

New Therapeutic Drug Monitoring without any Preparation

Applications of Temperature responsive
column "*AquaWay*" series

Business Development CellSeed Inc.

Contents of presentation

- 1. Company Profile**
2. Temperature-responsive Polymer
3. Temperature-responsive Reverse Phase HPLC column *Aqua Way "Phylic"*
4. Temperature-responsive Ion charged HPLC column *Aqua Way "Cation"*

Company profile

Foundation: May 2001 in Japan

Background: Spin-off from Tokyo Women's Medical
University

Number of employees: 42

Paid in Capital: 11 mil USD / Private

Business Domain:

- 1) Regenerative medicine
- 2) Cell cultureware
- 3) HPLC column

Contents of presentation

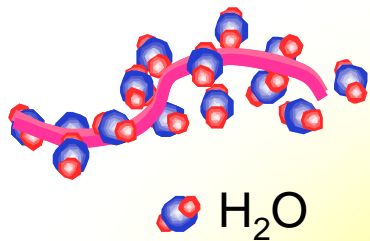
1. Company Profile
2. Temperature-responsive Polymer
3. Temperature-responsive Reverse Phase HPLC column *Aqua Way "Phylic"*
4. Temperature-responsive Ion charged HPLC column *Aqua Way "Cation"*

Temperature-responsive Polymer

Properties of Poly(*N*-isopropylacrylamide) in water

Hydration

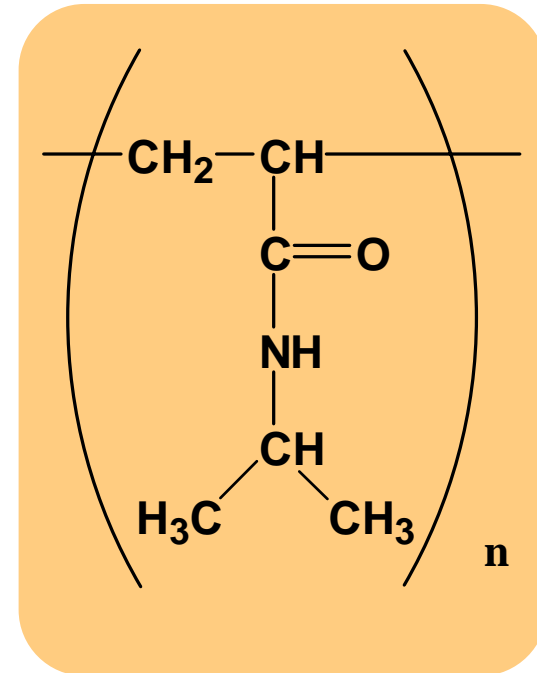
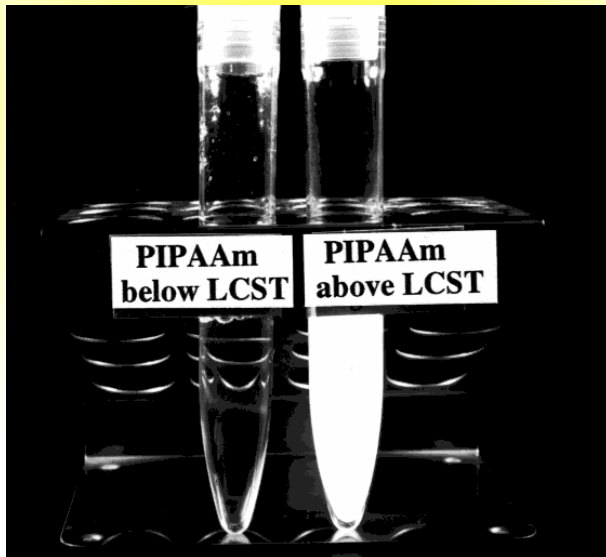
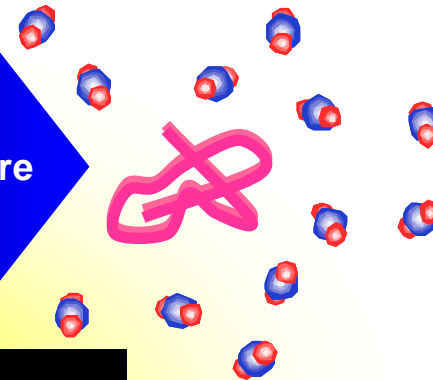
In polymer solution



Lower Critical
Solution Temperature
(LCST)

at 32

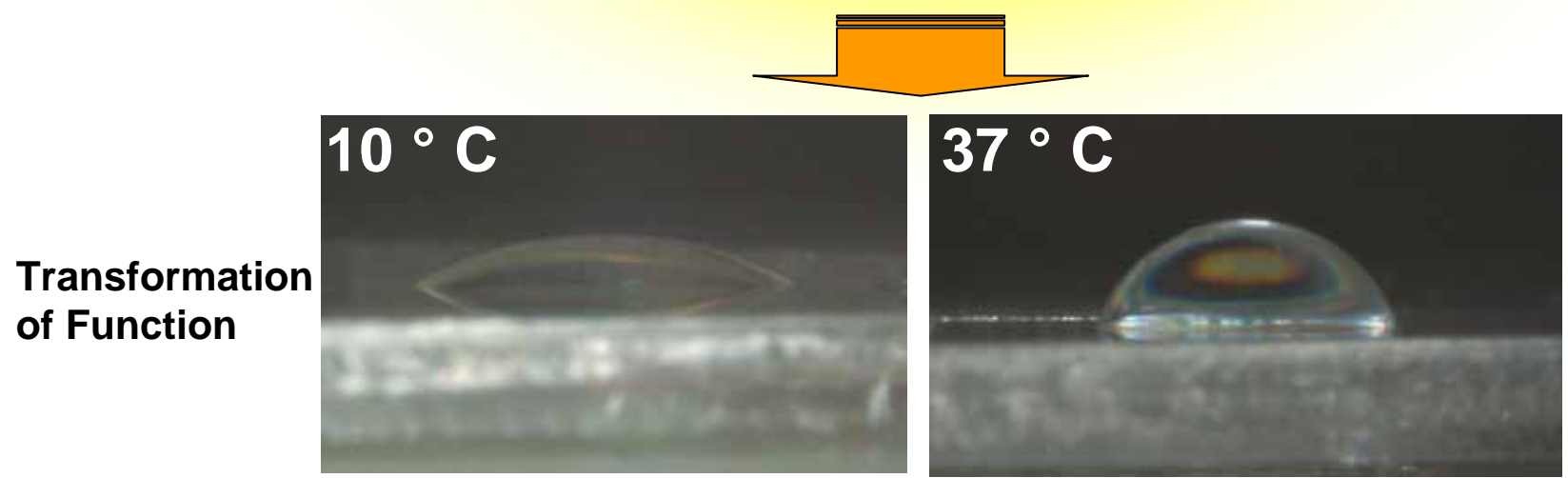
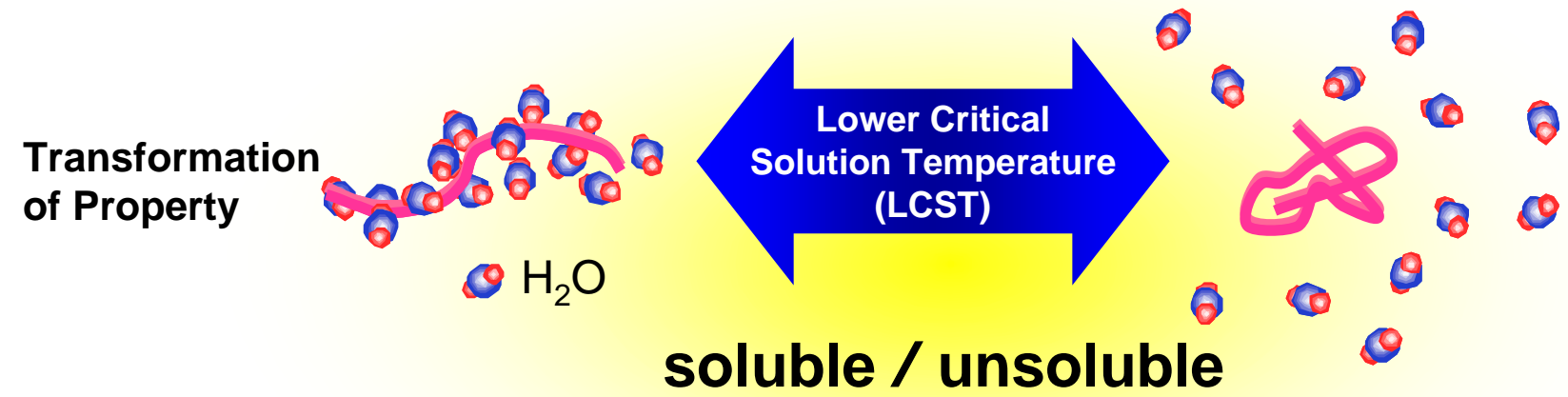
Dehydration



Poly(*N*-isopropylacrylamide)
(PIPAAm)

Transformation of Grafted Surface Temperature-Responsive Polymer

Poly(*N*-isopropylacrylamide): **PIPAAm**; **LCST = 32 ° C**



hydrophilic / hydrophobic

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3. **Temperature-responsive Reverse Phase HPLC column *Aqua Way "Phylic"***
4. Temperature-responsive Ion charged HPLC column *Aqua Way "Cation"*

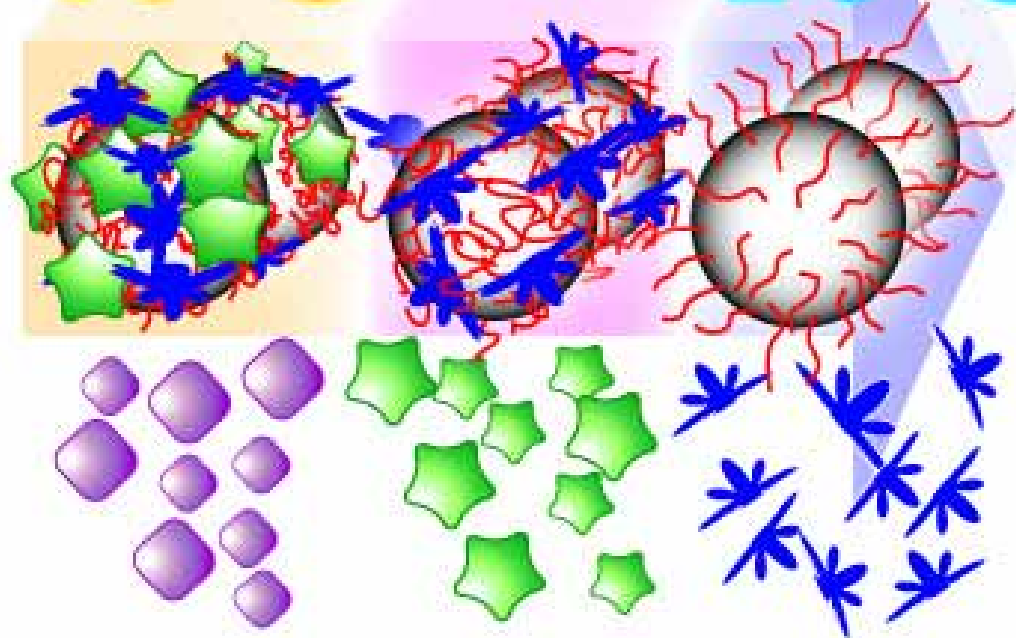
Hydrophobicity changes of *Aqua Way "Philoc"* by Temperature

