Designed Chemically Bonded-Silica Gels "*La Mer*" for both LC-MS and Quality Control

High pressure and high flow rate separation are not ideal chromatographic conditions to obtain stable, sensitive, and reproducible results. Gradient elution is commonly used for fast separation. The separation time in gradient elution is short, but this method requires a period of re-equilibration and, in general, it is not suitable for highly sensitive detection. A column-switching method is complicated because of the instrumentation needed. Once a system is established, however, a stable, reproducible, and highly sensitive chromatography can be achieved [1]. Therefore, a simple liquid-chromatographic separation requires stable, inert, and reproducible packing materials made from silica gel. New silica gels, the so-called pure silica gels, were introduced as matrices of bonded phases. These chemically modified products are inert to basic compounds, and chemical stability is expanded from pH 2.0 – 7.5 to pH 1.5 – 10. The lifetime of the bonded phases using pure silica gels exceeded 3 month for continuous operation in 1% trifluoroacetic acid and 50 mM disodium phosphate in 50 vol.% methanol solution. Therefore, the separation of peptides at low pH and ion-pair liquid chromatography at high pH have become common, and the maintenance of pumps has become more important for routine analysis [2].

Stability of bonded-phase silica gels

The stability in basic solution is critical; therefore, the stability tests were performed in 10 mM ammonium formate solution (pH 10.2) containing 30% acetonitrile suitable for LC-MS analysis. Newly developed pentyl (C5)-, octyl (C8)-, phenylhexyl (PhHx)-, and octadecyl (C18)-bonded silica gels were stable more than 2,500 h in this solution. The stability of C18, PhHx, and C5 columns demonstrate in following **Figures 1 - 3**, where retention time (tR) and peak symmetry (As) of phenol and pyridine were used as the practical indicators.

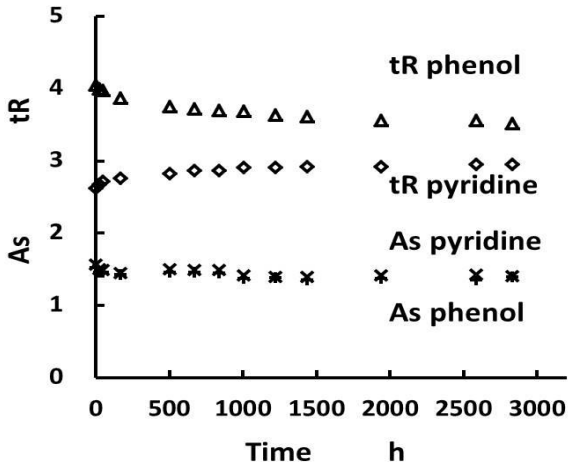


Figure 1. Stability of octadecyl-bonded silica gel in pH 10.2

Column: **Paci-3-C18**, 100 x 2.1 mm I.D.;
Eluent: 10 mM ammonium formate pH10.2 + acetonitrile (70+30);
Flow rate: 0.2 mL/min at 40ºC; Pressure: 8.7MPa;
tR: retention time min; As: Peak symmetry,

These designed bonded-phase silica gels synthesized from pure silica gel were stable at high pH eluent generally used for LC-MS operation. These bonded-phase silica gels were used for acidic drug analysis in reversed-phase liquid chromatography in 10 mM formic acid containing 50% methanol. These were also used for reversed-phase liquid chromatography for basic drugs in 10 mM ammonium formate (pH 2.88) containing 45% methanol. These chromatograms are demonstrated in following Figures.

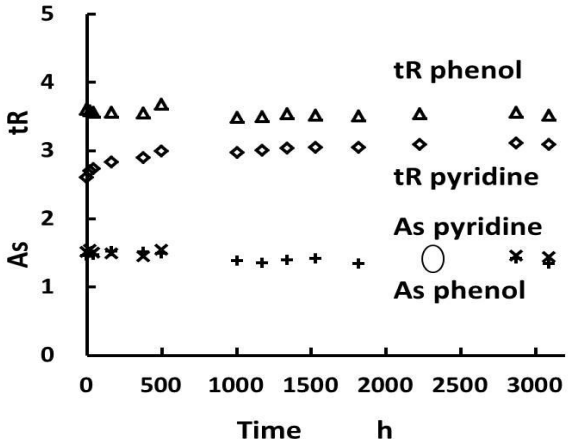


Figure 2. Stability of phenylhexyl-bonded silica gel in pH 10.2

Column: **Paci-3-PhHx**, 100 x 2.1 mm I.D.;
Eluent: 10 mM ammonium formate pH10.2 + acetonitrile (70+30)
Flow rate: 0.2 mL/min at 40ºC; Pressure: 8.7MPa;
tR: retention time min; As: Peak symmetry; O: calculation error

