## Note

# Analysis of the cinchona alkaloids by high-performance liquid chromatography

## Use as probes of activity towards basic compounds shown by reversed-phase columns

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Although it was recently estimated[1] that over 70% of high-performance liquid chromatographic (HPLC) separations are carried out in the reversed-phase mode with non-polar bonded phase columns, problems still exist when the technique is applied to basic compounds. These compounds can give rise to poor column efficiency and asymmetrical tailing peaks, attributable to interaction with underivatised silanol groups on the surface of the stationary phase[2].

In a recent review[3] it was noted that few comparative studies on the usefulness of various reversed-phase materials for the analysis of basic compounds are available[4]. In this investigation, the principal cinchona alkaloids quinine and quinidine are used as probes of column activity to compare the performance of six different commercial reversed-phase columns.

EXPERIMENTAL

The HPLC system consisted of two pumps (Model 100, Altex, Berkeley, CA, U.S.A.), a variable-wavelength UV detector with a time constant of 0.5 s and an 8-$\mu$l flow cell (Model LC3, Pye Unicam, Cambridge, U.K.), a valve injector equipped with a 5-$\mu$l loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.). The new columns used were $\mu$Bondapak $C_{18}$, 10-$\mu$m, 30 × 0.39 cm I.D. and Novapak $C_{18}$, 4-$\mu$m, 15 × 0.39 cm I.D. (Waters Assoc., Milford, MA, U.S.A.), LiChrosorb RP-8 Select B, 7-$\mu$m, 25 × 0.40 cm I.D. (E. Merck, Darmstadt, F.R.G.), Hypersil ODS, 5-$\mu$m, 25 × 0.46 cm I.D. (Shandon Southern, Runcorn, U.K.), Spherisorb ODS I, 5-$\mu$m and Spherisorb ODS II, 5-$\mu$m, 25 × 0.46 cm I.D. (Phase Separations, Queensferry, U.K.). Column efficiency ($N$) and asymmetry factors ($A_s$) were calculated at half and 10% of peak height using a microcomputer data station (Model 2000, Trivector, Sandy, U.K.) in conjunction with a BASIC program. The void volumes of the columns were calculated[5] using the formula $V_m = \pi d_c^2 \, L \varepsilon_{tot}/4$, where $d_c$ = column bore, $\varepsilon_{tot}$ = 0.75, $L$ = column length. All of the results reported represent the average value of triplicate sample injections.

TABLE I

COLUMN EFFICIENCY AND ASYMMETRY FACTORS FOR TOLUENE MEASURED IN METHANOL–WATER (3:2)

Detector UV at 254 nm; flow-rate, 1 ml min$^{-1}$. $d_p$ = Particle diameter; $V_R$ = retention volume; for other symbols see text.

| Column | $d_p$ ($\mu m$) | $L$ (cm) | $V_R$ (cm$^3$) | $k'$ | $N$ | $A_s$ |
|---|---|---|---|---|---|---|
| $\mu$Bondapak C$_{18}$ | 10 | 30 | 11.7 | 3.3 | 5800 | 1.2 |
| Novapak C$_{18}$ | 4 | 15 | 6.6 | 3.9 | 7000 | 0.95 |
| LiChrosorb RP-8 Select B | 7 | 25 | 8.8 | 2.7 | 7100 | 1.4 |
| Hypersil ODS | 5 | 25 | 15.0 | 3.8 | 15 000 | 1.4 |
| Spherisorb ODS I | 5 | 25 | 11.2 | 2.6 | 12 000 | 1.0 |
| Spherisorb ODS II | 5 | 25 | 18.2 | 4.8 | 16 000 | 1.4 |

RESULTS AND DISCUSSION

The columns were first tested by chromatography of a simple test compound (toluene) using a mixture of methanol–water (3:2) and the column efficiency and peak asymmetry factor was calculated (Table I). Direct comparison of performance is difficult due to extra column dispersion which may affect each column to a different degree depending on its physical characteristics. For example, simple calculations[6] show that the performance of the instrumentation used (which has a fairly standard specification) is entirely adequate for use with the 10-$\mu$m column but predict that the efficiency of the 4-$\mu$m column would not be fully realised in this test. These calculations were supported by experimental observation that the plate number of the 10-$\mu$m column measured using benzene (which had a retention volume about 2/3 that of toluene) was virtually unchanged, whereas a similar determination on the 4-$\mu$m column indicated a drop in plate number for benzene of about 20% relative to toluene. Use of different solvent strength mixtures for this test was not considered beneficial, due to the variation in solvent viscosity and its possible effect on column performance[7].