Hydrophobic surface
(40°C)
~Molecule attaching~

Hydrophilic surface
(10°C)
~Molecule releasing~

40°C

10°C



★ Molecules with high hydrophobicity

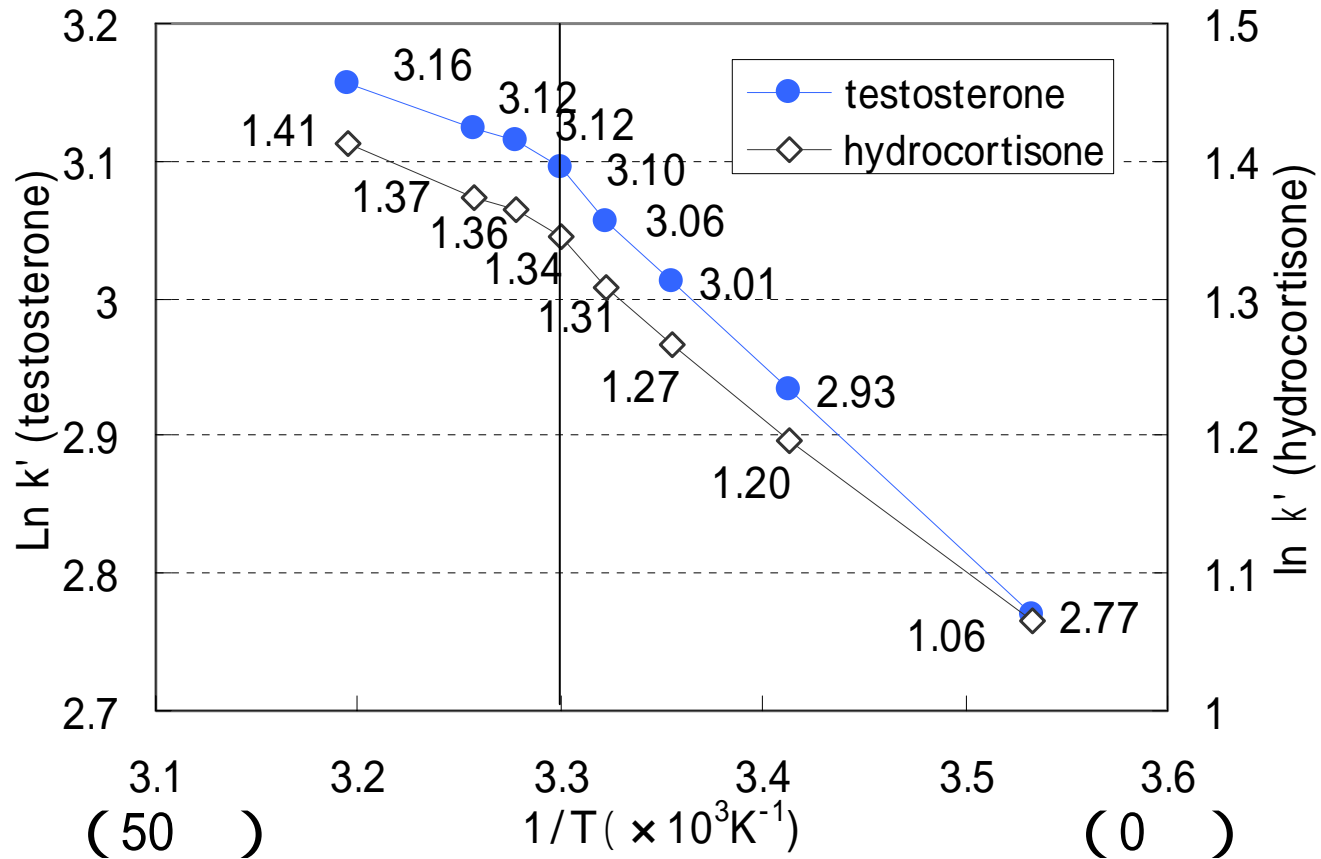
↕

◆ Molecules with high hydrophilicity

"Temperature-Responsive Chromatography Using Poly (N-isopropylacrylamide) - Modified Silica."
H.Kanazawa, K.Yamamoto, Y.Matsushima, N.Takai, A.Kikuchi, Y.Sakurai and T.Okano,
Analytical Chemistry 68,100-15 (1996).

Easy to Handle! Gradual Change of Hydrophobicity

Van't Hoff plots of steroids



Column: *AquaWay "Philoc"* 150 mm \times 4.6 mm I.D.

Mobile phase: water

Flow rate: 1.0 ml/min

Detection: UV 254 nm

Sample: hydrocortisone, testosterone t_0 = Uracil

Key features & benefits of *Aqua Way "Phylic"*

Temperature-responsive Polymer is synthesized and grafted onto silica beads.

Using only aqueous solution as a mobile phase.

Separation selectivity and retention time are controlled by column temperature.

Having analyzable condition which is unanalyzable by conventional ODS column.

No Denaturation of Targets

Same benefit as gradient elution can be achieved by temperature-gradient.

Application ~ Therapeutic Drug Monitoring ~

Problems of current method

EIA/ELISA

- Unanalyzable without antibody (new drug, lead compounds)
- Preparation process of removing blood serum is cumbersome
- Loss of target molecules during preparation phase

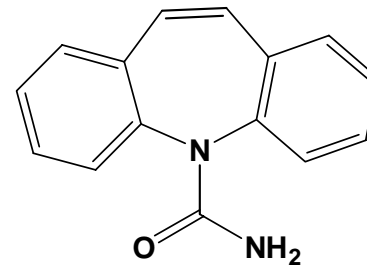
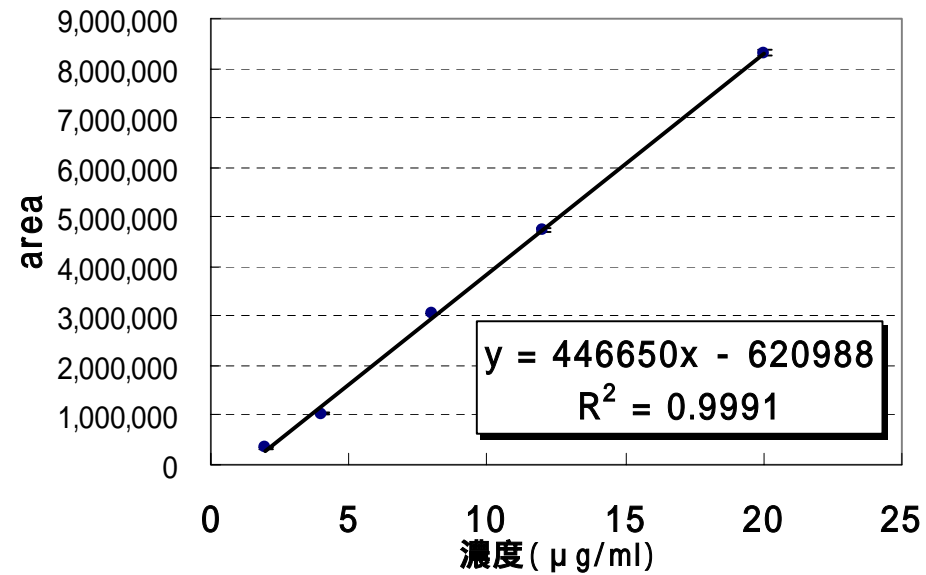
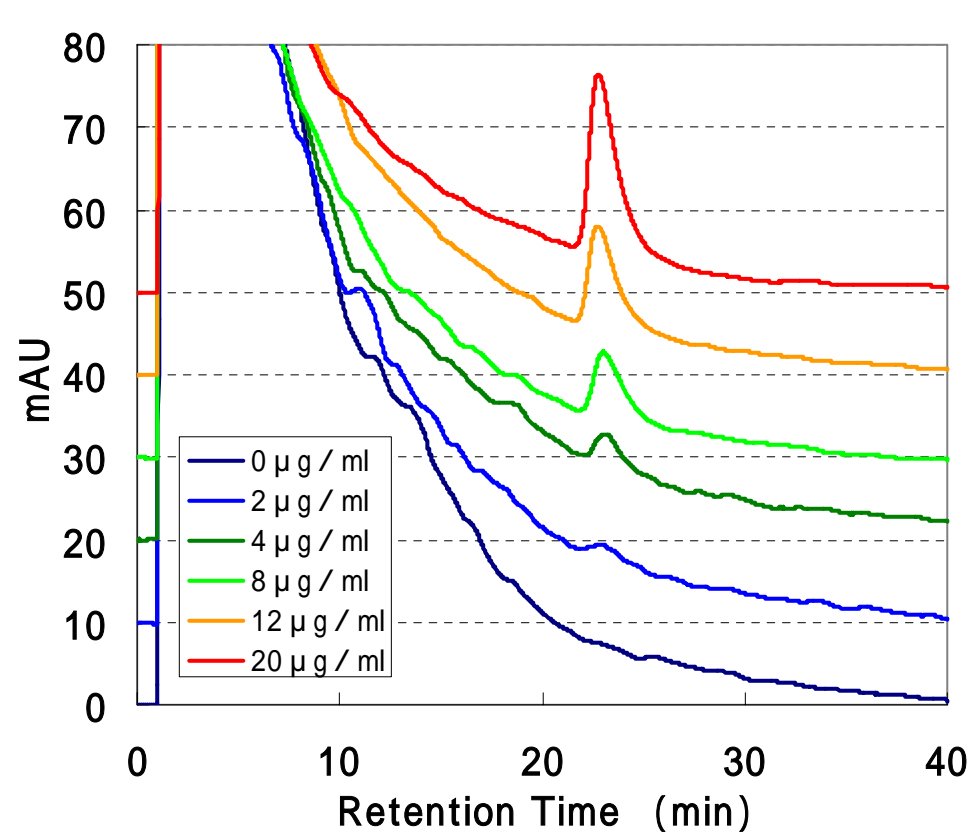
HPLC

- Cumbersome Process of removing blood serum
Loss of target molecules
- Column-Switching System is required (need at least two columns, and more than two mobile phases).

Aqua Way "Phylic" can analyze target molecules in blood serum without preparation

- **Avoid Loss of Target Compound**
- **Lower cost**

Analysis of Carbamazepine in Blood Serum



Carbamazepine
(antiepileptic drug)

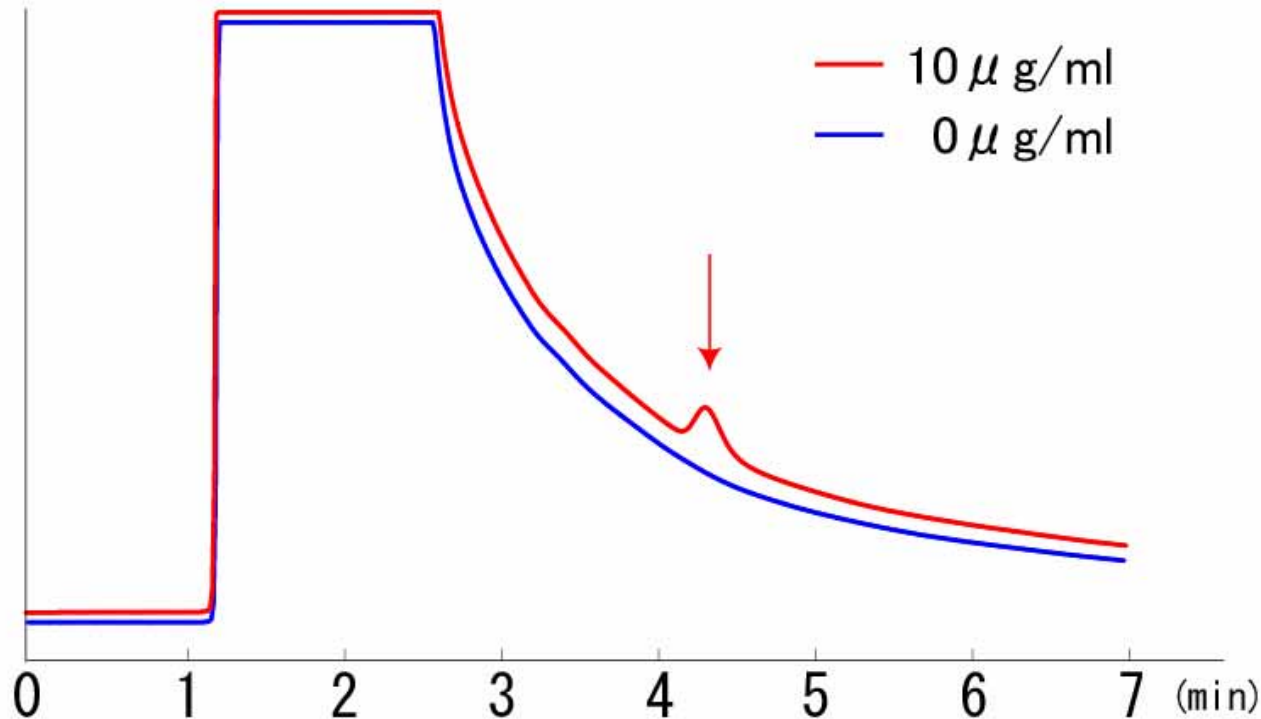
Column: *AquaWay "Phylic"* 150mm × 4.6mm I.D.

Mobile phase: 66.7 mM phosphate buffer (pH 7.0) Flow rate: 1.0ml/min

Temperature: 40 Detection: UV 220nm

Sample: Carbamazepine in Blood Serum

Analysis of Primidone in Blood Serum



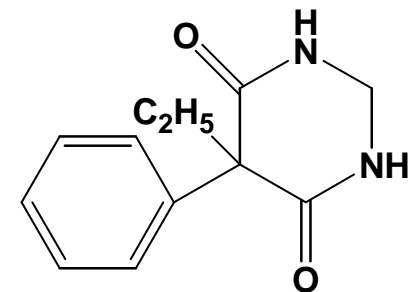
Column length: Aqua Way Philic 150 mm x 4.6 mm I.D.