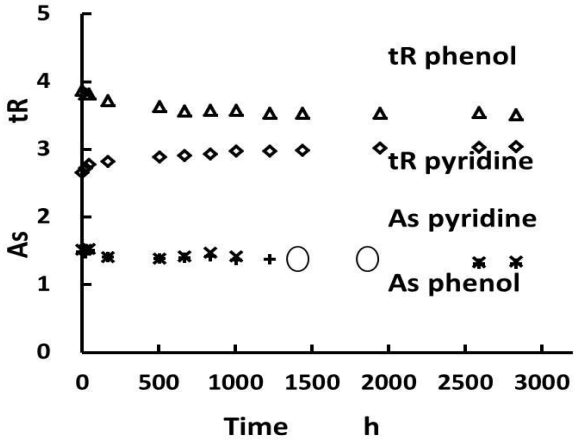


Figure 3. Stability of pentyl-bonded silica gel in pH 10.2

Column: **Paci-3-C5**,100 x 2.1 mm I.D.;
Eluent:10 mM ammonium formate pH10.2 + acetonitrile (70+30);
Flow rate: 0.2 mL/min at 40ºC; Pressure: 8.7MPa;
tR: retention time min; As: Peak symmetry; O: calculation error

Fast separation of acidic drugs in reversed-phase liquid chromatography

Elution order in reversed-phase liquid chromatography is basically based on hydrophobicity (log *P*: octanol-water partition coefficient) of analytes [3-5]. The log *P* values of acidic drugs were correlated with the log *k* values measured in an eluent containing 50% methanol and 10 mM formic acid (pH 2.9). The slope and intersection demonstrated the selectivity of these bonded-phases. The relation is summarized in **Figure 4**. The properties of these acidic drugs are collected in **Table 1**.

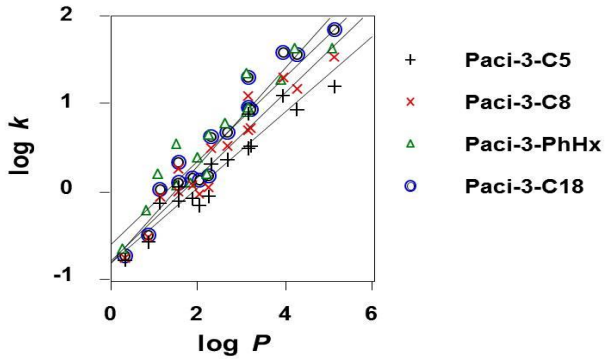


Figure 4. Retention strength of columns based on hydrophobicity of acidic drugs

Column size: 50 x 3.0 mm I.D.; Eluent: 50% methanol containing 10 mM formic acid (pH 2.88); Flow rate: 0.40 mL/min at 40°C.

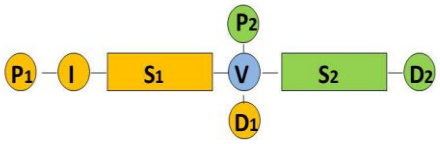
Table 1. Properties of acidic drugs

		Log *P*	p*K*a
1	Nicotinic acid	0.36	4.75
2	Sulfamethoxazole	0.86	6.16*
3	Chloramphenicol	1.14	7.49*
4	*p*-Hydroxybenzoic acid	1.58	4.54#
5	Nalidixiric acid	1.59	8.6
6	Benzoic acid	1.87	4.19
7	Furosemide	2.03	4.25*
8	Salicylic acid	2.26#	2.97#
9	Tolbutamide	2.34	5.16
10	Tolazamide	2.69	4.07*
11	Phenylbutazone	3.16	4.5
12	Naproxen	3.18	4.15
13	Probenecid	3.21	3.4
14	Ibuprofen	3.97	4.91
15	Indomethacin	4.27	4.5
16	Mefenamic acid	5.12	4.2

