A Dell Latitude C840 computer equipped with a 2 GHz processor and 1024 MB memory was used for data acquisition and analysis. The molecular properties of analytes and model phases and molecular interaction energy values were calculated by molecular mechanics (MM2), using version 5 of the CAChe program from Fujitsu, Tokyo, Japan. The standard parameters used were bond stretch, bond angle, dihedral angle, improper torsion, van der Waals, hydrogen bonds, and electrostatic bonds (MM2/MM3 bond dipoles). The van der Waals cut off distance was 9 Å. The energy unit was kcal/mol (1 kJ/mol = 4.18 kcal/mol). A Cricket-Graph program from Computer Associates (San Diego, CA) and Project Reader of the CAChe program were used for data analysis.

For the development of a QSRR in chromatography, the molecular interaction energy value (MI energy) was calculated between a model phase and an analyte. The optimized energy value was less than 0.00001 kcal/mol. MI energy was defined as the energy value of the complex subtracted from the sum of the energy values of the model phase and analyte.

$$\mathrm{MI} = \mathrm{FS}_{\mathrm{analyte}} + \mathrm{FS}_{\mathrm{modelphase}} - \mathrm{FS}_{\mathrm{complex}} - \mathrm{hb} \qquad (1)$$

where FS is energy value of the final structure (optimized structure), and hb is the hydrogen bond energy value of the analyte. The subtraction of hb is necessary for molecules with intra- molecular hydrogen bonding. The MI energy values of the molecular and ionized forms of the analytes are summarized in Table 1. The MI energy values correlated with the capacity ratios measured using liquid chromatography.

RESULTS AND DISCUSSION

Inductive Effect on pKa

The retention time was longer, however, in the new ion-exchange liquid chromatography. The precision was higher for the weakly retained

compounds in this liquid chromatography. In the previous experiment, the negative $\log k$ values had to be neglected due to the very short retention time. With the new guanidino-bonded phase, however, retention time was reasonably long and all measured capacity ratios were analyzed.

Furthermore, the dissociation constant (p*K*a) of these analytes was calculated from the capacity ratios measured using eluents of pH 3.0 to 9.0. The p*K*a values obtained from the experiment were very different from those reported in the literatures (see Table 1). The difference was partly influenced by the methanol concentration in the eluent, but the inductive effect between the guanidino group of the ion-exchanger and the analyte could also contribute to the difference. The relation between measured and reference values are given by the following equations:

$$\text{p}K\text{a (ion-exchange LC)} = 0.370\ (\text{p}K\text{a reference}) + 4.116, \quad r = 0.792, \quad n = 13, \tag{2}$$

$$\text{p}K\text{a (ion-exchange LC)} = 0.397\ (\text{p}K\text{a reference}) + 4.048, \quad r = 0.931, \quad n = 12, \tag{3}$$

The correlation coefficient improved from 0.792 to 0.931 after the elimination of nicotinic acid. The slopes of equations (2) and (3) indicate the selectivity of the guanidino-phase. The inductive effect of the ion-exchanger affects the shift of the measured p*K*a values, and the degree of the shift might depend on the ion-exchange capacity and ionic strength, as well as the ionic strength of the eluent used. The salting out effect at higher and lower pH was higher in the sodium-phosphate solution than in the sodium-acetate solution. In the sodium-acetate eluent, a strong acid (e.g., salicylic acid) was more retained than weaker acids. The maximum retention time was not obtained with a pH 3.22 solution.

The difficulty in standardizing the effect of an organic modifier is due to the current obstacles in quantitatively analyzing solvation. In this experiment, however, pH was measured before mixing with an organic modifier. Therefore, the relatively higher pH values should be a property of the ion-exchanger used.[13]

Quantitative *In Silico* Analysis of Log *k*

Using computational chemical calculations to analyze liquid chromatographic data, the direct interaction between a model phase and an analyte was calculated quantitatively as energy values using MM2 calculations. The quantitative analysis of retention in liquid chromatography is easy, because a homogeneous model phase can be used instead of a complicated protein model for studying docking mechanisms.