Eluent: 66.7 mM Phosphate buffer (pH7.0)

Flow rate: 1.0 ml/min

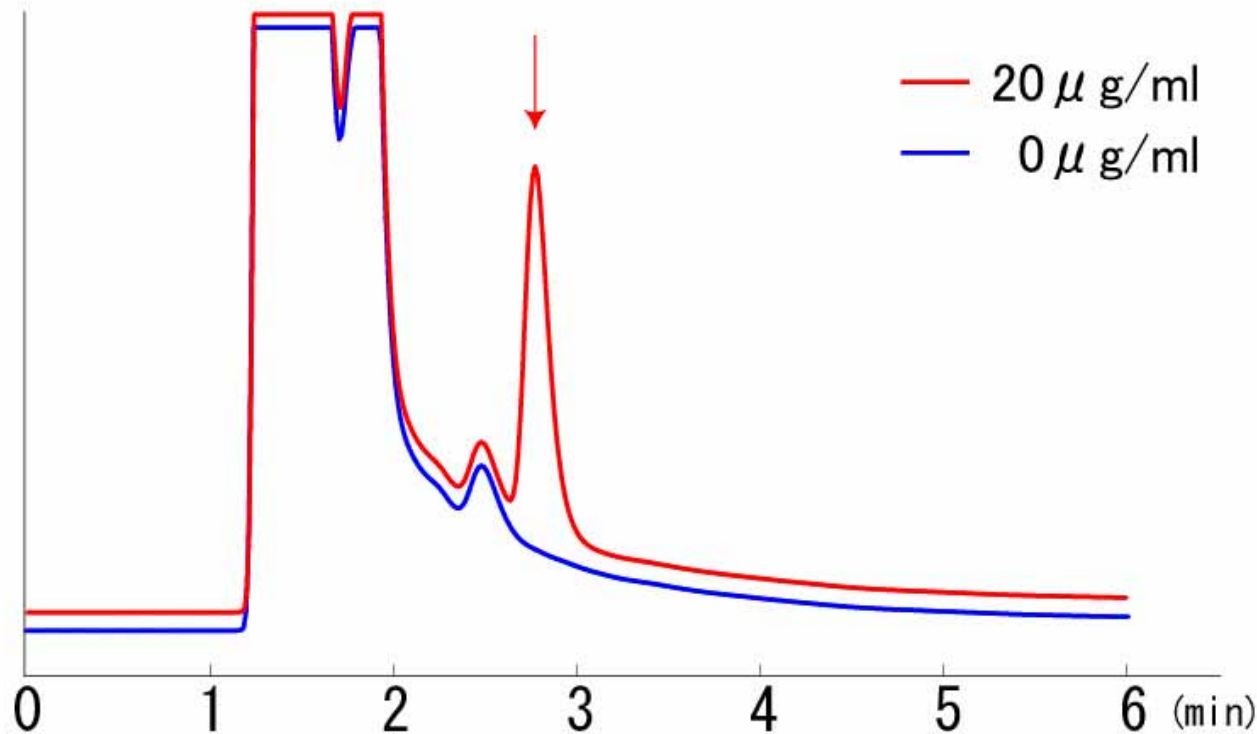
Detection: 220 nm Temperature: 40°C

Sample: Human Serum + primidone (0, 10 μ g/ml)



Primidone
(antiepileptic drug)

Analysis of Theophylline in Blood Serum



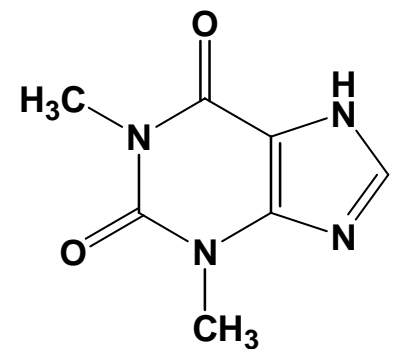
Column length: Aqua Way Philic 150 mm x 4.6 mm I.D.

Eluent: 66.7 mM Phosphate buffer (pH7.0)

Flow rate: 1.0 ml/min

Detection: 280 nm Temperature: 40°C

Sample: Human Serum + theophylline (0, 20 μ g/ml)

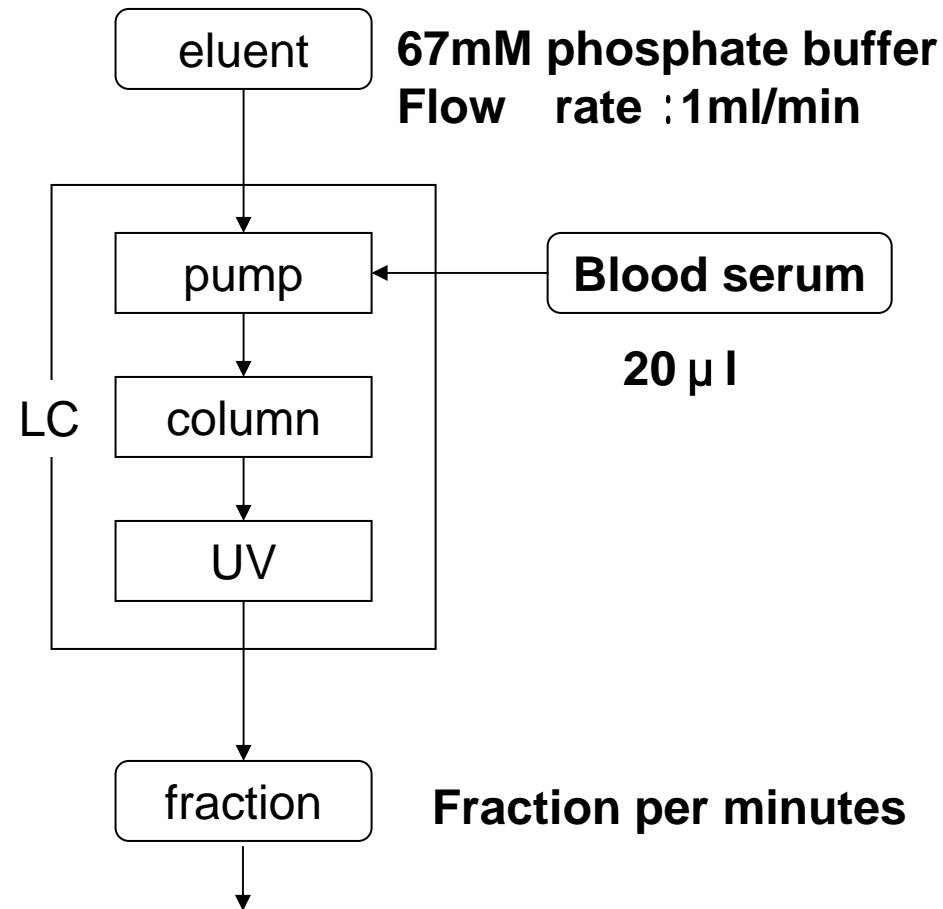
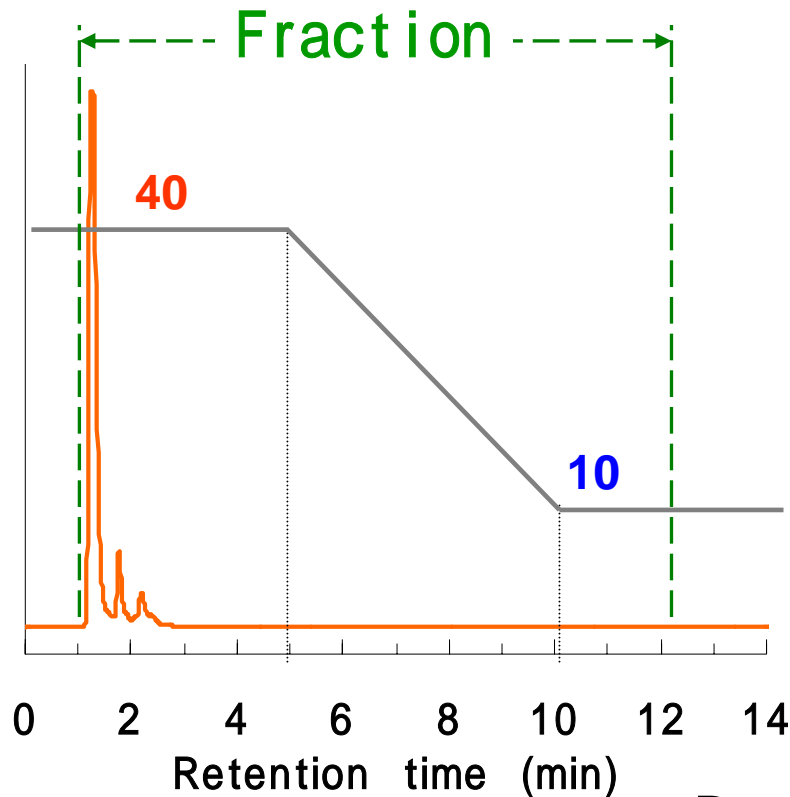