References : DrugBank, *predicted; #PubChem

The correlation coefficient between the results obtained using the octadecyl(C18)- bonded silica gel column and those of either the pentyl(C5)- or octyl(C8)-bonded silica gel columns were 1.00 (n = 16). The strength of hydrophobicity of these columns is C18 > C8 > C5. This indicated that these three columns did not have specific selectivity. When the shorter analytical time is required, generally increasing organic modifier concentration or using a shorter column is applied. However, such approach is called as accordion effect, and the resolution becomes poor. One solution for the shorter analysis time is construction of isocratic-elution and column-switching system.

One example; the retention time of mefenamic acid was 30 min in this chromatographic condition using a C18 column. Increasing organic modifier concentration from 50 to 60 or 70%, shortened the retention time to 9 or 3 min. The retention of these acidic drugs was basically related to their log *P* values in this condition. Therefore, one approach to shorten the analysis time was a selection of a weak hydrophobic column like C5. Compounds with higher *k* values could be analyzed using a C5, and those of lower *k* values could be analyzed using a C18. That is, a column switching technique [1] was preferred to separate wide range of hydrophobic compounds using a combination of weak and strong hydrophobic columns. A column switching system is shown in **Figure 5**.



P : Pump, I : Injector, V : Valve, D : Detector,
S1 : Separation column 1/ Analytical column 1
S2 : Separation column 2/ Analytical column 2

Figure 5 Basic column switching system

Injected sample (I) is separated by 1st separation column (S1) using analyte's specificity; then non-retain compounds are wasted through switching valve (V) or transferred to 2nd separation column (S2). When the same isocratic eluent is pumped from both pump 1 (P1) and 2 (P2), stable baseline can be obtained for reproducible and highly sensitive detection. Also a part of eluent from S1 column can be transferred to S2 for the further separation (hurt-cut operation). Effluent from both S1 and S2 are continuously monitored by detectors D1 and D2.

Isocratic-elution and column-switching separation of 12 acidic drug mixtures is shown in **Figure 6**, where sample solution was continuously injected at 15 min interval. Acidic drugs with high log *P* values were separated using a short 50mm C5 column and those with low log *P* values were separated using a combined the short C5 column with a longer 150 mm C18 column.

On the other hand, these mixtures were separated using a gradient elution technique. This analysis time was also 15 min. However, it required re-equilibration time between injections. The column re-equilibration interval was studied from reproducibility of retention time and peak area. The re-equilibration interval was 2, 4, and 6 min. The results are summarized in **Tables 2 and 3.** The chromatogram after 4 min re-equilibration indicated poor base line (see **Figure 7**). In this gradient, methanol concentration was increased from 52 to 80%. Higher the gradient slope, the resolution between compounds 9 and 10 became poor. Furthermore; higher methanol concentration

