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Salting effects in reversed mobile phases on chiral separation of benzonaphthazepine stereoisomers

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ABSTRACT

The salting-in effect of $NaClO_4$ in reversed mobile phases *versus* the salting-out effect of NaCl on the separation of benzonaphthazepine (BNA) stereoisomers was evaluated. The critical role of $NaClO_4$ in the mobile phase was examined. A hypothesis was proposed on the salting-in effect compared with the effect of ion-pair reagents. A mechanism of separation of BNA stereoisomers was suggested based on molecular modeling of BNA enantiomers and diastereomers.

INTRODUCTION

There is much research and development work reported on the salting effects on protein purification [1–3], ion-exchange chromatography [4–6], thin-layer chromatography [7–9], and headspace gas chromatography [10]. For instance, Roettger *et al.* [1] reported that aqueous mobile phases with high salt concentrations are often used to adsorb proteins onto mildly hydrophobic supports. Jandera *et al.* [5] described the separation of saccharides, aldehydes, ketones, ethers, and alcohols by salting-out and solubilization chromatography on ion-exchange columns. Cserhati *et al.* [8] studied salting-in and salting-out effects on reversed-phase thin-layer chromatography of 15 dansylated amino acid derivatives. Another interesting paper, by Nagai [10], reported the improved sensitivity of headspace gas chromatography by salting effects and its application to residual solvent analysis of medicines.

Recently Ishikawa and Shibata [11] reported that a reversed mobile phase system similar to the one described in this paper attained good chiral separation on propranolol, and $NaClO_4$ and other salts were added as anionic chaotropes in the mobile phase. The paper also reported that the use of conventional ion-pair reagents such as undecanesulfonate was not successful in an attempt to replace $NaClO_4$ salt. However, the authors explained the separation mechanism in terms of chaotropicity and tried to generalize the critical role of $NaClO_4$ as that of conventional ion-pair reagents.

This paper describes the use of $NaClO_4$ as salting-in agent to promote chiral selectivity of a oligocellulose tris(3,5-dimethylphenylcarbamate) stationary phase on benzonaphthazepine (BNA) stereoisomers. The behavior of analytes in terms of interactions with two different kinds of salts in the mobile phase and chiral stationary phase was examined. A hypothesis of the separation mechanism different from conventional ion-pair chromatography is proposed at the molecular level with the assistance of CAche a three-dimensional molecular modeling tool.

EXPERIMENTAL

Reagents

The following reagents were used: deionized water (MilliQ Reagent Water System, Milli-

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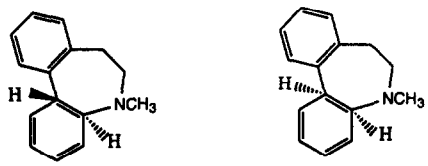

Fig. 1. Molecular structures of BNA stereoisomers.

pore), acetonitrile (ACN) (Optima, Fisher Chemical), sodium chloride (Certified ACS, Fisher Chemical), sodium perchlorate (HPLC grade, Fisher Chemical) and potassium dihydrogen phosphate (Certified ACS, Fisher Chemical).

HPLC system and chromatographic conditions

The chromatographic equipment consisted of a Waters Module I networked into a Waters 840 ExpertEase Station with a Chiralcel OD-R 25 cm × 4.6 mm I.D. column (10 μm particle size, manufactured by Daicel Chemical Industries) and a UV detector (210 nm). The flow-rate was 1.0 ml/min and temperature 40°C. The mobile phase was (0.25 to 0.75 *M* $NaClO_4$–0.02 *M* KH_2PO_4, pH 4.7)–acetonitrile (60:40). The samples were dissolved in acetonitrile–water (50:50).

RESULTS AND DISCUSSION

The molecular structures of the benzonaphthazepine (BNA) stereoisomers are shown in Fig. 1. BNA has two chiral centers, and therefore, two pairs of enantiomers and four stereoisomers, *i.e.*, (−)*trans*, (+)*trans*, (−)*cis*, and (+)*cis* isomers.

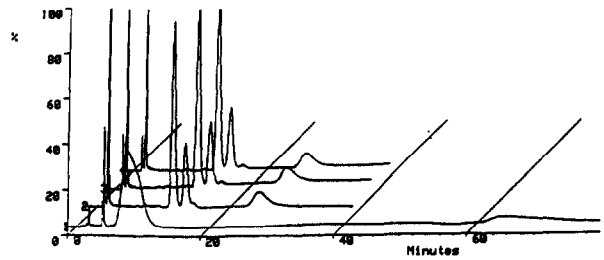

Fig. 2. The effect of the concentration of $NaClO_4$ on the separation of BNA. Chromatograms: 1 = 0 *M* $NaClO_4$; 2 = 0.25 *M* $NaClO_4$; 3 = 0.50 *M* $NaClO_4$; 4 = 0.75 *M* $NaClO_4$.