theophylline

bronchodilator

No Clogging by Injecting Serum Directly into *Aqua Way "Phylic"*

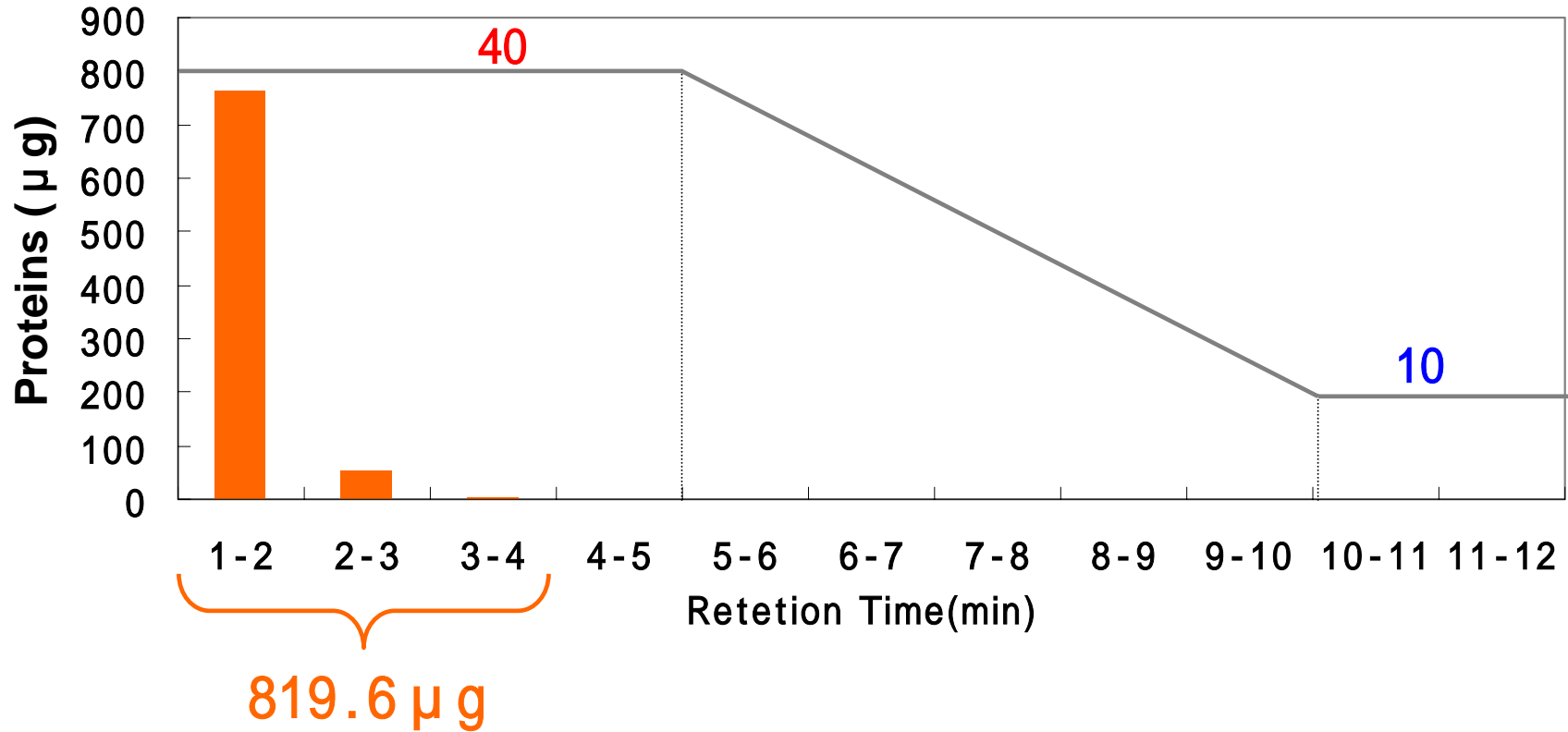
Recovery of blood serum



**Determine the Quantity of proteins
by Micro BCA Protein Assay**

No Clogging by Injecting Serum Directly into *Aqua Way "Philoc"*

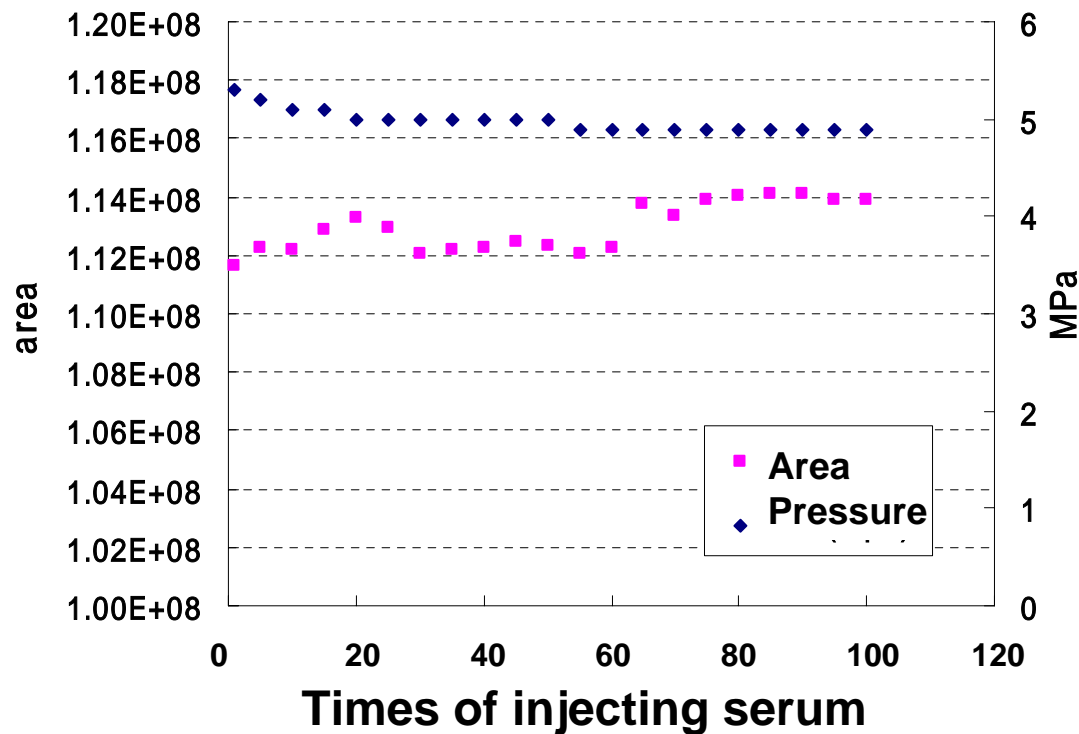
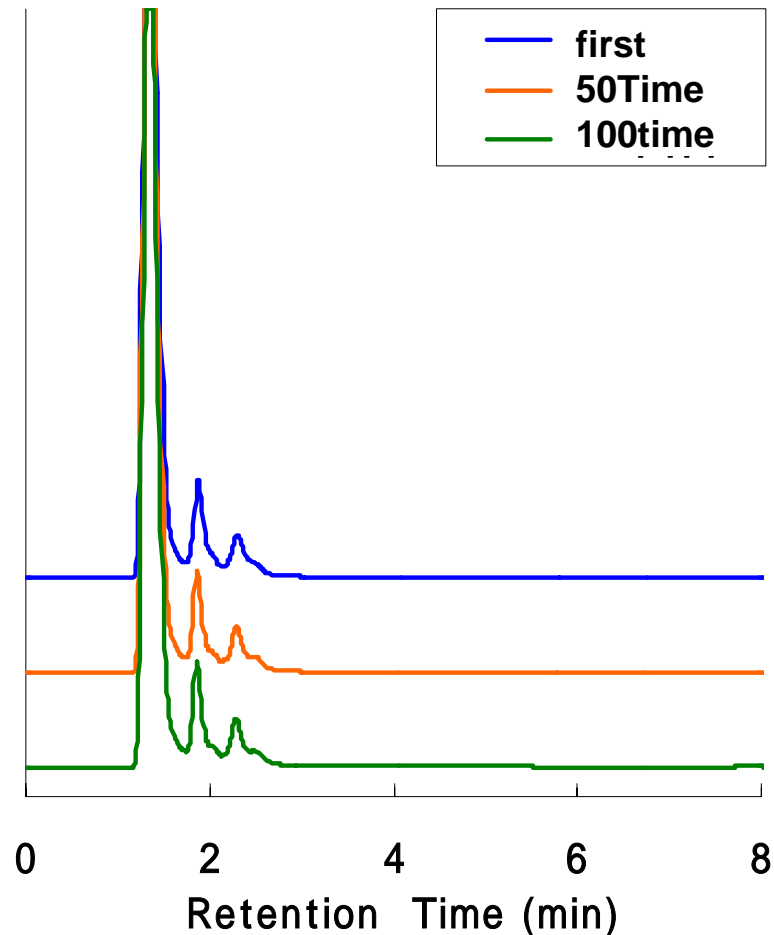
Proteins quantity of each fraction



$$\text{Recovery rate} = \frac{\text{recovery } 819.6 (\mu\text{g})}{\text{injection } 878.2 (\mu\text{g})} \times 100 = \boxed{93.3\%}$$

High Durability, No clogging over 100 times of serum injection!

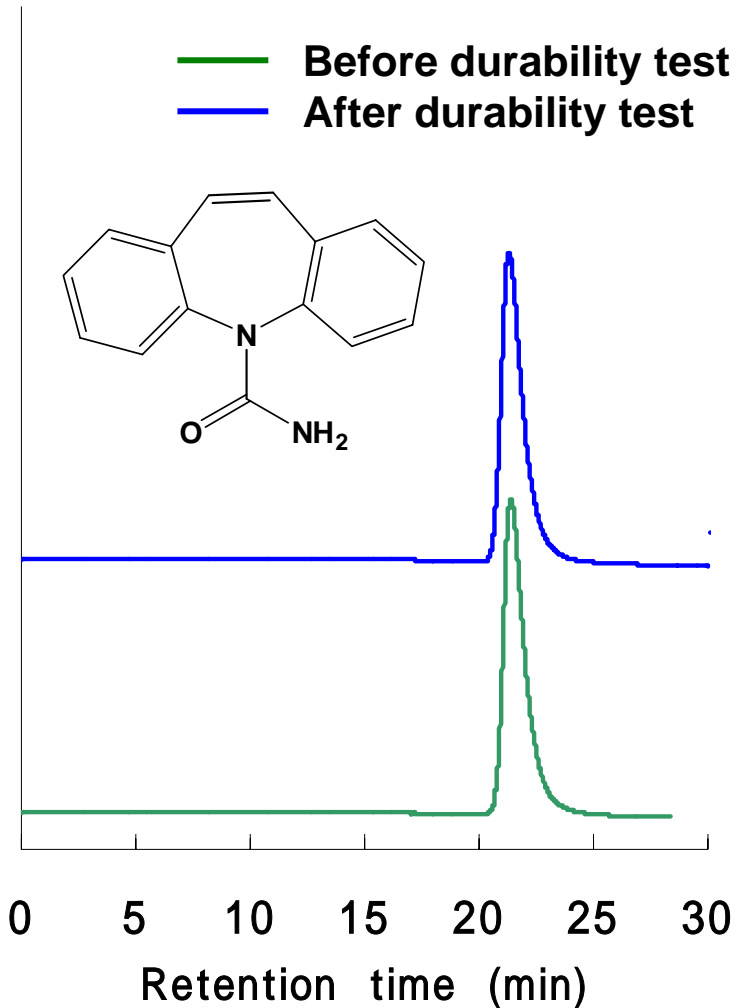
Analysis of Carbamazepine in serum



Column: *AquaWay "Phylic"* 150mm x 4.6mm I.D.
Mobile phase:
66.7mM phosphate buffer (pH7.0)
Flow rate: 1.0ml/min Temperature: 40
Detection: UV 280nm
Sample: blood serum (20 μ l)

No deterioration of *Aqua Way Philic* over 100 times of serum injection!

Analysis of Carbamazepine in serum



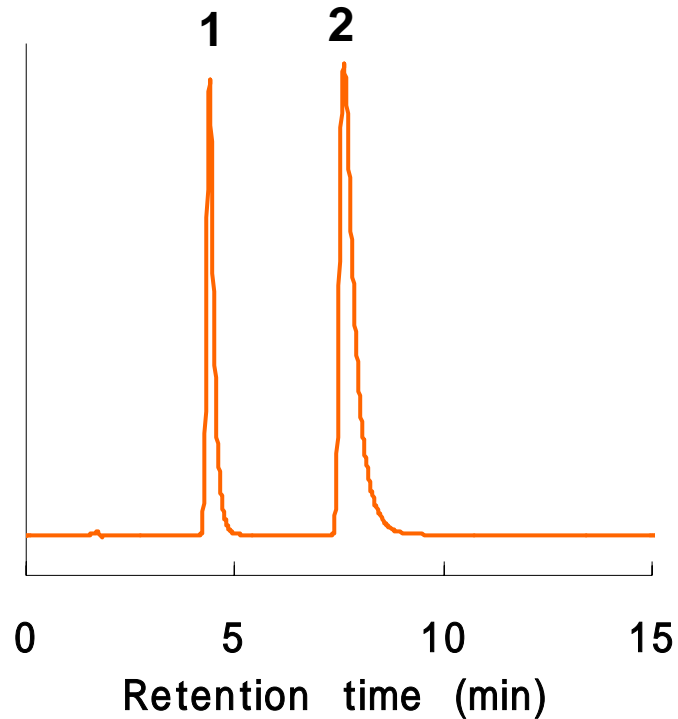
**Stable retention time of carbamazepine
Before and After durability test**



**Stable Surface Condition of the
Column, Scarce Adhesion of Serum**

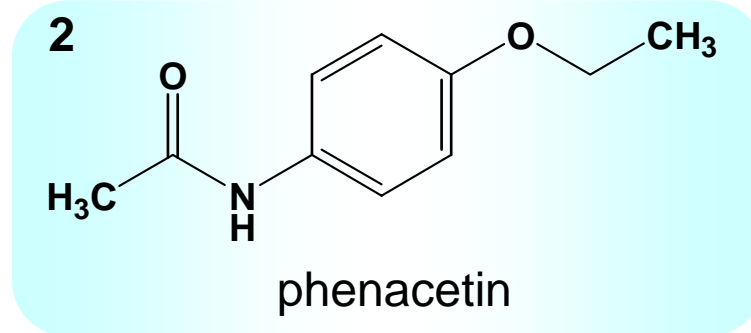
Column: *AquaWay "Philic"*
150mm x 4.6mm I.D.
Mobile phase:
66.7mM phosphate buffer (pH7.0)
Flow rate: 1.0ml/min Temperature: 40
Detection: UV 220nm
Sample: carbamazepine

Application ~ CYP Probe Substrates ~

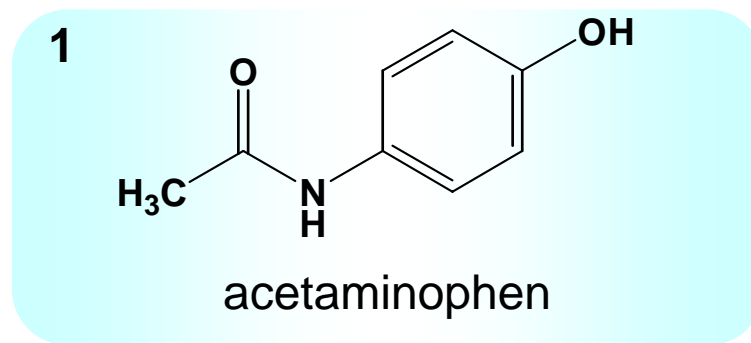


Column: *AquaWay "Philoc"* 150mm x 4.6mm I.D.
Mobile phase: 66.7mM phosphate buffer (pH7.0)
Flow rate: 1.0ml/min
Detection: UV 220nm
Temperature: 40
Sample: 1 acetaminophen 2 phenacetin

CYP = cytochrome P450

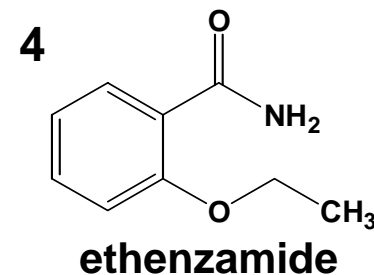
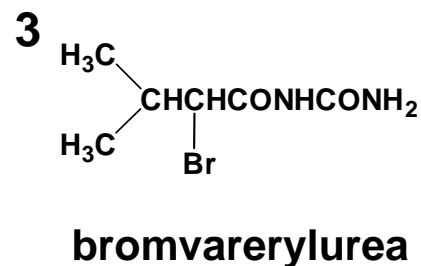
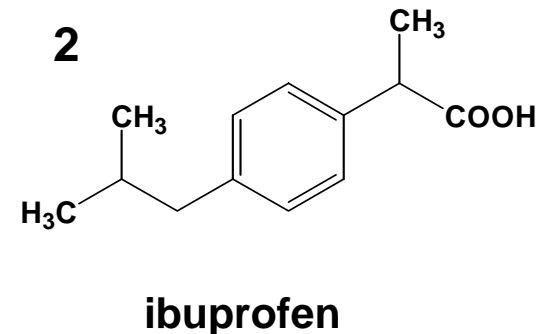
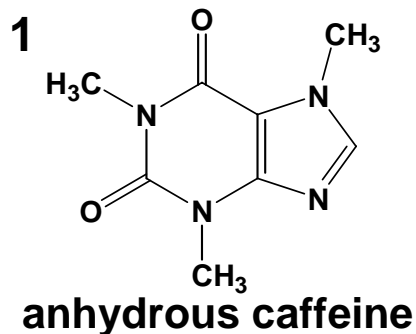
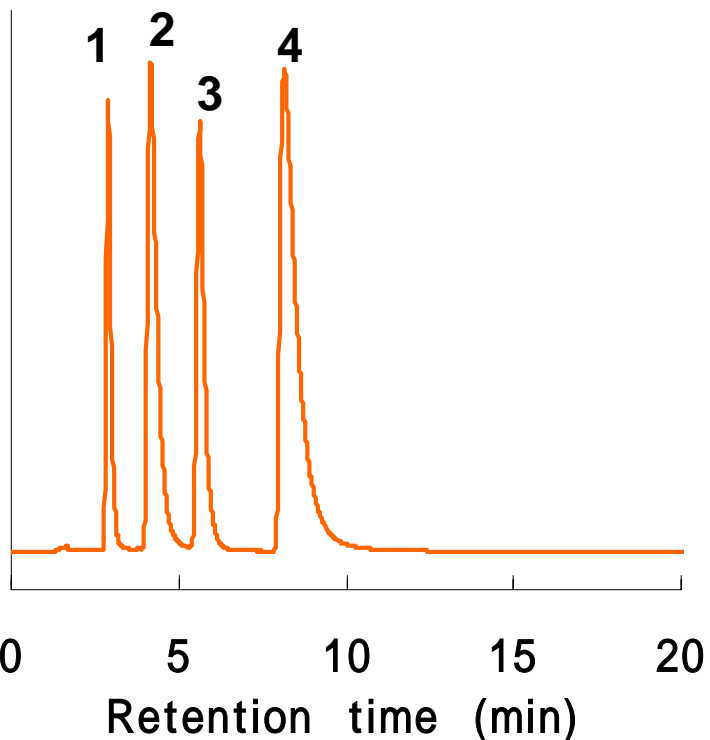