All columns tested contained octadecylsilyl (ODS) bonded packings except the LiChrosorb material which is a C$_8$ packing. The latter was included in the scheme, however, since it is especially recommended by the manufacturer for the chromatography of basic compounds.

TABLE II

COMPARISON OF PHOSPHATE AND ACETATE BUFFERS FOR ANALYSIS OF QUININE

Column, $\mu$Bondapak C$_{18}$. UV detector at 220 nm. Flow-rate, 1.0 ml min$^{-1}$. pH measured before organic modifier addition.

| Mobile phase | $V_R$ (cm$^3$) | $k'$ | $N$ | $A_s$ |
|---|---|---|---|---|
| 1% Acetic acid, pH 2.8 in methanol–water (25:75) | 23.0 | 7.6 | 1500 | 3.3 |
| 1% Acetic acid, pH 2.8 in acetonitrile–water (15:85) | 18.7 | 6.0 | 2100 | 3.2 |
| Acetonitrile–0.1 $M$ phosphate buffer, pH 3.0 (15:85) | 14.2 | 4.3 | 4500 | 1.8 |

The prior assessment of column performance with toluene allows subsequent broad comparisons to be made with performance for basic analytes.

Recently, Kiel et al.[8] showed that of the tricyclic antidepressants, the tertiary amine amitryptiline ($pK_a = 9.4$) gave particularly poor peak symmetry in reversed-phase separations, using a $C_8$ column in conjunction with acetonitrile and phosphate buffer. Quinine also contains a tertiary amino group ($pK_{a2} = 9.7$) and has in addition a less basic nitrogen atom in the quinoline ring system ($pK_{a1} = 5.1$)[9].

The reversed-phase chromatography of quinine was initially attempted using a mobile phase consisting of methanol, water and acetic acid as reported by Johnston et al.[10] in conjunction with the recommended μBondapak column. Column performance (Table II) was poor using this mobile phase, or when substituting acetonitrile as the organic modifier. It is possible that acetate ion associates with the hydrophobic column surface providing ion-exchange sites for the protonated alkaloids (or gives rise to an "ion-pair" effect) resulting in a complex retention mechanism. Furthermore, UV absorption by acetic acid precludes monitoring at the wavelengths which give optimum sensitivity for the alkaloids. A considerable improvement in performance was obtained by using a phosphate buffer at similar pH. The peak asymmetry factor of quinine was greatly reduced, and a decrease in the capacity ratio, $k'$, also results.

Table III shows the effect of ionic strength and pH on the peak shape of quinine. Use of 0.01 $M$ rather than 0.1 $M$ potassium dihydrogen phosphate adjusted to pH 3.0 with orthophosphoric acid gives increased retention and peak asymmetry. This feature can be attributed to the ion-exchange effect of underivatised silanol groups on the column. No improvement in column efficiency or peak symmetry was obtained by working at pH values below 3.0. Thus, pH 3.0 was preferred in further studies since Glajch et al.[11] have shown that phase stripping may occur between pH 2.0 and 3.0, although the effect is more serious when using methanol rather than acetonitrile as the organic modifier. It should be noted, however, that the buffering action of phosphate systems is poorer at higher pH values. Similar effects of pH and ionic strength were noted for the other columns.