$NaClO_4$ salt effects on separation

The critical role of $NaClO_4$ on the separation of four stereoisomers of BNA is shown in Table I.

Fig. 2 shows the overlay of four chromatograms Nos. 1–4, corresponding to the concentrations of $NaClO_4$ in the mobile phase from 0 to 0.75 *M*. The amazing separating power of $NaClO_4$ was clearly demonstrated and it was shown that the separation was concentration dependent. Without $NaClO_4$, the last peak eluted at 65 min and the critical pair of (+)*trans* and (−)*cis* isomers of BNA coeluted. As the concentration of $NaClO_4$ increased from 0.25 to 0.50 and 0.75 *M* (in terms of aqueous constituent), the run time did not increase, while the resolution factor increased from 0.8 to 1.0 and 1.4 correspondingly, which was further optimized to 1.6 in later experiments.

TABLE I

THE $NaClO_4$ EFFECT ON SEPARATION

Mobile phase: 0–0.75 *M* $NaClO_4$, pH 4.7–ACN (60:40), 1 ml/min.

$NaClO_4$ (*M*)	t_R (min)				R_s (−)*cis* and (+)*trans*
	(−)*trans*	(−)*cis*	(+)*trans*	(+)*cis*	
(1) 0.00					no separation
(2) 0.25	10.9	13.0	14.4	24.4	0.8
(3) 0.50	12.0	13.5	15.0	25.0	1.0
(4) 0.75	12.7	14.1	16.0	25.2	1.4

TABLE II

SALTING-IN EFFECT OF $NaClO_4$ ON $CHCl_3$

	Into water	Into water–ACN (1:1)
Add $CHCl_3$	$CHCl_3$ lower phase	$CHCl_3$ rises to top
Add $NaClO_4$	$CHCl_3$ dissolves in water	$CHCl_3$ dissolves, one phase
Add NaCl	$CHCl_3$ lower phase	$CHCl_3$ mixed in ACN, upper phase, water lower phase

Research has indicated that addition of certain kinds of salt into a hydrocarbon solute–water solution increases the hydrophobic effect by electrostriction of water that decreases the solubility of hydrocarbons and thus promotes their association [12]. These kinds of salts are known as salting-out agents. On the other hand, addition of other salts in a hydrocarbon solute–water solution will decrease the hydrophobic effect, by breaking up the electrostriction of water that increases the solubility of hydrocarbons and thus demotes their association. The latter are referred to as salting-in agents. The salting effects on non-electrolytes can be measured by the salting coefficient [13]. In a diluted solution the linear term usually suffices:

$$\log S^0/S = K_s C_s \quad (1)$$

where S^0 is the solubility of solute in pure solvent, S the solubility of solute in salt–solvent system, K_s the salt–non-electrolyte interaction (or salting) parameter, and C_s the molar concentration of the solute.

The experiments given in Table II are designed to see salting-in effects of $NaClO_4$ salt on a hydrocarbon–water solution in a qualitative way at a higher concentration (compared to the diluted concentration of the salt in the mobile phase).

As we noticed, the addition of $NaClO_4$ in a $CHCl_3$–water or a water–ACN solution turns a heterogeneous solution into a homogenous one. This is an indication of the salting-in effect of $NaClO_4$ on a $CHCl_3$–water or a water–ACN system, promoting solubility of $CHCl_3$ in the system. However, NaCl does not have the same salting-in effect as $NaClO_4$, leaving $CHCl_3$ in the heterogeneous solution. Referring to eqn. 1, we would conclude that the salting parameter K_s of $NaClO_4$ in the system is negative since $S^0 < S$. To see if there is the same kind of salting effect of $NaClO_4$ in a BNA–water–ACN system, the experiment described in Table III was carried out.