drug-metabolizing enzyme **CYP1A2** **metabolism**



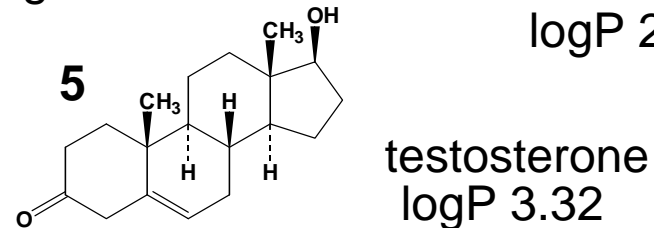
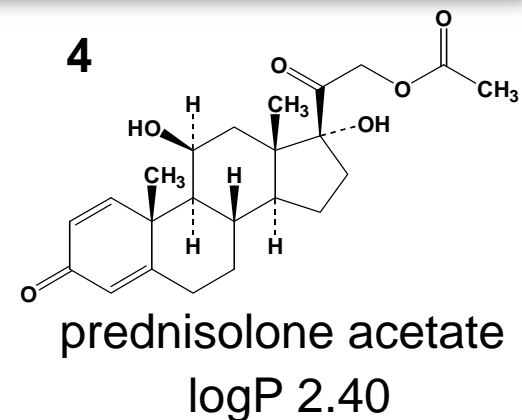
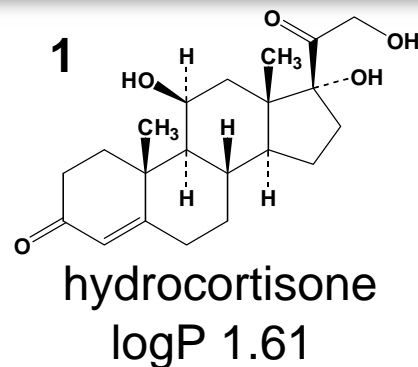
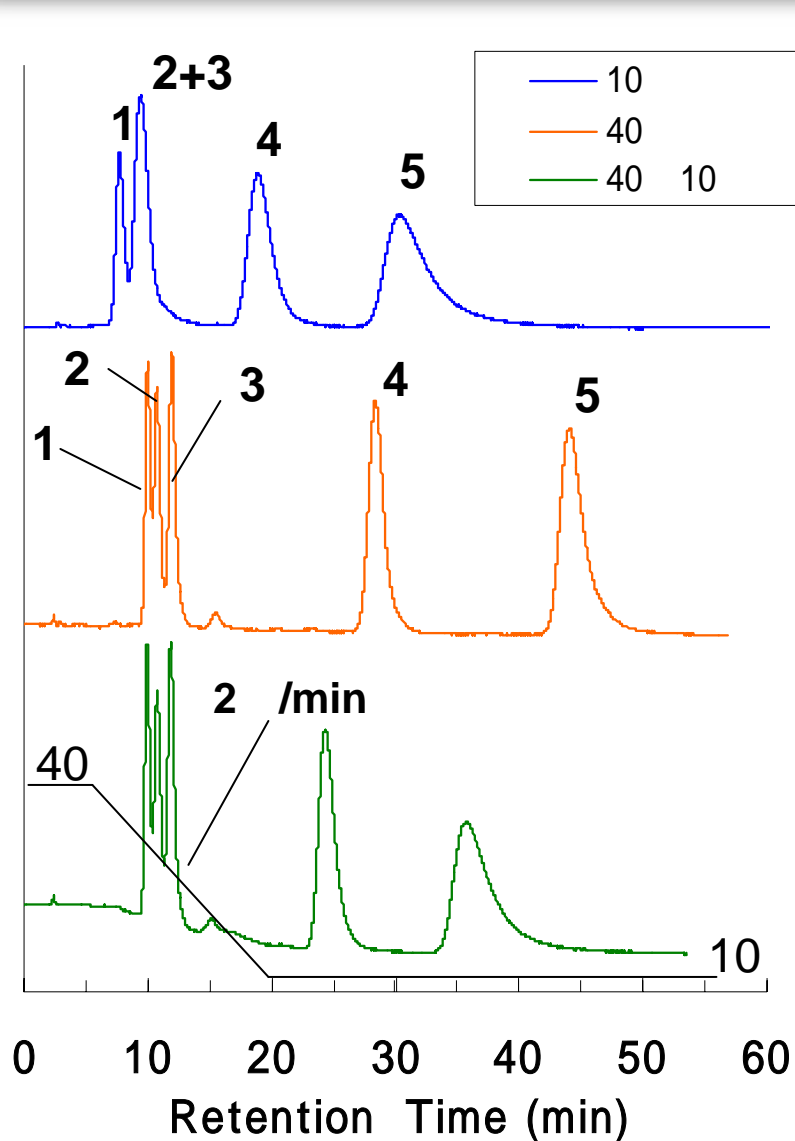
Application

~ OTC drugs ~



Column: AquaWay "Phylic" 150mm x 4.6mm I.D.
Mobile phase: 66.7mM phosphate buffer (pH7.0)
Flow rate: 1.0ml/min
Detection: UV 230nm
Temperature: 40
Sample: 1 anhydrous caffeine 2 ibuprofen
 3 bromovalerylurea 4 ethenzamide

Application ~ Separation of Steroids ~



Column: AquaWay "Philoc"
150 mm x 4.6 mm I.D.

Mobile phase: water

Flow rate: 1.0 ml/min

Detection: UV 254 nm

Sample: 1 hydrocortisone

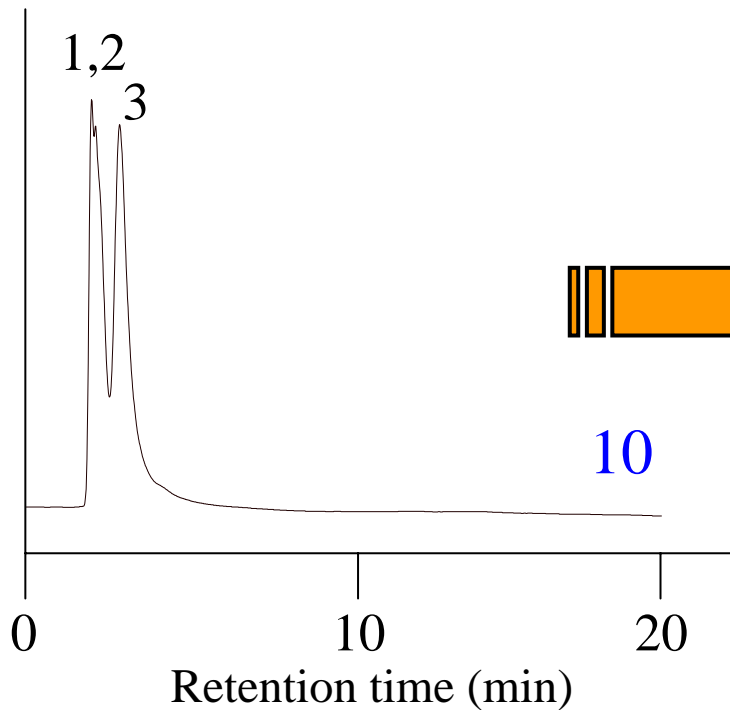
2 cortisone

3 prednisolone

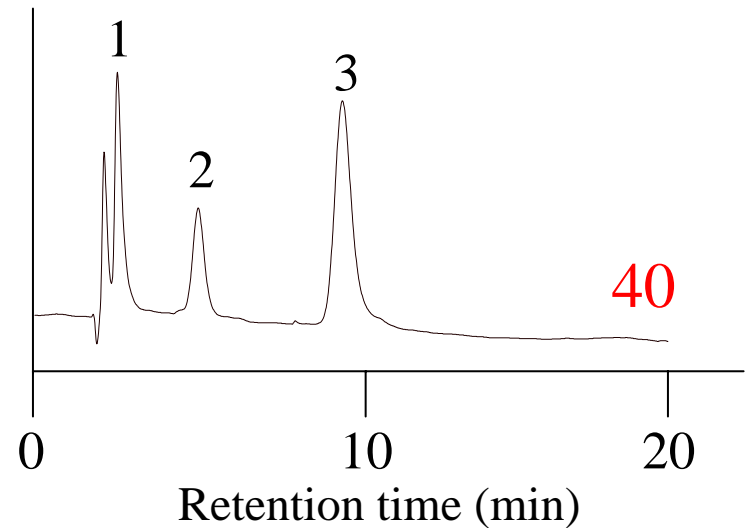
4 prednisolone acetate

5 testosterone

Application ~ Separation of Peptides ~



HPLC conditions
Detection: UV (210 nm)
Eluent: **0.9 w/v% NaCl solution**
Flow rate: 1.0 mL/min



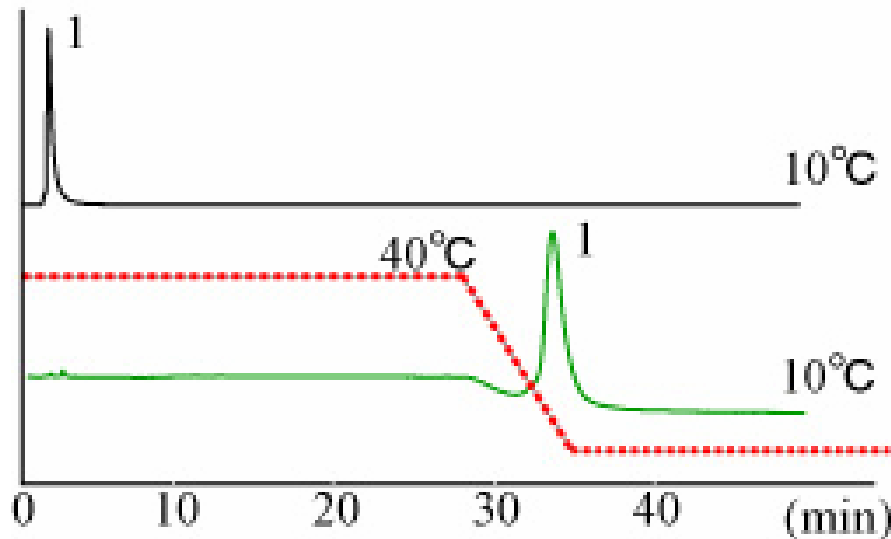
- 1. Insulin chain A (79.97 $\mu\text{g/mL}$)
- 2. β -Endorphin fragment 1-27 (60.44 $\mu\text{g/mL}$)
- 3. Insulin chain B (859.8 $\mu\text{g/mL}$)

Peptides are analyzable by the difference in number of hydrophobic amino-acid residue of each molecule.

Kanazawa, et al. *Anal. Bioanal. Chem.* **378**, 46 (2004).

Application ~ Protein ~

Protein



Column: 150mm x 4.6mm I.D.
Mobile phase: 66.7mM phosphate buffer (pH7.0)
Flow rate: 1.0ml/min
Detection: UV 280nm
Column oven: Aqua Way Gradienter
Temperature gradient speed: 4°C/min
Sample: 1) Chymotrypsinogen A

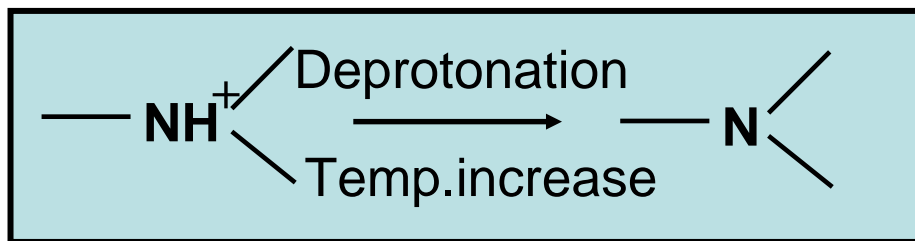
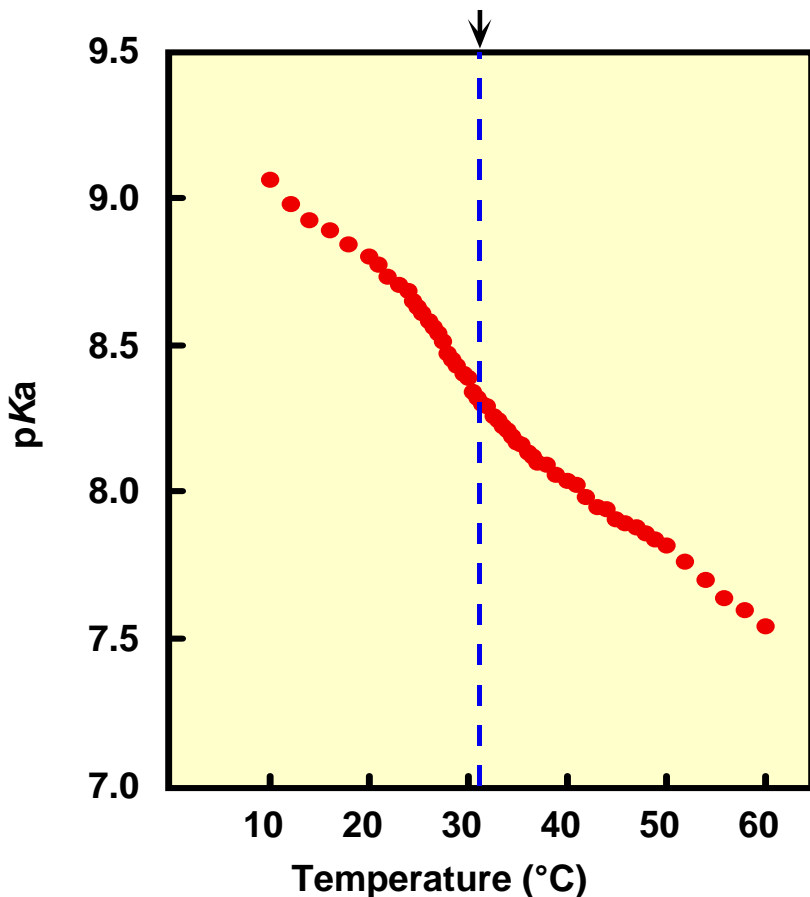
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pKa Changes of Amino Groups in Cationic Temperature-Responsive Polymers

