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# SEPARATION OF HOMOLOGOUS AND ISOMERIC ALKALOIDS RELATED TO NICOTINE ON A β-CYCLODEXTRIN-BONDED PHASE

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## SUMMARY

Nicotine and thirteen structurally related alkaloids were separated with a β-cyclodextrin-bonded phase column. The retention of homologous compounds tends to increase as the size of the homolog increases. Structural isomers in which the pyridine nitrogen is in the 4-position are always retained the longest. Both retention and efficiency are very pH dependent. Conversely, selectivity does not change significantly with pH.

## INTRODUCTION

The separation and quantification of nicotine (I) and related alkaloids are necessary in a number of important areas. These include studies involving structure–reactivity relationships[1–4], tobacco alkaloid metabolism and biosynthesis[5–7] and plant breeding control[8]. A variety of separation schemes have been proposed for a relatively small number of nicotine alkaloids, particularly nicotine, cotinine and some of their metabolites. The earliest reported methods involved thin-layer and gas chromatography (GC)[9–15]. More recently, solvent extraction followed by GC–mass spectrometry and high-performance liquid chromatographic (HPLC) methods have been shown to be sensitive and efficient[5,8,16–23]. The HPLC-based separations utilized either reversed-phase or ion-exchange packings. In most studies only two to four compounds needed to be resolved. The two most complex separations reported involved a mixture of eight natural tobacco alkaloids[22] and a mixture of nicotine and twelve metabolites[23].

Stable cyclodextrin-bonded phases have been shown to be effective LC stationary phases for the separation of a variety of enantiomers, diastereomers, structural isomers and routine compounds[24–30]. β-Cyclodextrin (β-CD) is known to form tight inclusion complexes with many compounds that contain two to four rings[31]. Therefore, it is reasonable to assume that β-CD-bonded phases might be able to discriminate between such structurally similar alkaloids as examined in this study (all of which contain at least two ring moieties).

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We have recently observed the enantiomeric resolution of a number of racemic nicotine analogs using HPLC with β-CD-bonded phases[32]. While this study focused exclusively on chiral recognition and enantiomeric resolution, and while mixtures of the nineteen analogs and nicotine were not examined, the values of the observed capacity factors, $k'$, suggest that the separation of these compounds from each other is possible[32]. In a second study, we examined the reversed-phase LC separation of twelve tobacco alkaloids and metabolites that differ considerably in the functional groups present using a β-CD-bonded phase[33]. Specific attention was placed on developing a basic equilibrium-driven model to explain the effect of mobile phase composition and in particular pH on retention and selectivity.

In the work described here, the separation behavior of fifteen nicotine analogs was analyzed. Special attention was devoted to the separation of structural isomers and homologous compounds, as these types of analytes are known to be difficult to separate by conventional reversed-phase LC. Therefore, many of the comparisons made here are between compounds that differ either solely in the location of the nitrogen atom or by a single methylene unit. No separation data or techniques have been described previously for over half of the compounds in this study.

## EXPERIMENTAL

### *Methods*

All separations were done at room temperature (21°C) with a Shimadzu LC-6A liquid chromatograph. The compounds were detected at 254 nm with a variable-wavelength detector with an 8-μl flow cell. All samples were dissolved in acetonitrile or methanol (depending on the mobile phase composition) prior to manual injection. Columns (25 × 0.46 cm I.D.) containing β-CD bonded to 5-μm silica were obtained from Advance Separation Technologies (Whippany, NJ, U.S.A.). The void volume of the column was determined by injecting neat methanol. The peak–trough combination caused by the change in refractive index was used as a marker. Flow-rates, solvent compositions and pHs are given in the respective tables and figures.

### *Materials*

HPLC-grade methanol, acetonitrile, triethylamine and water were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Buffers were prepared by making a 1% solution of triethylamine in water and adding glacial acetic acid until the desired pH was obtained. The alkaloids were obtained as follows. Nicotine (I) is commercially available from a variety of suppliers and was used without further purification; XI[34], XII[35] and 2-(2-pyridyl)pyrroline (XV)[35] were prepared using the method of Hu *et al.*[36] or of Seeman *et al.*[37]; II, III[35] and IV[35] were prepared by the sodium cyanoborohydride reduction[36] of XI, XII and XV, respectively; V[35], VI[35] and XIV[38] were prepared by either Clarke–Eschweiler methylation[37,39] or butyllithium–iodomethane alkylation[40] of IV, III and XIII, respectively. Preparations of VII[39,41], VIII[42], IX[43] and XIII[34] were reported previously; X was prepared by both the method of Büchel and Korte[44] or by a combination of methods described previously [34–40]. (*S*)-(−)-Nicotine was optically pure, (*S*)-(−)-VII was partially racemized and II–VI, VIII–X, XIII and XIV were racemic mixtures.