The results show that although BNA does not dissolve in water alone in the presence of either of the salts (this is probably because of the limited solubility of BNA in water), $NaClO_4$ does help BNA get into the solution in a water–ACN system. It was further observed, that addition of NaCl to a BNA–water–ACN solution first will cause the organic layer to phase out from a homogeneous solution, and that sub-

TABLE III

SALTING-IN EFFECT OF $NaClO_4$ ON BNA

	Into water	Into water–ACN (1:1)
Add BNA	BNA is suspended	BNA dissolves
Add more BNA	–	Solution is turbid
Add $NaClO_4$	BNA is suspended	Solution is clear
Add NaCl	BNA is suspended	Solution separates into two phases

sequent addition of $NaClO_4$ will bring the two phases together again into a homogeneous solution. This is to say that it is the type of the salt, not the quantity of the salt, that makes BNA soluble in the system, and obviously $NaClO_4$ salts-in BNA into the solution, while NaCl salts-out BNA from the solution.

The different salting effects of $NaClO_4$ and NaCl on the separation of BNA in the chromatographic system were further investigated and the results are shown in Table IV.

Figs. 3–5 show the chromatograms generated from mobile phases with two different kinds of salts under identical chromatographic conditions

Under almost identical chromatographic conditions, $NaClO_4$ plays a distinctive role in the separation of the four isomers with satisfactory resolution, but NaCl can not resolve the (−)*trans* and (−)*cis* isomers. Further attempts were made to separate the (−)*trans* and (−)*cis* isomers in NaCl mobile phase: variables included concentration of the salt, percentage of ACN, flow-rate, etc. None of them produced acceptable results. In one of the attempts we decreased the percentage ACN in the mobile phase in order to separate the (−)*trans* from the (−)*cis* isomer through the increased retention of BNA in the stationary phase. The result is shown in Fig. 5.

The (−)*trans* isomer still coelutes with the (−)*cis* isomer and the resolution between other peaks is also lost because of the 10% decrease of ACN.

Obviously, the properties of different salts give significantly different chromatographic effects. The salting-in effect of $NaClO_4$ makes BNA stereoisomers more soluble in the mobile

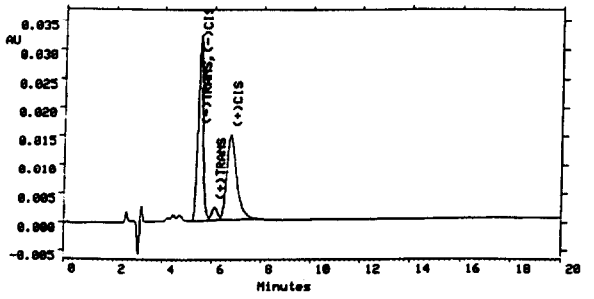

Fig. 3. The effect of the NaCl mobile phase on the separation of BNA isomers.

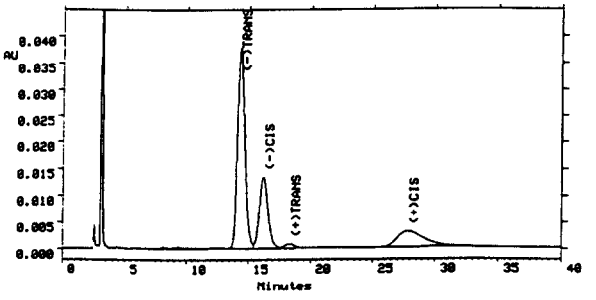

Fig. 4. The effect of $NaClO_4$ on the separation of BNA isomers.

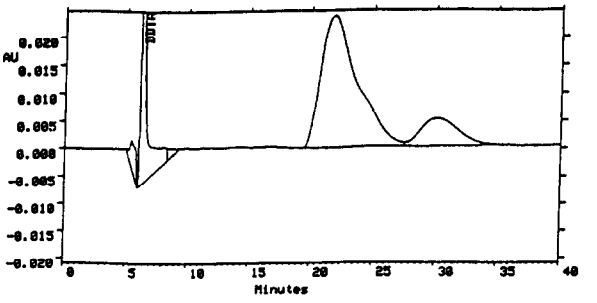

Fig. 5. Chromatogram after a decrease of %ACN in the NaCl mobile phase. Mobile phase: 0.75 *M* NaCl, pH 4.7–ACN (70:30), 0.5 ml/min.

TABLE IV

COMPARISON OF THE EFFECTS OF $NaClO_4$ AND NaCl IN MOBILE PHASE

Mobile phase: (0.75 *M* $NaClO_4$ or NaCl, pH 4.7)–ACN (60:40), 1 ml/min.