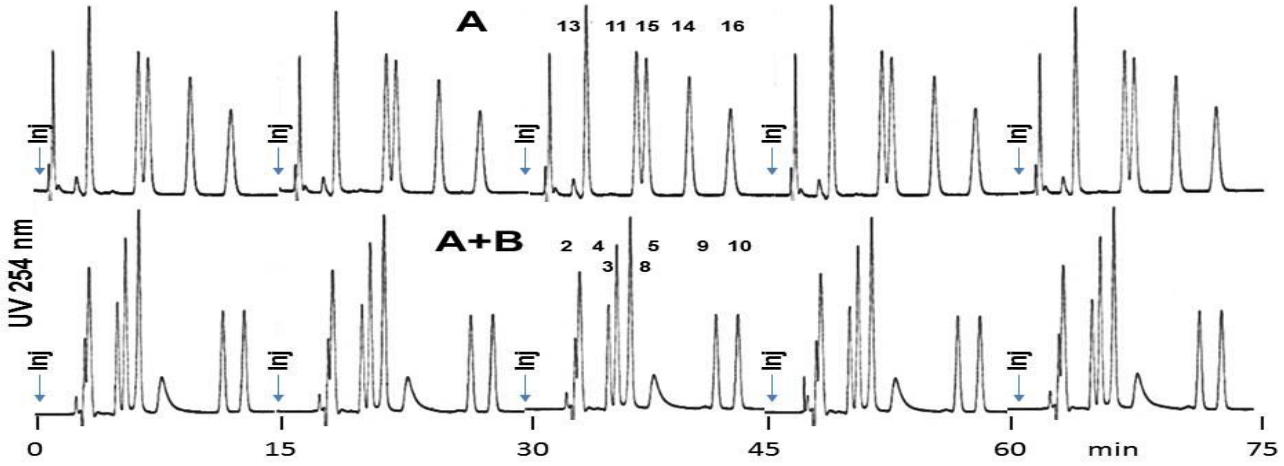


Figure 6 Rapid isocratic-elution and column-switching separation of acidic drugs
Column A: **Paci-3-C5** 50 x 3 mm I.D., Column B **Paci-3-C18** 150 x 3 mm I.D.;
Eluent: 10 mM formic acid (pH 2.88) containing 52% methanol; Flow rate: 0.40 mL/min at 40°C.
Peak number: see Table 1.

of eluent B shortened the analysis hour, but resolution between 11 and 15 became poor. Shorter re-equilibration interval made the poor reproducibility of retention time of weakly retained compounds. The column re-equilibration interval required at least 5 min in this gradient elution system. As the result, it required 20 min for one analysis. Further, reproducibility of peak area was poor in the gradient. See standard deviation of peak area in Table 3. Therefore; above isocratic and column-switching technique is recommended for establishing a quality control system to obtain reproducible and sensitive results.

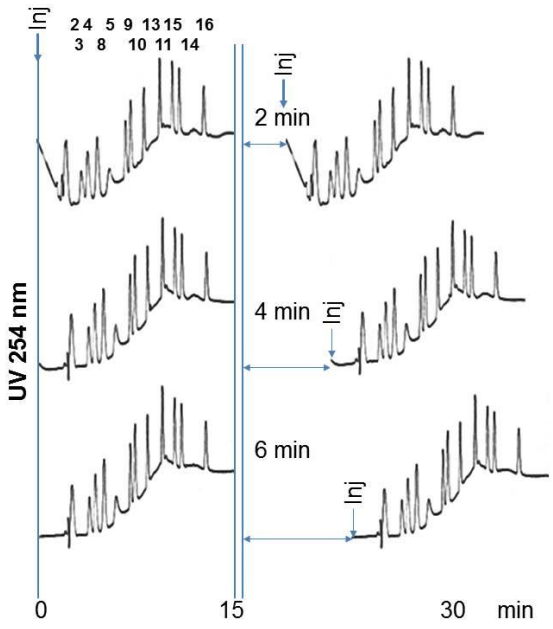


Figure 7. Gradient elution of acidic drugs
Column: **Paci-3-C18**, 150 x 3.0 mm I.D.; Eluent: A52 – B80% methanol containing 10mM formic acid (pH 2.88); Flow rate: 0.40 mL/min at 40°C.; Peak number: see Table 1

Table 2 Reproducibility of retention time depending on reconditioning hour in gradient elution (n = 3)

	Injection interval min		
Sample No	2	4	6
2	2.291	2.467	2.482
3	3.417	3.800	3.820
4	3.905	4.261	4.282
8	4.630	4.925	4.947
5	5.560	5.863	5.873
9	6.770	6.952	6.968
10	7.168	7.312	7.325
13	8.168	8.257	8.267
11	9.356	9.407	9.413
15	10.297	10.337	10.345
14	10.821	10.864	10.871
16	12.688	12.725	12.732

Table 3 Reproducibility of peak area measured by isocratic and gradient elution (n = 3). Attn. reconditioning hour of gradient was 6 min.

Sample	Isocratic		Gradient	
No	area	SD %	area	SD %
2	81691	1.03	81105	0.55
3	49115	0.68	44349	0.09
4	77663	0.14	69080	0.16
8	100377	0.14	88725	0.24
5	46811	0.09	51992	1.16
9	56187	0.40	51601	1.46
10	60559	0.20	64637	1.97
13	61618	0.21	52575	0.74
11	55404	0.18	58764	1.01
15	58452	0.12	5297	0.18
14	56797	0.29	52523	0.51
16	49193	0.25	47090	0.58

Fast separation of basic drugs in reversed-phase liquid chromatography

Elution order in reversed-phase liquid chromatography of basic drugs is also based on hydrophobicity (log *P*: octanol-water partition coefficient) [3-5]. The log *P* values were correlated with log *k* values measured in an eluent containing 50% methanol and 10 mM ammonium formate (pH 10.2). The slope and intersection demonstrated the selectivity of these bonded-phases. The relation is summarized in **Figure 8**. The properties of these basic drugs are collected in **Table 4**.