## RESULTS AND DISCUSSION

The structure and separation data for fourteen related alkaloids are given in Tables I and II. The ability of the β-CD-bonded phase to discriminate between structurally similar compounds is evident. It is important to note that many of the solutes examined are racemic mixtures and were not resolved into their constituent enantiomers under the conditions examined here. Some of the peak broadening observed could be due to the racemic nature of these solutes.

Because I–XIV are structurally very closely related, a wide variety of structure–chromatographic property comparisons could be investigated. Some of the most interesting comparisons were examined and evaluated as shown in the tables.

A number of generalizations can be immediately made. For example, the effect of pH on retention and selectivity is seen by comparison of the data in Tables I and II. In all instances, the capacity factor ($k'$) is considerably greater when the pH of the buffer is 7.1 rather than 4.1. This is due to the alkaloids going from their protonated form at pH 4.1 to their unprotonated, free base form at pH 7.1. The hydrophobic core of the β-CD binds the nicotinoid free base more tightly than its protonated form. The more hydrophobic the alkaloid, the greater is the increase in retention caused by the increased pH (*cf.*, N′-benzylnornicotine, for example).

In all cases studied, increasing the size of the molecule increases $k'$. This is true for both major structural variations, *e.g.*, compare N′-benzylnornicotine (VIII) with nicotine (I), or any of the nornicotines (II–IV) with their corresponding nicotines (I, V and VI) or 6-ethylnicotine (VII) with nicotine.

Fig. 1 shows the complete separation of the homologs: 1-methyl-2-(3-pyridyl)-azetidine (IX) (four-membered ring), nicotine (I) (five-membered ring) and N′-methylanabasine (X) (six-membered ring) in 15 min. Interestingly, the relative retention appears to be directly controlled by the size of the saturated ring (Table I). Presumably, the larger ring size produces a tighter inclusion complex, which results in a longer retention time. Examples of other facile homolog separations include nicotine from nornicotine and 2-phenylpyrrolidine from 1-methyl-2-phenylpyrrolidine (Table I). Fig. 2 shows another example of the separations possible using these procedures.

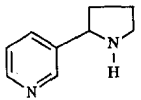
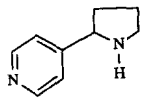
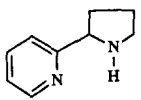
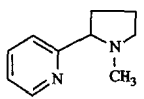
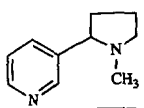
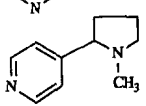
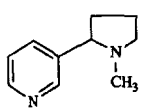
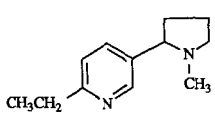
There are some very subtle selectivity changes also. Table III compares the three isomeric nicotines with the three isomeric nornicotines. Nicotine and 4-isonicotine each have $k'$ values *ca.* 0.45 units greater than nornicotine and 4-isonornicotine, respectively, at both pH 4.1 and 7.1. However, 2-isonicotine has a slightly greater $k'$ at pH 4.1 than does 2-isonornicotine but the order is reversed, and significantly so, at pH 7.1. The change in selectivity for the 2-iso series indicates that it is likely that the inclusion complex for the protonated *versus* unprotonated compound is very different, whereas the 3- and 4-iso series are expected to be similar.

