Journal of Chromatography, 260 (1983) 184–188
Elsevier Science Publishers B.V., Amsterdam — Printed in the Netherlands

CHROM. 15,555

Note

Analysis of cinchona alkaloids by high-performance liquid chromatography

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(First received July 28th, 1982; revised manuscript received November 23rd, 1982)

The cinchona alkaloids, which occur primarily in the bark of *Cinchona* and *Remijia* species are still widely used as pharmaceuticals, as bitter flavourings in the food industry and as chiral reagents in organic synthesis. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been the most successful methods for the separation of these compounds. TLC procedures have been recently reviewed[1]; the best separation of a complex cinchona alkaloid mixture by HPLC appears to have been that obtained by Bauer and Untz[2]. For our work[3], we have required a procedure giving greater reproducibility of retention time than is described for this method. In addition, the method requires accurate measurement of the mobile phase water content by the Karl-Fischer procedure. Here we report a reproducible method for the analysis of these alkaloids, using a silica column. The proposed adsorption method is briefly compared with results achieved on bonded-phase octadecylsilyl (ODS) columns.

EXPERIMENTAL

All analyses were performed using a high pressure pump (Model 110A; Altex, Berkeley, CA, U.S.A.), a 20-μl loop injector (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and a variable wavelength UV detector (Model LC3; Pye Unicam, Cambridge, Great Britain). The silica column used was a 250 × 4.6 mm I.D. Hypersil, 5 μm, column (Shandon Southern, Runcorn, Great Britain) in conjunction with a guard column. The column was used daily in the described solvent systems for more than a year without ill effect, although the basic eluent was replaced with an inert solvent (acetone) for overnight storage. Temperature control was achieved with a home-made glass jacket covering the entire column assembly, which was connected to a thermostat water circulator bath. Quinine, quinidine, cinchonine and cinchonidine were obtained from Sigma (St. Louis, MO, U.S.A.), hydroquinidine from Fluka (Buchs, Switzerland), hydroquinine, hydrocinchonidine and quinidinone were a gift from D. J. Millin, AFP consultants, Great Britain. Hydrocinchonine was synthesised via catalytic hydrogenation (Pd/C) of the parent alkaloid[4]. The veracity of all compounds was checked by mass spectrometry.

0021-9673/83/$03.00

RESULTS AND DISCUSSION

Excellent separations of the major cinchona alkaloids quinine, quinidine, cinchonine and cinchonidine were obtained in about 20 min using hexane–dichloromethane–methanol–diethylamine (66:31:2.6:0.5) as the mobile phase. Complete separation of the parent alkaloids and their dihydro derivatives in a single run is possible in about 40 min by adjustment of the solvent ratios. To save time in routine analysis however, we have generally used a slight solvent adjustment (2.0% methanol, 0.65% diethylamine) to give the separation in Fig. 1, achieved in about 30 min. Good peak symmetry and resolution is shown for all compounds except hydrocinchonine (relative retention 0.96, quinine 1.00), which is only partially resolved from quinine under these conditions. The hexane–dichloromethane ratio, and the concentration of diethylamine in the mixture both give profound selectivity effects which can be used to manipulate the separation. We have also noted the importance of the nature of the basic additive; for example substitution of triethylamine for diethylamine gives substantially inferior results.

We have found irreproducible retention to be caused by three main factors.

Use of water as a polar modifier. Water has a low solubility in the type of solvent necessary for this separation. It is difficult to prepare eluents with reproducible water content and the amount may change due to slow equilibration with container walls[5]. Our solvent system does not give rise to such problems. In addition, the methanol content appears to give rise to a favourable "buffering" action against the presence of small variable amounts of water in the mobile phase. The level of

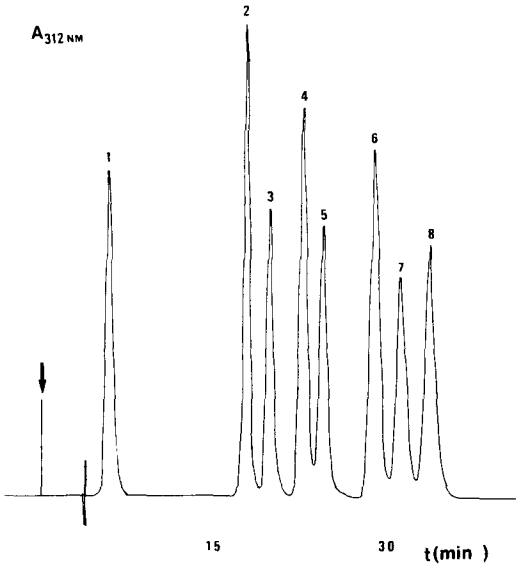

Fig. 1. Separation of cinchona alkaloids on 5-μm Hypersil column. UV detector at 312 nm. Solvent: hexane–dichloromethane–methanol–diethylamine (66:31:2.0:0.65). Peaks: 1 = quinidinone; 2 = quinidine; 3 = cinchonine; 4 = hydroquinidine; 5 = cinchonidine; 6 = quinine; 7 = hydrocinchonidine; 8 = hydroquinine, all 100 mg l^{-1}; flow-rate, 1.0 ml min^{-1}; temperature, 22°C.

water in HPLC grade solvents is thus generally low enough such that they can be used without any special treatment in this separation.