Table 4 Properties of basic drugs

	Chemicals	log *P*	p*K*a
1	Theobromine	-0.78	9.9
2	Allopurinol	-0.55	9.4
3	Caffeine	-0.07	14.0
4	Theophylline	-0.02	8.6
5	Isoproterenol	0.08	7.9
6	Scopolamine	0.98	7.75
7	Triamterene	0.98	6.2
8	Homatropine	1.45	9.9
9	Carbamazepine	1.98	13.9
10	Procain	2.14	8.11
11	Lidocaine	2.26	7.9
12	Quinine	3.44	4.1

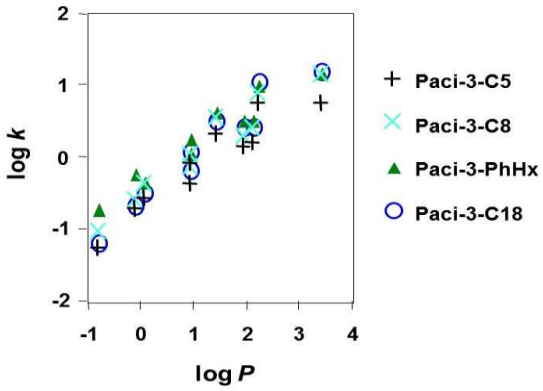


Figure 8. Retention strength of columns based on hydrophobicity of basic drugs

Column size: 50 x 3.0 mm I.D.; Eluent: 50% methanol containing 10 mM ammonium formic acid (pH 10.2); Flow rate: 0.40 mL/min at 40°C.

The strength of hydrophobicity of these bonded phases was PhHx ≥ C18 ≥ C8 > C5 columns. The correlation between log *P* and log *k* values were 0.97 (PhHx), 0.97 (C18), 0.97 (C8), and 0.95 (n = 10). The reason of the low correlation coefficient compared to those for acidic drugs is that these basic drugs were not molecular form based on their p*K*a values in the eluent used for LC-MS operation. The log *k* values measured using the C18 column was similar to those measured on the C8 column. This means these compounds did not require longer alkyl-chain bonded phases because of their molecular size. The requirement differs from that of carotenoids whose molecule shape is quite long.

The correlation coefficient between the results obtained using octadecyl(C18)- bonded silica gel column and those obtained using either pentyl(C5)- or octyl(C8)-bonded silica gel columns were 1.00 (n = 10). The result indicated that these three columns have no specific selectivity. The difference is the strength of hydrophobicity. When the shorter analytical time is required, increasing organic modifier concentration or using a shorter column was performed. However, such approach is called as accordion effect, and the resolution becomes poor. One solution to perform the shorter analysis time is construction of isocratic-elution and column- switching system. The example of chromatograms is shown in **Figure 9**. Basic drugs having longer retention times due to their log *P* values were separated using a short (50 mm) C5 column, and those weakly retained on the C5 column due to their low log *P* values were separated using a combined the 50 cm C5 column and a 150 cm C18 column. These mixtures were separated within 15 min.

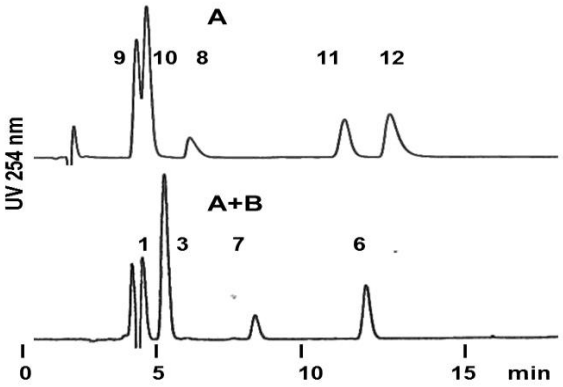


Figure 9 Quick separation of basic drugs using isocratic and column switching elution

Column A: **Paci-3-C5**, 50 x 3.0 mm I.D., Column B: **Paci-3-C18**, 150 x 3.0 mm I.D.;
Eluent: 45% methanol containing pH 10.2 ammonium formate;
Flow rate: 0.40 mL/min; at 40°C; Peak number: see Table 4.