For QSRR of reversed-phase liquid chromatography, the contact surface area was the most important factor. Selection of the model phase was difficult,

but a simple model phase was satisfactory for the measurement of albumin-acidic drug binding affinity in ion-exchange liquid chromatography.[14] A simple model guanidino-bonded phase was constructed to investigate acidic compound guanidino phase interactions. The model phase contained 1117 atoms, 1470 bonds, and 8432 connectors. Twelve hexyl guanidino groups and twelve hexyl groups bound on a double layer like carbon phase that kept the rigid basic structure. The analyte was located in the center of the guanidino phase, and the complex was optimized using MM2 calculations. The optimized energy value was less than 0.00001 kcal/mol. An example of a complex form is shown in Figure 1, where ibuprofen is adsorbed on the guanidino phase.

The pH effect on molecular interactions can be examined experimentally using liquid chromatography. The capacity ratio in an eluent of a given pH can be predicted from the following equation:[15]

$$k = (k\mathrm{m} + k\mathrm{i}([\mathrm{H}^+]/[K]))/(1 + ([\mathrm{H}^+]/[K])), \quad (4)$$

where km and ki are capacity ratios of the molecular and ionized forms of the analytes. $[\mathrm{H}^+]$ is the concentration of hydrogen ions in the eluent and $[K]$ is the dissociation constant of the analyte. The km and ki were replaced with MI energy values calculated using molecular mechanics; the km value was replaced with the MI energy value of the molecular form of the analyte (MIm) and the ki value was replaced with using the MI energy value of the ionized form of the analyte (MIi). The following equation is used for further discussion:

$$\mathrm{MI} = (\mathrm{MIm} + \mathrm{MIi}([\mathrm{H}^+]/[K]))/(1 + ([\mathrm{H}^+]/[K])), \quad (5)$$

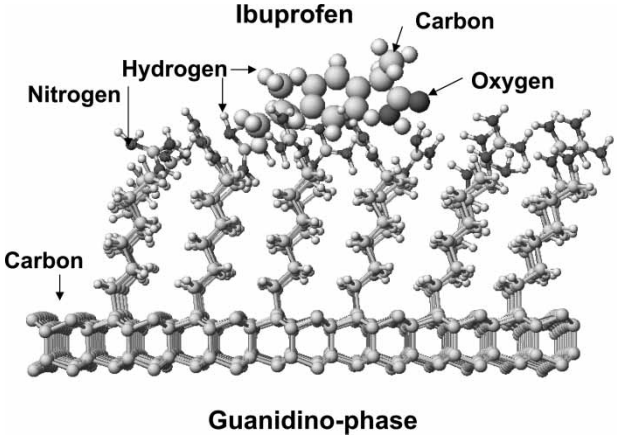

Figure 1. Docking of ibuprofen on guanidino-phase. Small white ball: hydrogen; large white ball: carbon; large gray ball: nitrogen; large black ball: oxygen. Atom size of ibuprofen is twice of guanidino-phase.

This led us to examine how to obtain the relative dissociation constant in ion-exchange liquid chromatography. The relative p*K*a values measured in this anion-exchange liquid chromatography were shifted to higher values compared to the reference values as given in equations (2) and (3). The original p*K*a values measured by titration were affected by the organic modifier concentration and the inductive effect of the ion-exchange groups of the bonded phase. Therefore, the MI at a given pH was first calculated using the measured p*K*a and equation (5). The contribution of intramolecular hydrogen bonding energy was high for salicylic acid, and therefore, the final molecular interaction energy value was reduced by the original hydrogen bonding energy value of the analyte, as shown in equation (1).