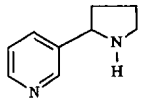
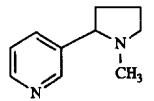
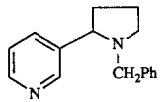
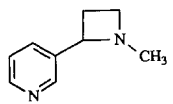
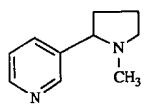
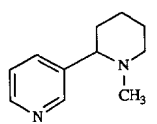
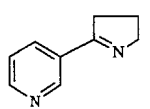
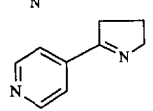
In conclusion, it is clear that the combined effects of size, $pK_a$ and geometry govern the retention of these tobacco alkaloids on β-CD-bonded phases. Increasing the size of an alkaloid by addition of one or more methylene units or methyl groups gives a more hydrophobic compound and a tighter inclusion complex. This always results in an increased relative retention. The $pK_a$ of the solute and the location of the amine functionality (geometry) govern the ability of the alkaloid to hydrogen bond to the cyclodextrin molecule while complexed.

It is well known that hydrogen bonding is an important factor that affects the

TABLE I

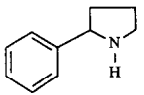
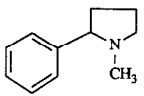
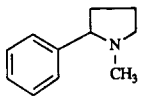
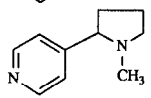
STRUCTURES AND CHROMATOGRAPHIC DATA (pH = 4.1) FOR A SERIES OF ALKALOIDS RELATED TO NICOTINE

| *No.* | *Compound*[a] | | *k'* | α | $R_s$ | *Mobile phase*[b] | *Column*[c] |
|---|---|---|---|---|---|---|---|
| II |  | Nornicotine | 0.279 | | | | |
| | | | | 1.32 | 1.50 | | |
| III |  | 4-Isonornicotine | 0.367 | | | 0:100 | B |
| | | | | 1.40 | 1.66 | | |
| IV |  | 2-Isonornicotine | 0.514 | | | | |
| V |  | 2-Isonicotine | 0.658 | | | | |
| | | | | 1.12 | 1.00 | | |
| I |  | Nicotine | 0.739 | | | 0:100 | B |
| | | | | 1.31 | 183 | | |
| VI |  | 4-Isonicotine | 0.863 | | | | |
| I |  | Nicotine | 0.406 | | | | |
| | | | | 1.91 | 4.80 | 6:95 | B |
| VII |  | 6-Ethylnicotine | 0.775 | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| II |  | Nornicotine | 0.241 | | | | |
| | | | | 1.87 | 3.15 | | |
| I |  | Nicotine | 0.451 | | | 5:95 | B |
| | | | | 2.75 | 7.67 | | |
| VIII |  | N′-Benzylnornicotine | 1.24 | | | | |
| IX |  | 1-Methyl-2-(3-pyridyl)azetidine | 0.513 | | | | |
| | | | | 1.38 | 2.53 | | |
| I |  | Nicotine | 0.706 | | | 0:100 | B |
| | | | | 1.40 | 2.68 | | |
| X |  | N′-Methylanabasine | 0.981 | | | | |
| XI |  | Myosmine | 0.967 | | | | |
| | | | | 1.89 | 3.96 | 5:95 | B |
| XII |  | 4-Isomyosmine | 1.83 | | | | |

*(Continued on p. 174)*

TABLE I *(continued)*

| *No.* | *Compound*[a] | | *k'* | α | $R_s$ | *Mobile phase*[b] | *Column*[c] |
|---|---|---|---|---|---|---|---|
| XIII |  | 2-Phenylpyrrolidine | 0.653 | 1.22 | 3.00 | 5:95 | A |
| XIV |  | 1-Methyl-2-phenylpyrrolidine | 0.792 | | | | |
| XIV |  | 1-Methyl-2-phenylpyrrolidine | 0.875 | 1.49 | 2.37 | 0:100 | A |
| VI |  | 4-Isonicotine | 1.30 | | | | |

[a] (*S*)-(−)-Nicotine (I) was optically pure, (*S*)-(−)-VII was partially racemized and II–VI, VIII–X, XIII and XIV were racemic mixtures.

[b] Numbers refer to the vol.-% of acetonitrile in aqueous triethylammonium acetate (1%) buffered to pH 4.1. The flow-rate was 1 ml/min.

[c] A = one 25-cm β-CD column; B = two 25-cm β-CD columns.