The combination of isocratic-elution and column-switching system with narrow inter-diameter (3 mm) column demonstrated the reproducible retention time and peak area. Furthermore, shorter analysis time was achieved that gradient elution could not achieved. In gradient elution, require skill to read peak area because of base-line change. However, base-line is flat in isocratic elution. Continuous injection can be performed in isocratic elution, but gradient elution required re-equilibration interval. Larger the organic modifier concentration change, longer the re-equilibration interval. Consider a combination of isocratic-elution and column-switching system to perform reproducible and short analysis time chromatography for your regular chromate-gram-phic work. This technique has also advantage for easy maintenance of LC system and finding machine trouble.

Selectivity of Phenylhxyl-bonded siica gel

Another potential of column selection is utilizing PhHx-bonded silica gel column. The hydrophobicity of PhHx column is similar to that of C8 column, but it demonstrated selective separation between tolbutamide (9) and tolazamide (10) (Rs = 3.0), compared to the results obtained using the C8 column, no clear separation was observed (see **Figure 10**).

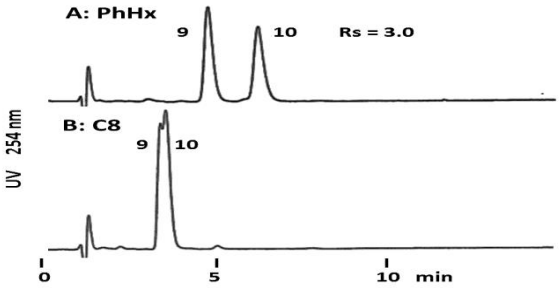


Figure 10 Selective separation of tolbutamide (9) and tolazamide (10) on phenylhexyl-phase
Column A: **Paci-3-PhHx** 50 x 3.0 mm I.D.,
B: **Paci-3-C8**, 50 x 3.0 mm I.D.;
Eluent: 10 mM formic acid containinig 50% methanol;
Flow rate: 0.4 mL/min at 40°C;
Peak number: see Table 1.

How to construct a quick and stable HPLC separation system?

We have seen how column switching can expedite separation substances of interest, using techniques that take advantage of dependence on the differences in hydrophobicity of analytes; dependence on the differences in Coulombic force of analytes; dependence on the molecular size of analytes; dependence on the steric hindrance of analytes; affinity and enzymatic reactions; and application of mass spectrophotometric analysis. Here, we demonstrated how column switching can expedite separation substances of acidic and basic drugs using dependence on the differences in their hydrophobicity. There have been major advances in design and development of bonded phases made from silica gels. These bonded phases have both advantages of silica and organic polymers (i.e.high mechanical strength and extended pH stability). Selection of columns permited to measure albumin-drug binding affinity without albumin [6].

Computational chemistry is a useful tool for designing new materials, for studying molecular interactions, and for predicting molecular properties [6]. The combination of computational chemical analysis with chemical and physical analyses will enhance the development of packing materials for chromatography. We will continuously develop new bonded phases for further support for quick liquid chromatographic separation. This computational approach is based on modeling analysis of enzyme reactivity. Such *in silico* analysis also supports for drug discovery [7, 8].

References

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Price list Paci-3-C18, Paci-3-PhHx, Paci-3-C8, Paci-3-C5, Paci-3-HxNy

Column size	Price	Column Size	Price	Column size	Price
50 x 4.6 mm ID	¥ 40,000	50 x 3.0 mm ID	¥ 40,000	50 x 2.1 mm ID	¥ 40,000
150 x 4.6 mm ID	¥ 40,000	150 x 3.0 mm ID	¥ 40,000	100 x 2.1 mm ID	¥ 40,000
250 x 4.6 mm ID	¥ 40,000	250 x 3.0 mm ID	¥ 40,000	50 x 2.1 mm ID	¥ 40,000

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