Poly(IPAAm-co-BMA-co-DMAPAA)
IPAAm/BMA=95/5, DMAPAA 7.5 mol%

LCST in distilled water

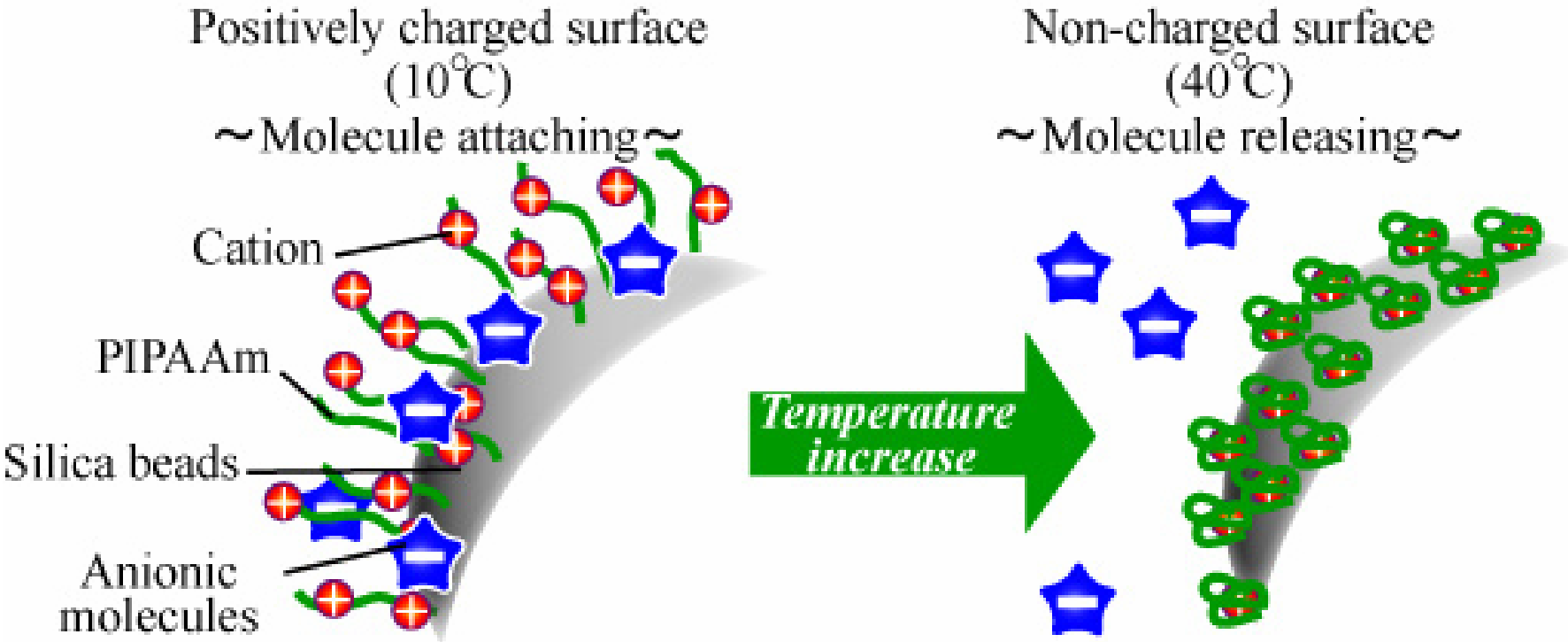


- pKa Decrease is induced by Temperature rise

Increasing hydrophobicity leads to lowered dielectric constant and progression of deprotonation.

→ ***Aqua Way "Cation" can control charge density by temperature***

Charge Density Changes of Aqua Way "Cation" by Temperature



Key feature & benefits of *Aqua Way "Cation"*

Cation Introduced temperature-responsive polymer is synthesized and grafted onto silica beads.

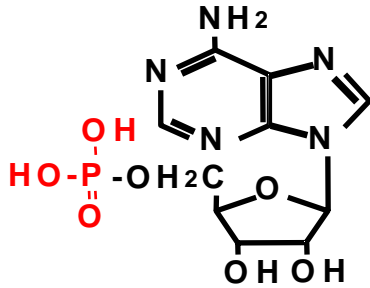
Charge density can be controlled by temperature while keeping elution constant such as an aqueous mobile solution.

No gradient elution of salt concentration is required.

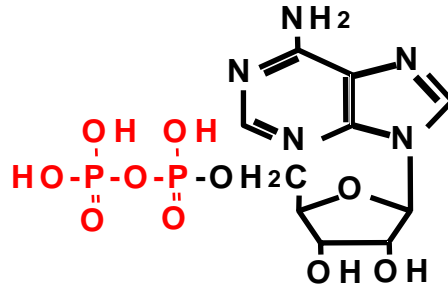
No denaturation of Targets

Maintenance of the column is easy since elution gradient using high salt concentration buffer is not required.

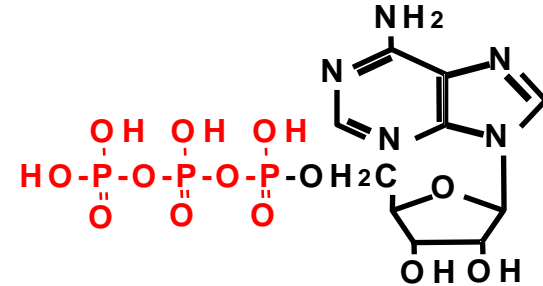
Structure of Adenosine Phosphates



AMP



ADP



ATP

Table properties of solutes

	M.W.	pK _a	log P*
AMP	347.2	3.80	- 3.52
ADP	427.2	3.90	- 4.00
ATP	551.1	4.06	- 4.60

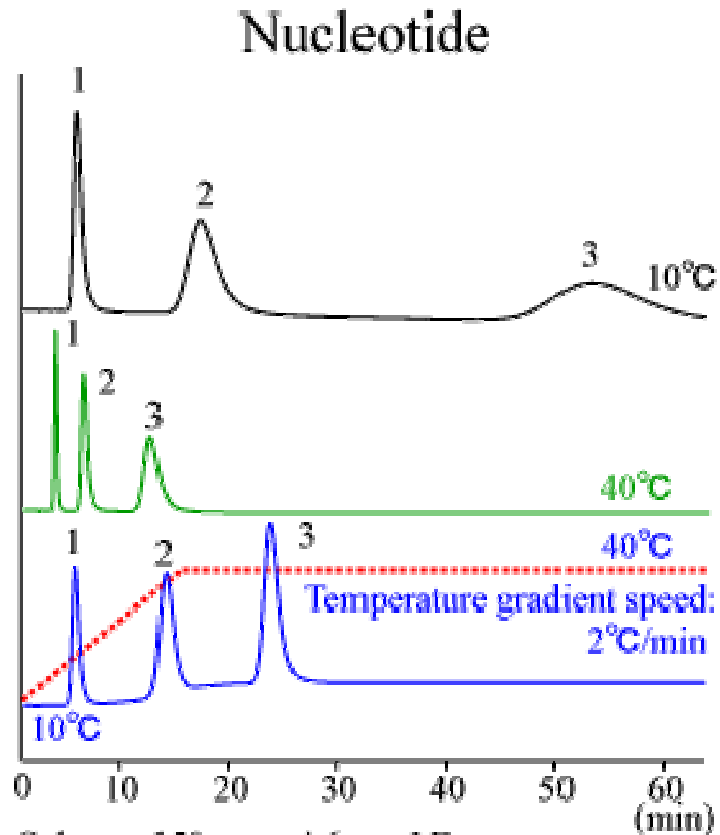
hydrophile

* P ; Partition coefficient of system.

n-octanol/water

Kikuchi, Okano

Application ~ Separation of Nucleotides ~



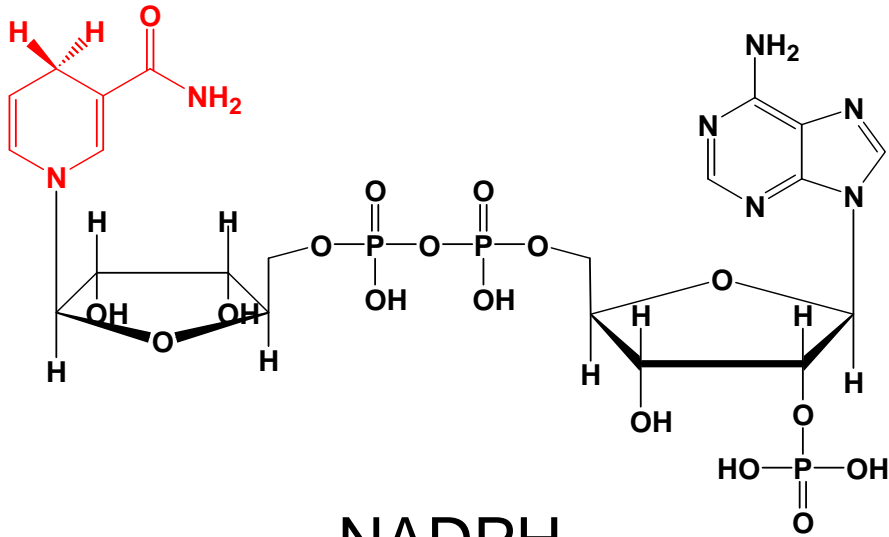
Column: 150mm x 4.6mm I.D.
Mobile phase: 66.7mM phosphate buffer (pH7.0)
Flow rate: 1.0ml/min
Detection: UV 254nm
Sample: 1) AMP
 2) ADP
 3) ATP

The number of phosphate groups for respective samples affect the retention time.

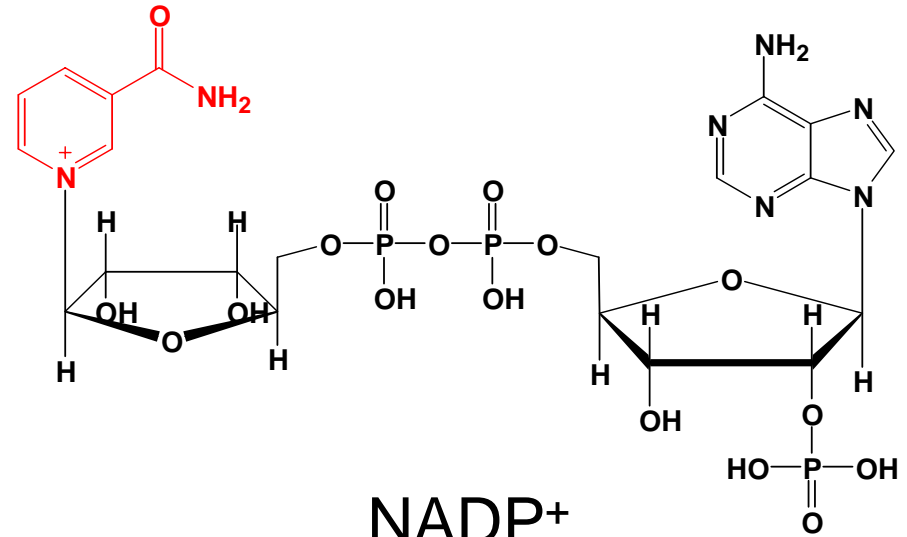
The surface of the grafted silica show charge properties at lower temperature and as temperature increase, transform to non-charge surface properties.

Lower the temperature, the longer the retention time.