TABLE II

STRUCTURES AND CHROMATOGRAPHIC DATA (pH = 7.1) FOR A SERIES OF ALKALOIDS RELATED TO NICOTINE[a,b]

| *No.* | *Compound* | *k'* | *Mobile phase*[c] |
|---|---|---|---|
| II | Nornicotine | 1.13 | 5:95 |
| III | 4-Isonornicotine | 1.31 | |
| IV | 2-Isonornicotine | 1.81 | |
| V | 2-Isonicotine | 0.87 | 10:90 |
| I | Nicotine | 1.53 | |
| VI | 4-Isonicotine | 1.79 | |
| I | Nicotine | 3.05 | 5:95 |
| VII | 6-Ethylnicotine | 5.24 | |
| II | Nornicotine | 1.42 | 5:95 |
| I | Nicotine | 3.06 | |
| VIII | N'-Benzylnornicotine | 9.79 | |
| IX | 1-Methyl-2-(3-pyridyl)azetidine | 2.24 | 5:95 |
| I | Nicotine | 3.01 | |
| X | N'-Methylanabasine | 3.61 | |
| XI | Myosmine | 3.28 | 5:95 |
| XII | 4-Isomyosmine | 3.35 | |
| XIII | 2-Phenylpyrrolidine | 1.12 | 20:80 |
| XIV | 1-Methyl-2-phenylpyrrolidine | 1.16 | |
| XIV | 1-Methyl-2-phenylpyrrolidine | 1.12 | 20:80 |
| VI | 4-Isonicotine | 1.52 | |

[a] (*S*)-(−)-Nicotine (I) was optically pure, (*S*)-(−)-VII was partially racemized and II–VI, VIII–X, XIII and XIV were racemic mixtures.

[b] Two 25-cm β-cyclodextrin columns were used in sequence.

[c] Numbers refer to the vol.-% of acetonitrile in aqueous triethylammonium acetate (1%) buffered to pH 7.1. The flow-rate was 1.0 ml/min.

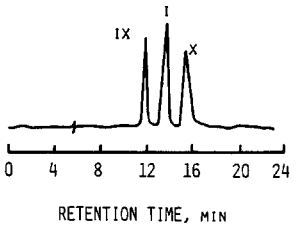



Fig. 1. Chromatogram showing the separation of 1-methyl-2-(3-pyridyl)azetidine (IX), nicotine (I) and N'-methylanabasine (X). Flow-rate, 1.0 ml/min; other separation conditions are given in Table I.

TABLE III

COMPARISON OF RETENTION BEHAVIORS

| *Compound* | *k'* | | | | | |
|---|---|---|---|---|---|---|
| | *pH = 4.1* | | | *pH = 7.1* | | |
| | *Positional substitution* | | | *Positional substitution* | | |
| | *2* | *3* | *4* | *2* | *3* | *4* |
| Nornicotine analog | 0.51 | 0.28 | 0.37 | 1.81 | 1.13 | 1.31 |
| Nicotine analog | 0.66 | 0.74 | 0.86 | 0.87 | 1.53 | 1.79 |
| Difference[a] | 0.15 | 0.45 | 0.49 | −0.94 | 0.40 | 0.48 |

[a] *k'* (N-methylpyrrolidine analog) − *k'* (NH analog).

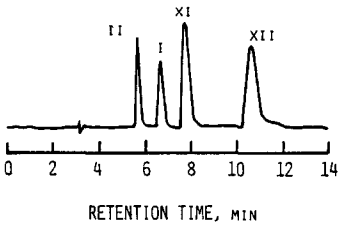



Fig. 2. Chromatogram showing the separation of nornicotine (II), nicotine (I), myosmine (XI) and 4-isomyosmine (XII). Flow-rate, 1.0 ml/min; other separation conditions are given in Table I.

size of a solute's binding constants; it appears that hydrogen bonding is largely responsible for the observed selectivity between isomers in which the position of the nitrogen heteroatom in the pyridine is different[32,33,45]. In these instances, both the size and conformation of the compounds in the uncomplexed state are essentially identical[46,47]. The increased selectivity of β-CD stationary phases over other more traditional reversed-phase packings (*e.g.*, $C_{18}$, $C_8$) is a direct result of the inclusion complexation process, which is considerably more restrictive than partitioning to a relatively featureless *n*-alkane-bonded phase.

In summary, HPLC using β-CD-bonded phases is an excellent and convenient technique for the separation of homologous and isomeric tobacco alkaloids and related compounds. Future work will involve the examination of the mechanisms and structural causes for some of the selectivities found, *e.g.*, in the 2-iso series.

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