$$\log k\,(\text{pH } 3.0) = 0.065\ (\text{MI, pH } 3.0) - 0.751, \quad r = 0.830, \quad n = 14,$$
$$\log k\,(\text{pH } 4.0) = 0.078\ (\text{MI, pH } 4.0) - 0.945, \quad r = 0.896, \quad n = 14,$$
$$\log k\,(\text{pH } 5.0) = 0.078\ (\text{MI, pH } 5.0) - 1.163, \quad r = 0.867, \quad n = 14,$$
$$\log k\,(\text{pH } 6.0) = 0.062\ (\text{MI, pH } 6.0) - 1.401, \quad r = 0.756, \quad n = 14,$$
$$\log k\,(\text{pH } 7.0) = 0.071\ (\text{MI, pH } 7.0) - 2.320, \quad r = 0.824, \quad n = 14,$$
$$\log k\,(\text{pH } 7.4) = 0.070\ (\text{MI, pH } 7.4) - 2.450, \quad r = 0.843, \quad n = 14,$$
$$\log k\,(\text{pH } 8.0) = 0.073\ (\text{MI, pH } 8.0) - 2.697, \quad r = 0.858, \quad n = 14,$$
$$\log k\,(\text{pH } 9.0) = 0.073\ (\text{MI, pH } 9.0) - 2.850, \quad r = 0.866, \quad n = 14.$$

Further study was performed using equations (1), (3), and (5) and the reference p*K*a values listed in Table 1.

$$\log k\,(\text{pH } 3.0) = 0.065\ (\text{MI, pH } 3.0) - 0.750, \quad r = 0.830, \quad n = 14,$$
$$\log k\,(\text{pH } 4.0) = 0.078\ (\text{MI, pH } 4.0) - 0.936, \quad r = 0.898, \quad n = 14,$$
$$\log k\,(\text{pH } 5.0) = 0.079\ (\text{MI, pH } 5.0) - 1.173, \quad r = 0.902, \quad n = 14,$$
$$\log k\,(\text{pH } 6.0) = 0.065\ (\text{MI, pH } 6.0) - 1.503, \quad r = 0.814, \quad n = 14,$$
$$\log k\,(\text{pH } 7.0) = 0.071\ (\text{MI, pH } 7.0) - 2.338, \quad r = 0.834, \quad n = 14,$$
$$\log k\,(\text{pH } 7.4) = 0.071\ (\text{MI, pH } 7.4) - 2.454, \quad r = 0.847, \quad n = 14,$$
$$\log k\,(\text{pH } 8.0) = 0.073\ (\text{MI, pH } 8.0) - 2.698, \quad r = 0.859, \quad n = 14,$$
$$\log k\,(\text{pH } 9.0) = 0.073\ (\text{MI, pH } 9.0) - 2.850, \quad r = 0.866, \quad n = 14.$$

These correlation coefficients were not high compared with those obtained with reversed-phase liquid chromatography. The predicted p*K*a values from the reference values measured by titration can be used if a p*K*a conversion equation, like equation (3), is obtained systematically for a specific ion-exchanger. This approach is still difficult, however, due to the uncontrolled inductive effect of the ion-exchange groups.

The measurement of HSA-drug binding affinity is an important process in drug discovery. The binding conformation and site have been analyzed using spectroscopic methods, especially NMR. Rapid measurement of the binding

affinity has been performed using capillary electrophoresis[16,17] and liquid chromatography using an immobilized HSA affinity column.[18–20] The reproducibility of capillary electrophoresis is not satisfactory, however, due to Faraday's law. The purity and stability of the protein are not compatible with a chemically synthesized packing material. The capacity ratios measured at pH 7.4 in the above system were related to HSA-acidic drug binding affinity measured by a modified Hummer-Dreyer method using purified HSA. The correlation coefficient between the binding affinity measured using purified HSA (97% pure) and the measured $\log k$ at pH 7.4 was 0.910 ($n = 5$), and that measured previously by two liquid chromatographic systems was 0.882 ($n = 15$).[11]

CONCLUSION

Chromatography *in silico* using a model phase is practical for studying the retention mechanisms; furthermore, the elution order can be predicted even in ion-exchange liquid chromatography. It is difficult, however, to predict relative p*K*a values in ion-exchange liquid chromatography. It seems that the compounds are dissociated based on their p*K*a, as measured by titration. A computational chemical optimization based on the molecular properties of analytes is practical in liquid chromatography, and for studying protein-drug binding affinity.

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