Structure of NADP⁺ and NADPH

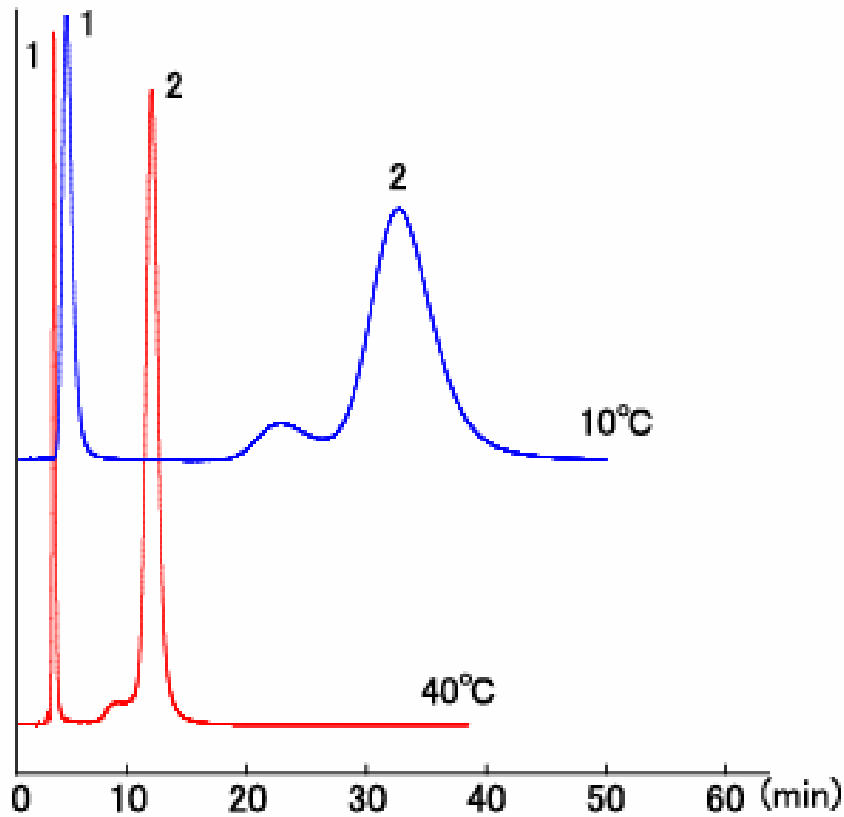


NADPH



NADP⁺

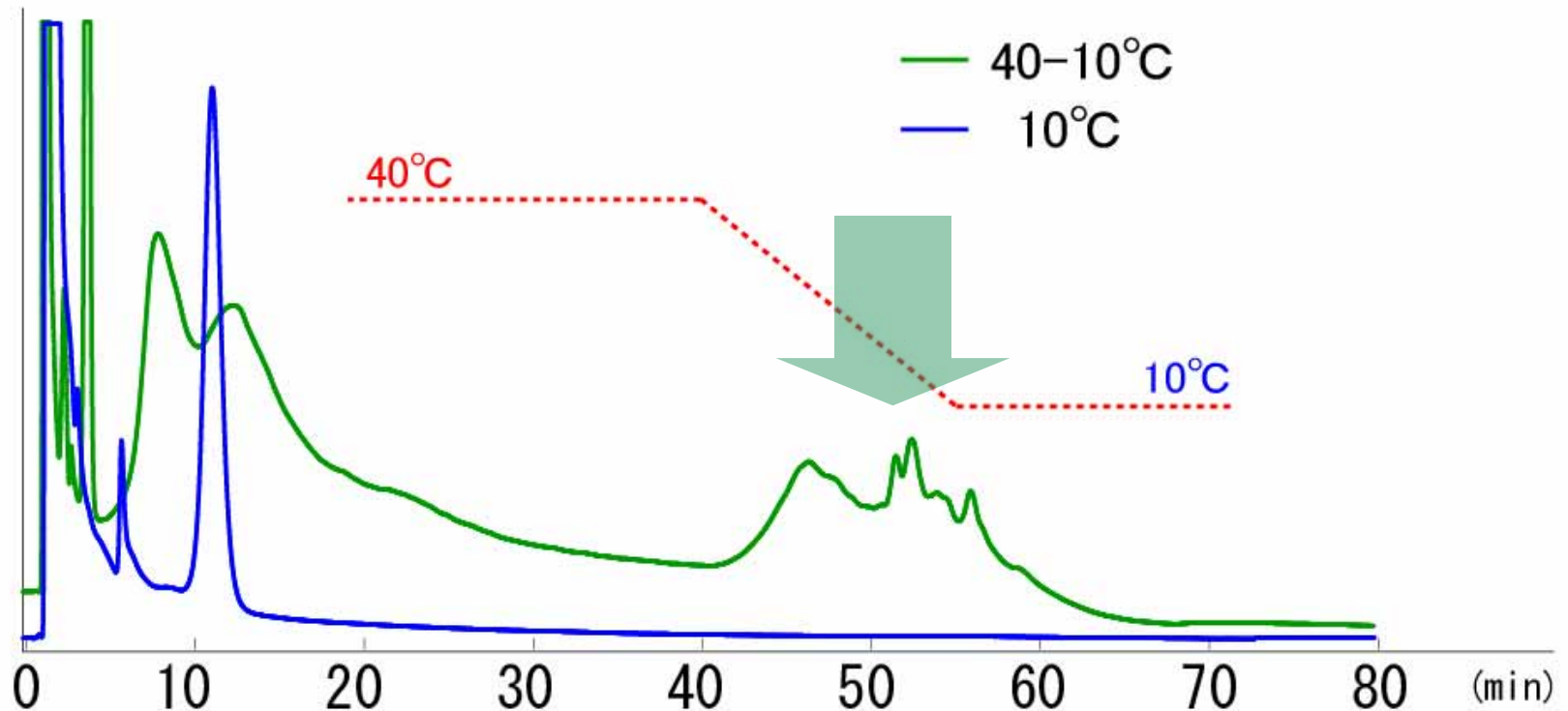
Application ~ Separation of NADP⁺ and NADPH ~



Column: 4.6 mm I.D. x 150 mm
Mobile phase: 66.7 mM phosphate buffer (pH7.0)
Flow rate: 1.0 ml/min
Detection: UV 260 nm
Sample: 1) NADP⁺
 2) NADPH

- NADP⁺ and NADPH are recognizable.
- Retention time are controlled by temperature.
- The gradient elution of high salt concentration is not required for separation.

blood serum



Column length: Aqua Way Cation 150 mm x 4.6 mm I.D.

Eluent: 33mM Phosphate buffer (pH7.0)

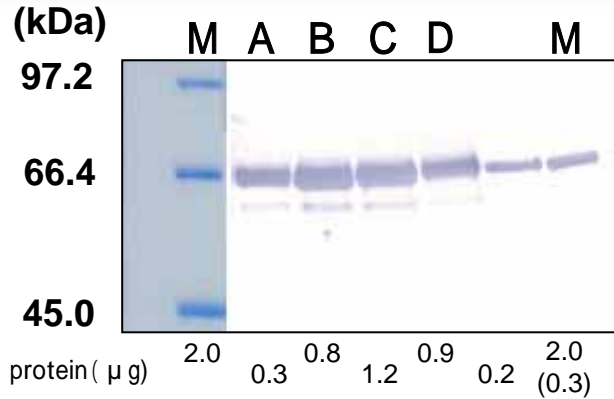
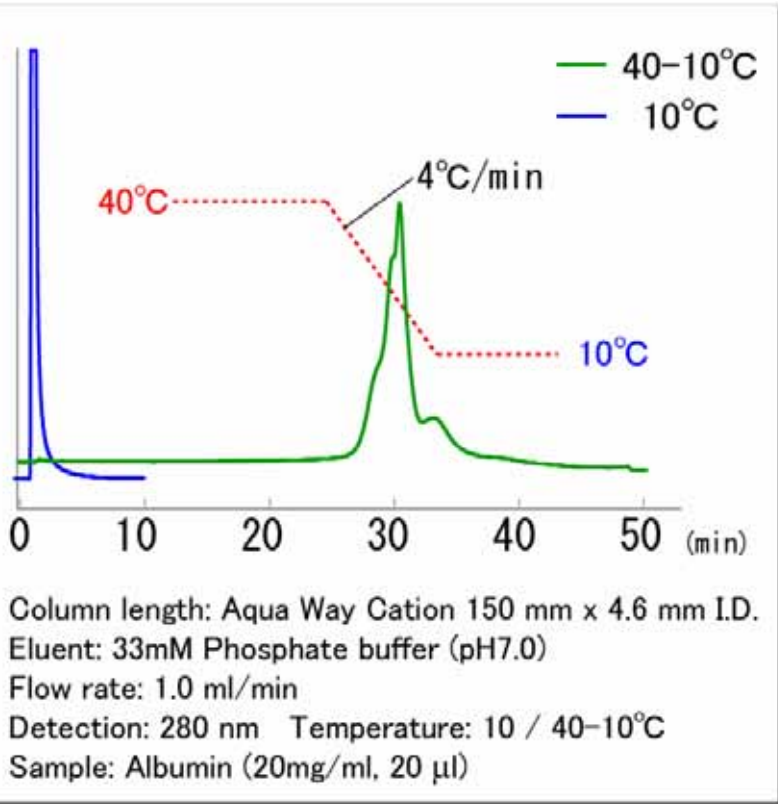
Flow rate: 1.0 ml/min

Detection: 280 nm Temperature: 10 / 40-10°C

Sample: Human Serum (20 μ l)

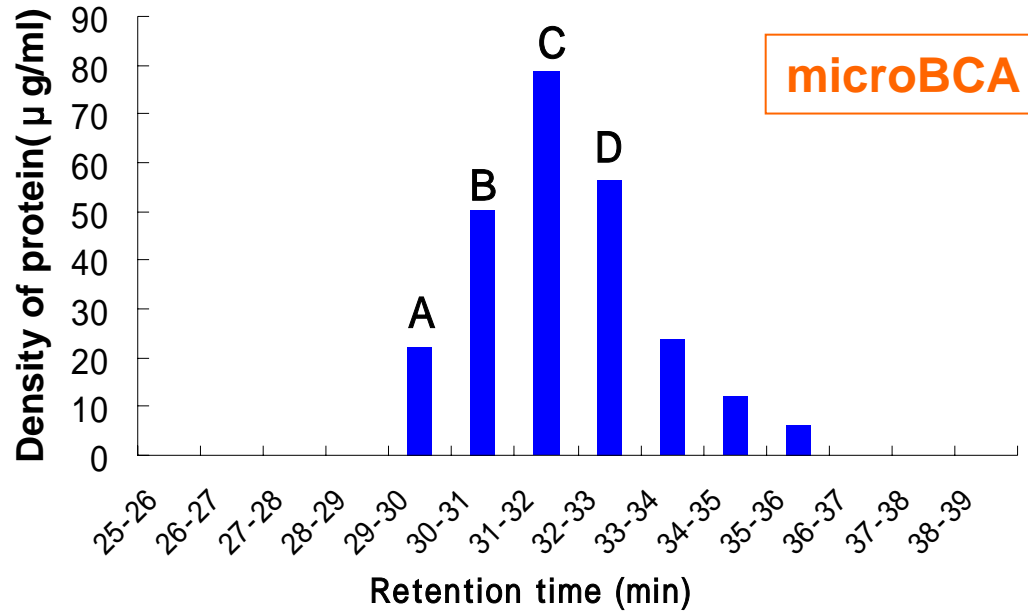
Application ~ Albumin ~

UV chart



Western blotting

= albumin



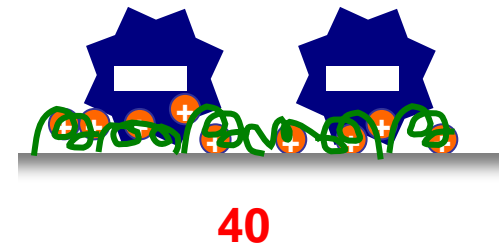
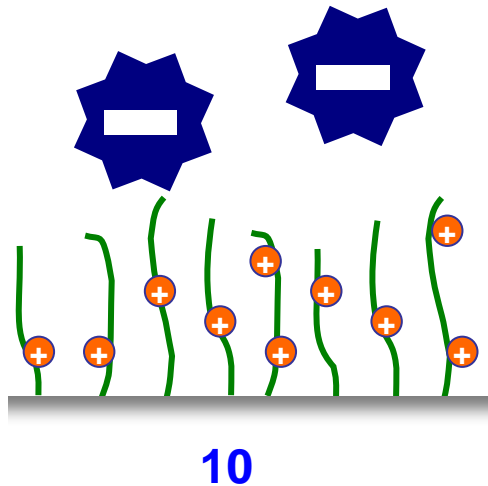
microBCA

Application ~ Albumin ~

electrostatic interaction and shrinkage of polymer have an effect on retaining albumin.

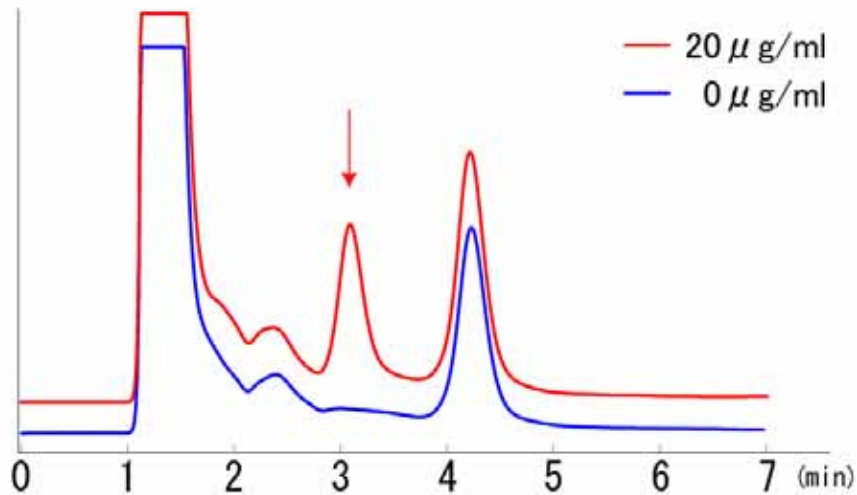


- 40 Shrinkage of polymer allows electrostatic interaction
- 10 Extension of polymer inhibits electrostatic interaction



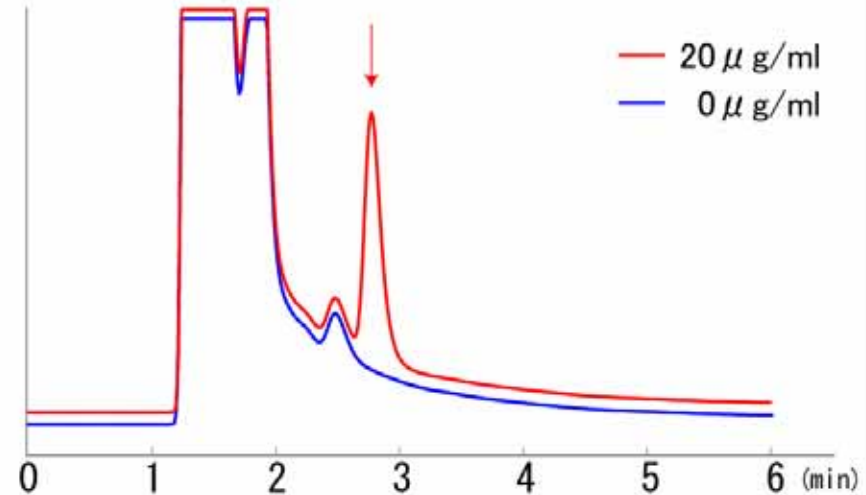
Analysis of Theophylline in Blood Serum

Aqua Way Cation



Column length: Aqua Way Cation 150 mm x 4.6 mm I.D.
Eluent: 66.7 mM Phosphate buffer (pH7.0)
Flow rate: 1.0 ml/min
Detection: 280 nm Temperature: 35°C
Sample: Human Serum + theophylline (0, 20 µg/ml)

Aqua Way Philic



Column length: Aqua Way Philic 150 mm x 4.6 mm I.D.
Eluent: 66.7 mM Phosphate buffer (pH7.0)
Flow rate: 1.0 ml/min
Detection: 280 nm Temperature: 40°C
Sample: Human Serum + theophylline (0, 20 µg/ml)

Summary of *Aqua Way* (1)

Benefit

Aqua Way "Phylic"

No preparation process of removing serum!