Table IV shows a comparative study for alkaloid analysis for six columns using acetonitrile–0.1 $M$ potassium dihydrogen phosphate, pH 3.0 (15:85). Results for both quinine and its stereoisomer quinidine are shown. Included is the value of $k'$ for nitrobenzene using dry hexane as eluent, a procedure which has been used as an indicator of underivatised silanol groups[12]. A value of $k' < 0.5$ is supposed to indicate a low level of residual silanols. Wide differences are shown in the performance of the

TABLE III               '

EFFECT OF BUFFER STRENGTH AND pH ON COLUMN PERFORMANCE

Column: LiChrosorb RP-8 Select B. Other conditions as for Table II.

| Mobile phase | $V_R$ ($cm^3$) | $k'$ | $N$ | $A_s$ |
|---|---|---|---|---|
| Acetonitrile–0.01 $M$ potassium dihydrogen phosphate, pH 3.0 (1:9) | 43.5 | 17.4 | 1800 | 2.6 |
| Acetonitrile–0.1 $M$ potassium dihydrogen phosphate, pH 3.0 (1:9) | 39.0 | 15.5 | 4100 | 1.7 |
| Acetonitrile–0.1 $M$ potassium dihydrogen phosphate, pH 2.5 (1:9) | 20.8 | 7.8 | 3800 | 1.9 |
| Acetonitrile–0.1 $M$ potassium dihydrogen phosphate, pH 2.0 (1:9) | 12.3 | 4.2 | 3500 | 1.8 |

TABLE IV

PERFORMANCE OF SIX DIFFERENT REVERSED-PHASE COLUMNS FOR ALKALOID ANALYSIS USING ACETONITRILE–0.1 $M$ POTASSIUM DIHYDROGEN PHOSPHATE pH 3.0 (15:85)

qd = Quinidine; qn = quinine. Analysis conditions as for Table II.

| Column | $V_R$ (cm³) | | $k'$ | | $N$ | | $A_s$ | | $k'_{nitrobenzene}$ * |
|---|---|---|---|---|---|---|---|---|---|
| | qd | qn | qd | qn | qd | qn | qd | qn | |
| $\mu$Bondapak C$_{18}$ | 13.0 | 14.2 | 3.8 | 4.3 | 4600 | 4500 | 1.5 | 1.8 | 0.9 |
| Novapak C$_{18}$ | 10.8 | 12.6 | 7.1 | 8.4 | 4500 | 4300 | 3.0 | 2.9 | <0.5 |
| LiChrosorb RP-8B | 11.6 | 13.0 | 3.9 | 4.5 | 4200 | 4100 | 1.9 | 1.7 | 1.6 |
| Hypersil ODS | 21.0 | 24.0 | 5.7 | 6.7 | 500 | 500 | >5 | >5 | <0.5 |
| Spherisorb ODS I | 35.6 | 40.5 | 10.4 | 12.0 | 2200 | 2200 | 2.9 | 3.1 | 1.9 |
| Spherisorb ODS II | 11.6 | 12.7 | 2.7 | 3.1 | 7600 | 7200 | 2.3 | 2.7 | <0.5 |

* Measured in dry hexane.

various columns. A much greater inter-column variation in $k'$ for the alkaloids is evident than for toluene. Furthermore, although some columns give efficiency and peak symmetry values for the basic compounds which approach the values shown for toluene (e.g. $\mu$Bondapak C$_{18}$), the performance of other columns (e.g. Hypersil ODS) is seriously affected. The asymmetry factors for the alkaloids show no correlation with values of $k'$ recorded for nitrobenzene using hexane as eluent. However, it is interesting to note that two of the columns giving values of $k'$ for nitrobenzene >0.5 yielded the best peak symmetry for the alkaloids ($\mu$Bondapak ODS, LiChrosorb RP-8B). The accessibility and not merely the number of silanol groups may be the important factor determining their affect on basic analytes[13].