Effect of temperature. Temperature fluctuations in the chromatographic system were also identified as a source of irreproducible retention. Fig. 2 shows a plot of ln k' against $1/T$ for the four major alkaloids over the temperature range 22–32°C, obtained from triplicate sample injections. In this system, retention *increases* with increasing temperature. This result may be typical of separations where the moderator plays a dominant rôle in the adsorption of mildly polar solutes[6,7]. The increase in retention may be explained by decreasing amounts of modifier being adsorbed on the silica surface as temperature is raised, thereby increasing exposure of the surface functionality. This hypothesis was illustrated by the observation that increasing temperature at constant methanol concentration had a similar effect to decreasing the methanol content at constant temperature. It was shown that no significant selectivity effects could be obtained by temperature variation. It is thus adequate merely to maintain the column sufficiently above ambient such that temperature control can be achieved.

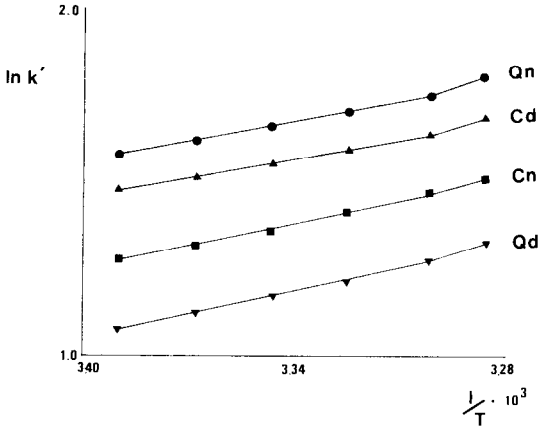

Fig. 2. Effect of temperature on retention of major cinchona alkaloids. Qd = Quinidine; Cd = cinchonidine; Cn = cinchonine; Qn = quinine. Solvent: hexane–dichloromethane–methanol–diethylamine (66:31:2.6:0.5).

Sample size. A small *continuous* increase in retention of 2–3% was noted as sample size was gradually decreased from 4 μg to 20 ng injected alkaloid. These levels are far below those considered to lead to column overloading. The results are explained in terms of competition amongst sample molecules themselves for column adsorption sites. It is thus important for the most precise work to have reasonably well-matched samples and standards.

With observation of the above precautions, a relative standard deviation (R.S.D.) of less than 0.5% in the retention of each alkaloid was observed for ten injections of a mixture containing the four major alkaloids over a 12-h period. For ten similar injections over a 3-month period, the R.S.D. was still below 1% which represents a ten-fold improvement on previously reported results[2].

Although our work mostly involves the determination of low levels of alkaloids

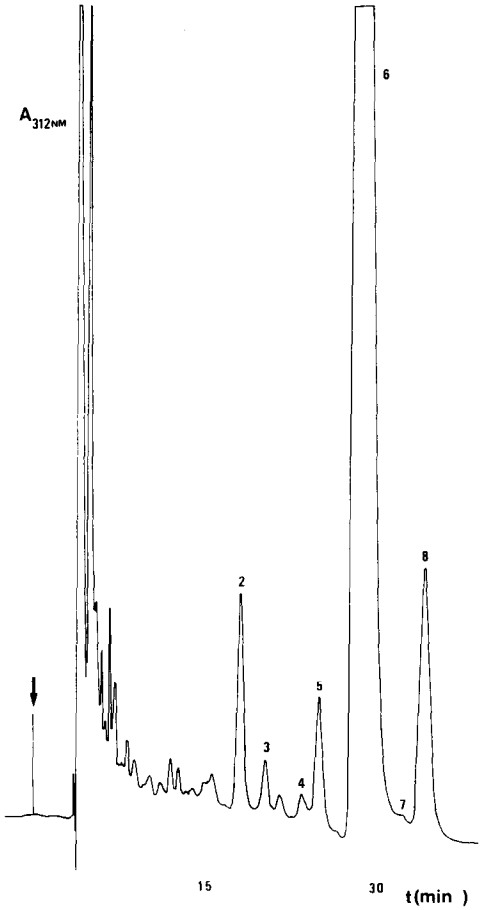

Fig. 3. Analysis of alkaloids in a chloroform extract of cinchona bark. Solvent and peak identity as Fig. 1.

in cell suspension cultures[3], Fig. 3 shows the application of the method to the determination of the alkaloids in a chloroform extract of cinchona bark.

Comparison with separations on bonded-phase ODS columns

A large number of reports deal with the assay of quinidine and its metabolites by HPLC using ODS columns, associated with the use of quinidine as a cardiac anti-arrhythmic, *e.g.*, refs. 8–10. The separation of the four major alkaloids on ODS columns has also been noted[11,12]. Typically, buffered methanol–water or acetonitrile–water mixtures are employed. The great majority of these separations have been performed on (Waters Associates) μBondapak columns. We have experienced difficulty in attempting to improve these separations by transfer to high-efficiency 5-μm columns (Hypersil ODS, Spherisorb ODS) due to poor peak symmetry, despite these being highly end-capped materials. The problems associated with the analysis of hydrophobic amines on ODS columns have recently been discussed[13–15]. While peak asymmetry is largely overcome by inclusion of an amine

additive in the acidic mobile phase, we have generally found that resolution of complex alkaloid mixtures is inferior to that given by the proposed adsorption system. However, further investigations of the ODS column system are currently in progress.

ACKNOWLEDGEMENTS

The author thanks Dr. C. S. Hunter and A. J. Barraclough whose biological studies have encouraged this work, Dr. D. J. Millin for the gift of standards and the Polytechnic Research Committee for financial support.

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* *Editor's Note:* See also A. Hobson-Frohock and W. T. E. Edwards, *J. Chromatogr.*, 249 (1982) 369.