Salt	t_R (min)				R_s (−)*cis* and (+)*trans*
	(−)*trans*	(−)*cis*	(+)*trans*	(+)*cis*	
NaCl	5.6	5.6	6.1	6.8	no separation
$NaClO_4$	14.5	16.6	18.4	27.8	1.5

phase so that a chiral stationary phase can selectively retain four stereoisomers in an optimal condition. The salting-out effect of NaCl promotes hydrophobic association of stereoisomers themselves more than the retention of stereoisomers to the chiral stationary phase so that the chiral stationary phase cannot discriminate between the (−)*trans* and (−)*cis* isomers.

Based on this observation, it is proposed that the salting-in agent in this chiral system works in quite a different way from what a conventional ion-pair reagent does in a non-chiral reversed-phase system. In a non-chiral reversed-phase system, ionic solutes are paired with organic ion-pair reagents to increase the hydrophobic interaction in the mobile phase and, therefore, the retention of the solutes in the stationary phase (by increasing the affinity of solutes to the stationary phase). In this chiral system, an inorganic salt is introduced into the mobile phase to increase the hydrophilic interaction of the stereoisomers (by increasing the solubility of the solute in the mobile phase) and to provide optimal conditions for the chiral stationary phase to work with. In a sense, the salting-in agent works more with the mobile phase, while the ion-pair reagent works more with the stationary phase. However, the salting-in agent cannot be solely responsible for the separation. The synergy is only achieved by the combination of an appropriate eluting strength of composition of the organic and aqueous phases, a proper concentration of the salting-in agent, the "magic" pH effect, and the chiral recognition of the stationary phase.

The chemistry of the chiral stationary phase Chiralcel OD-R was first revealed as the oligomer cellulose tris(3,5-dimethylphenyl carbamate) on a 10-μm silica gel [14], however, there exist no exact data about its molecular mass. The mechanism of chiral recognition of Chiralcel OD-R was proposed as a stacking model [15] in general, since the column shows non-conventional sensitivity to non-planarity, and steric hindrance. From the chemistry of Chiralcel OD-R, it is not difficult to locate many spots in the stationary phase where stacking can easily take place between aromatic rings and between methyl groups and aromatic rings.

Proposed separation mechanism based on molecular modeling

Using computer and conventional molecular modeling tools, we are able to see something at the molecular level.

The computer software for molecular modeling used in this study is the three-dimensional CAChe version 3.0.4. It gives the lowest energy conformation of the molecule. Fig. 6 shows two pairs of enantiomers of BNA in the lowest energy conformations.

The upper pair are the *trans* enantiomers, and the lower pair are the *cis* enantiomers. Unfortunately, in two dimensions we can not appreciate the beauty of the magnificent stereoscopic effects on CAChe's "three-dimensional" CRT screen. The conformations of BNA shown above are in their calculated lowest energy according to the quantum mechanics; Fig. 7 illustrates the energy levels *versus* different conformations of the BNA *cis* isomer.

The position of the filled circle indicates the lowest energy point (8.07 kcal/mol) among many other conformations. The two pairs of BNA enantiomers in Fig. 6 are all at their lowest energy conformations.

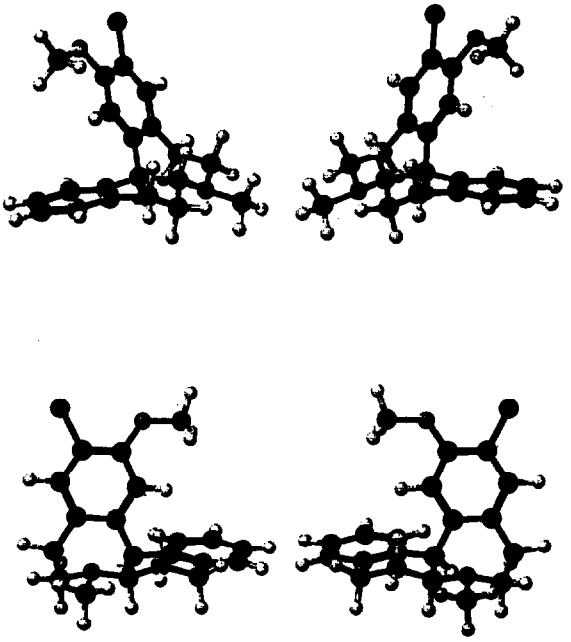

Fig. 6. BNA molecular structures.