Direct comparison between the columns is difficult because they differ in so many ways (e.g. phase loading, nature of base silica etc.). However, the Spherisorb
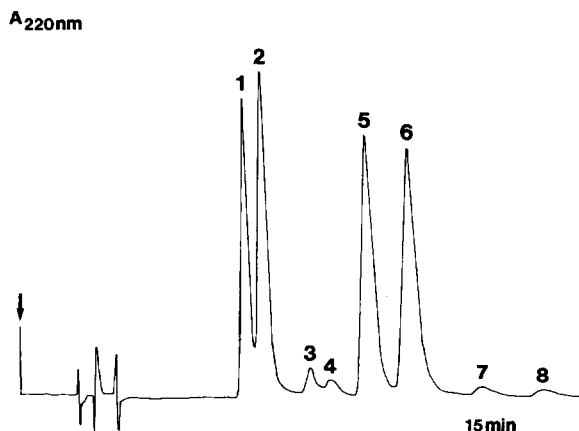
A$_{220nm}$



Fig. 1. Separation of major cinchona alkaloids and their dihydro derivatives on 7-$\mu$m LiChrosorb RP-8 Select B column. Mobile phase: as for Table IV. Peaks: 1 = cinchonine; 2 = cinchonidine; 3 = hydrocinchonine; 4 = hydrocinchonidine; 5 = quinidine; 6 = quinine; 7 = hydroquinidine; 8 = hydroquinine.
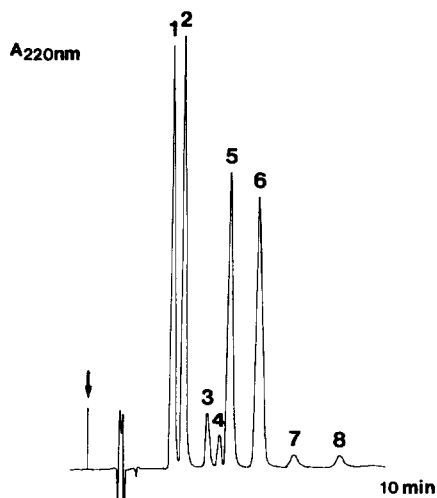
Fig. 2. Separation of major cinchona alkaloids and their dihydro derivatives on 4-$\mu$m Novapak $C_{18}$ column. Peak identities as Fig. 1. Mobile phase: 7% acetonitrile in 0.05 $M$ hexylamine adjusted to pH 3.0 with orthophosphoric acid. Flow-rate = 1.0 ml min$^{-1}$.

ODS I and ODS II columns are manufactured from the same base silica. Spherisorb ODS I has, according to the manufacturer, a "controlled" number of free silanol groups with a carbon coverage of 7% (w/w) whereas Spherisorb ODS II has a lower level of residual silanols and a carbon coverage of 12% (w/w). The $k'$ values for the alkaloids are, however, about four times greater on the more lightly loaded column, indicating that interaction with the silanol groups is the dominant retention mechanism.

The complete separation of the four major cinchona alkaloids and their dihydro derivatives was achieved with reasonable peak symmetry ($A_s < 2.0$) on two of the columns ($\mu$Bondapak $C_{18}$ and LiChrosorb RP-8 B) using mixtures of phosphate buffer and acetonitrile. An example of such a separation is shown in Fig. 1. For the other columns, it was necessary to incorporate a long-chain amine additive in the mobile phase to achieve good results. Fig. 2 shows an optimised separation of the alkaloids in only 8 min, obtained with the Novapak column and an eluent containing hexylamine phosphate buffer. The peak asymmetry factors for quinine and quinidine were 1.0, which is virtually identical to the result obtained for toluene with this column. Excellent alkaloid separations can even be obtained with the Hypersil column, as shown previously[14]. The retention of the alkaloids is greatly reduced using these conditions, as shown by the low acetonitrile concentrations necessary to achieve elution. This decrease can be attributed to the adsorption of protonated hexylamine on the column surface and repulsion of the similarly charged alkaloid molecules and/or competitive silanol masking effects[15].

CONCLUSIONS

Commercial reversed-phase columns show wide differences in properties when basic analytes are chromatographed. Whereas some column materials give peak sym-

metry and column efficiency values for basic compounds which approach the performance of the column shown under the conditions used by manufacturers in test chromatograms supplied with the column, the use of suitable additives to improve the performance of other materials is essential. In either case, analysis of the cinchona alkaloids is achieved rather more simply than in some recently published methods[16,17].

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