Avoid loss of target compounds

No necessity of organic solvent!

- 1. Avoid denaturation of targets**
- 2. Stable back ground**

Aqua Way "Cation"

No gradient elution of high salt concentration needed!

Use only an aqueous mobile solution

No cumbersome reactivation is required!

Avoidance of denaturation of targets

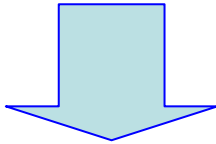
High reproducibility of result

Summary of *Aqua Way* (2)

Advantage

Aqua Way "Phylic"

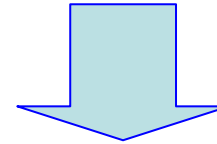
vs
ODS



ODS columns are not competitor to *Aqua Way "Phylic"*. Because "*Phylic*" can separate molecules under conditions which may evoke clogging when ODS columns is used. For example serum must be removed from samples for ODS columns whereas such is not required for "*Phylic*". ODS columns are difficult to separate hydrophobes when only a water is used as a mobile phase.

Aqua Way "Cation"

vs
Ion-exchange column



Ion-exchange columns are not competitor to *Aqua Way "Cation"*. Because "*Cation*" can separate molecules without using gradient elution of high salt concentration. Ion-exchange columns need cumbersome process of reactivation by washing with water, but "*Cation*" is not necessary to do it.

Application Map of *Aqua Way*

Sample Condition

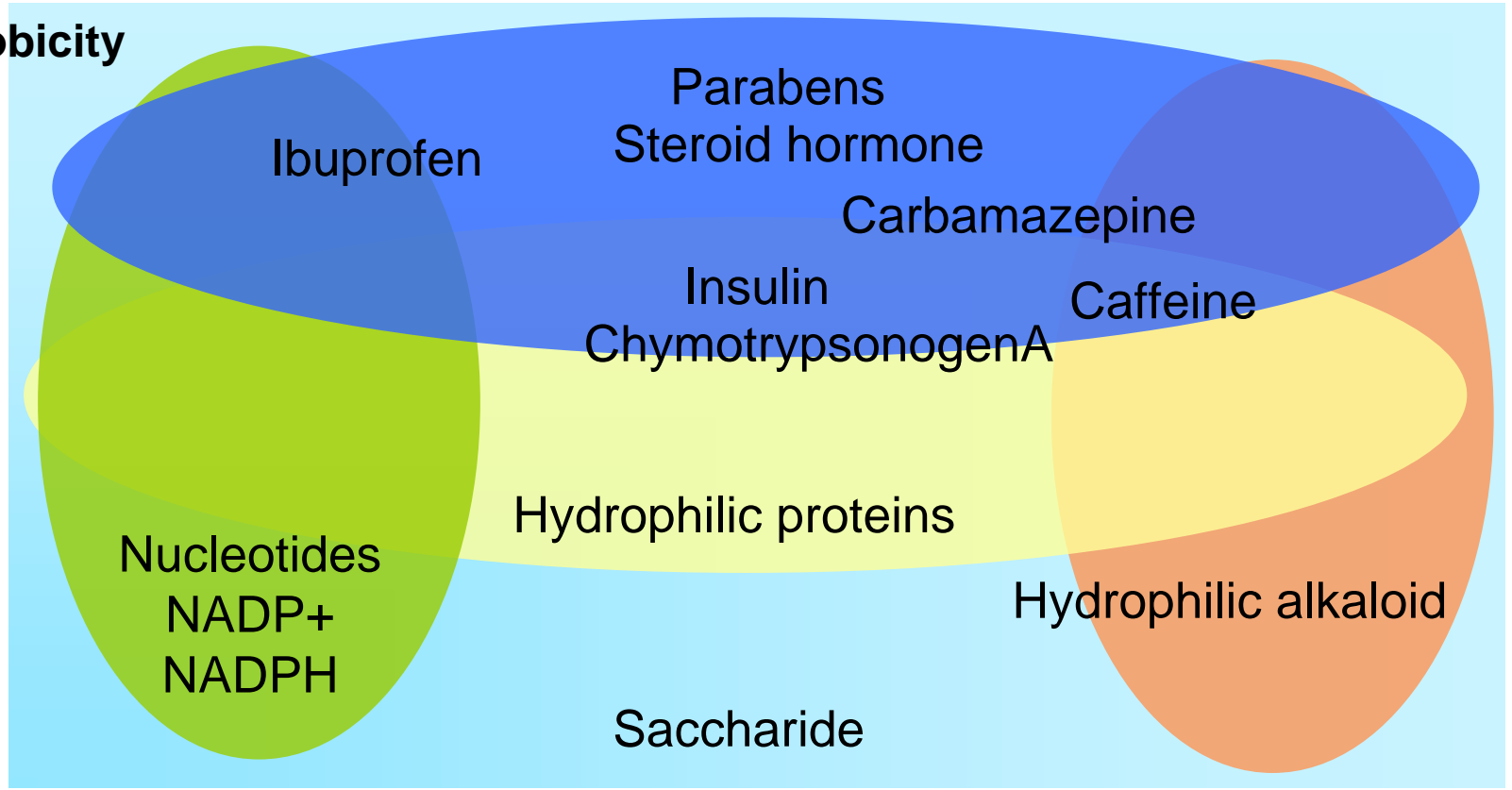
electric charge

- ← neutral → +

Hydrophobicity

hydrophobicity

Hydrophilicity



Philic **Phobic** **Cation** **Anion**

Product Configurations of *Aqua Way* series

CellSeed www.cellseed.com

New! Aqua Way[®] "Philic"

CellSeed's magic: Reverse-phase HPLC column controlled by temperature

100% aqueous solution! Control retention time/selectivity by column temperature.

- Temperature-responsive polymer (PIPAAm) is synthesized and grafted to anisotropic silica.
- The surfaces of the grafted silica show *hydrophobic properties at higher temperature and, as temperature decreases, transform to hydrophilic surface properties.*
- Separation selectivity and retention are controlled by controlling column temperature. Higher the temperature, the longer the retention time.

Hydrophobic surface (10°C) → Molecule attaching → Hydrophilic surface (10°C) → Molecule releasing →

PIPAAm
Target molecules
Silica beads

Temperature ↑
Polarities ↓

Small molecules

Chromatogram showing peaks at 10°C and 40°C. Column: 150mm x 4.6mm ID. Mobile phase: Water. Flow rate: 1.0ml/min. Temperature: 10°C and 40°C. Sample: 1. Polystyrene, 2. Hydrocortisone, 3. Hydrocortisone acetate.

Experience the gradient elution-like effect by mere control of temperature!

40°C 10°C

Adsorption with high hydrophobicity
Adsorption with high hydrophilicity

Protein

Chromatogram showing peaks at 10°C and 40°C. Column: 150mm x 4.6mm ID. Mobile phase: 10% MeCN phosphate buffer pH 6.5. Flow rate: 1.0ml/min. Temperature: 10°C and 40°C. Sample: 1. BSA, 2. Lysozyme.

http://www.cellseed.com/English/english_aquawayphilic.html

Pre-packed column	type	<i>Philic, Cation</i>
	length	5, 15, 25 cm
	ID	4.6 mm
Bulk Beads (particle size 5 μ m, pore size 120)		5, 10g *1
Trial set (Pre-packed column)		Philic+Cation (5cm)

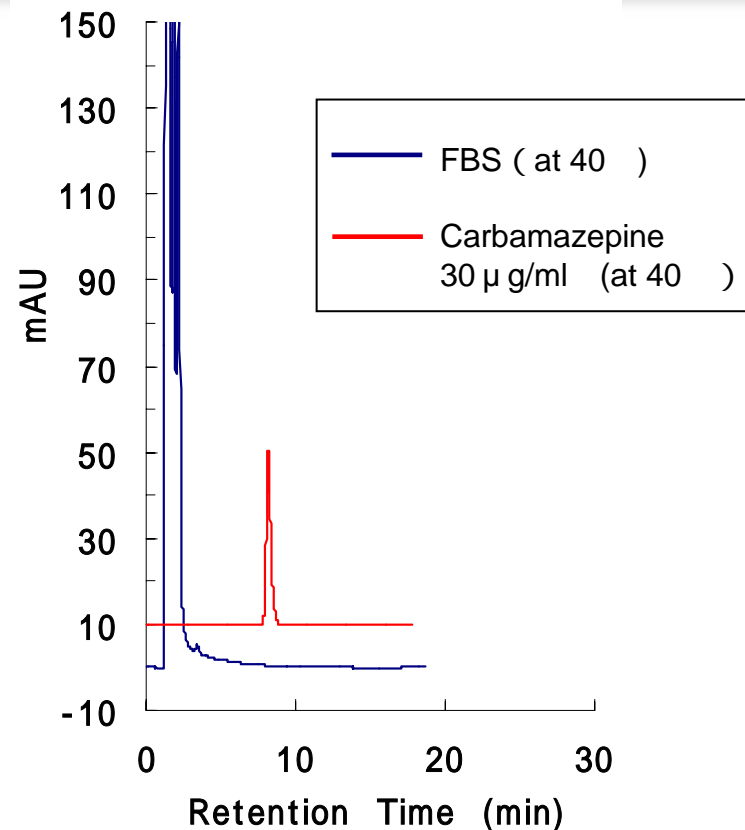
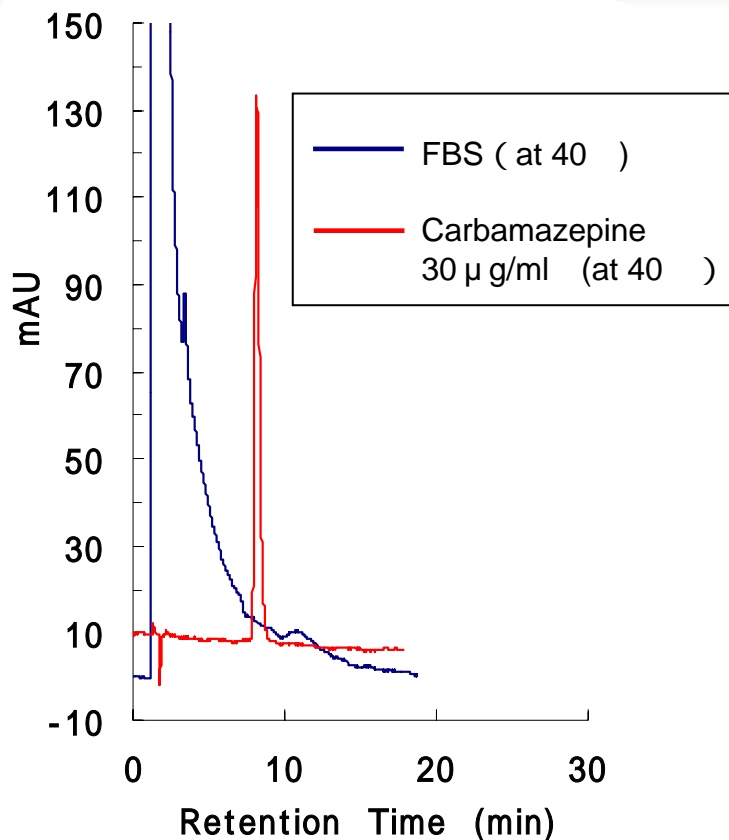
*1 The beads can be customized upon request



Aqua Way gradienter[®]

- Column oven that is best suited to Aqua way column
- Columns manufactured by Tosoh or Waters can be also used.

(Reference) Analysis of Carbamazepine in blood serum in case of using organic solvent as a mobile phase



Column: "Philoc" 150mm × 4.6mm I.D.
Mobile phase:
180mM Ammonium Acetate:CH₃CN (85:15)
Flow rate: 1.0ml/min
Detection: UV 220nm

Column: "Philoc" 150mm × 4.6mm I.D.
Mobile phase:
180mM Ammonium Acetate:CH₃CN (85:15)
Flow rate: 1.0ml/min
Detection: UV 254nm

Thank you for your kind attention.