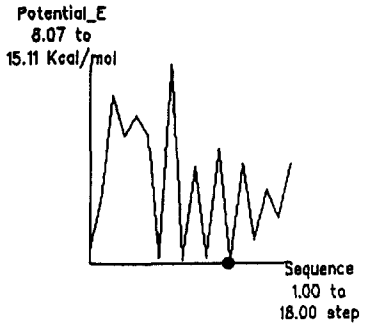

Fig. 7. The energy levels associated with the conformations of *cis*-BNA.

From Fig. 4, the elution order of the four BNA isomers is (−)*trans*, (−)*cis*, (+)*trans*, then (+)*cis*. Note that the enantiomers are segregated by their diastereomers. This seems inexplicable in that enantiomers having exactly the same chemical and physical properties do not follow each other in elution order.

By studying the molecules at the most stable conformation, it is found that because the two diastereomer molecules possess so-called "matching structures", the *cis* isomer can be stacked perfectly on the top of the *trans* isomer molecule without significant increase in physical space compared to a single molecule, just as placing one stackable chair on the top of another does not occupy a two-chair space. Two enantiomer molecules do not possess "matching structures" and cannot even be placed close with all efforts.

When a *trans* molecule encounters a *cis* molecule, they are attracted to each other by a hydrophobic force and matching conformations, and are then stabilized by sharing π electrons between two pairs of benzene rings. Their conformations fit each other so well that the increase in the size of the two stacked molecules is hardly noticeable, so they move in pairs freely in and out of the mobile phase until they get trapped by the stacking mechanism in a cavity of the proper size of the chiral stationary phase, where they get further discriminated by their subtle conformational differences.

In contrast, the enantiomer molecules do not possess the properties of matching conformations, and therefore they are moving around individually, get picked up at random by the chiral stationary phase, and are then separated widely. That gives the possible explanation why an enantiomer does not immediately follow its mirror image but a diastereomer does in this system.

Notice also from the chromatogram in Fig. 4 that the *trans* isomer elutes first, followed by the *cis* isomer. If we study their subtle conformational differences further, it is not difficult to see that the angle between two benzene rings in the *trans* isomer (60.659°) is about 6° less than that in the *cis* isomer (66.639°). So the *cis* isomer has more steric hindrance than does the *trans* isomer, and *cis* isomer's mobility in chiral stationary phase is more likely restricted and therefore more retained in the stationary phase.

CONCLUSIONS

Baseline resolution of BNA stereoisomers was achieved using a salting-in agent in a reversed mobile phase. The study of the molecular structures of BNA with computer molecular modeling tools suggests a chiral separation model for BNA at the molecular level. The proposed hypothesis of the separation mechanism based on the knowledge of the structures is in good agreement with the observed experimental results.

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REFERENCES

1 B.F. Roettger, J.A. Myers, M.R. Ladisch and F.E. Regnier, *Biotechnol. Prog.*, 5 (1989) 79–88.
2 C.J.O.R. Morris, *Trends Biochem. Sci.*, 1 (1976) N207–N208.
3 M.K. Gil'manov and T.K. Kozhanov, *Tr. Inst. Bot. Akad. Nauk Kaz. SSR*, 29 (1971) 216–226.
4 P. Jandera and J. Churáček, *J. Chromatogr.*, 98 (1974) 1–54.

5 P. Jandera and J. Churáček, *J. Chromatogr.*, 98 (1974) 55–104.
6 P. Jandera and J. Churáček, *J. Chromatogr.*, 86 (1973) 423–449.
7 R.L. Munier, *Qual. Plant. Mater. Veg.*, 16 (1968) 12–34.
8 T. Cserhat and Z. Illes, *J. Liq. Chromatogr.*, 14 (1991) 1495–1510.
9 G. Vučković, D. Miljević, T.J. Janjić and M.B. Ćelap, *J. Chromatogr.*, 609 (1992) 427–431.
10 Y. Nagai, *Bunseki Kagaku*, 41 (1992) 491–495.
11 A. Ishikawa and Shibata, *J. Liq. Chromatogr.*, 16 (1993) 859–878.
12 R. Breslow, *J. Am. Chem. Soc.*, 113 (1991) 4340.
13 J.E. Gordon, *The Organic Chemistry of Electrolyte Solutions*, Wiley, NY, 1975.
14 Y. Okamoto, M. Kawashima and K. Hatada, *J. Chromatogr.*, 363 (1986) 173.
15 K. Tachibana, *Theoretical Mechanism of Daicel Cellulose-Based Chiral Stationary Phase HPLC Columns, Chiral Technologies, Seminar at Schering-Plough, Bloomfield, NJ, July 1, 1992.*