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Note

Selective effects of reversed-phase column packings in high-performance liquid chromatography of steroids

E. C. NICE and M. J. O'HARE

Unit of Human Cancer Biology, Ludwig Institute for Cancer Research, Royal Marsden Hospital, Sutton, Surrey SM2 5PX (Great Britain)

We have recently published a series of high-performance liquid chromatographic (HPLC) systems in which a C_{18} reversed-phase (RP) column packing with gradient elution is used to separate steroid hormones in biological samples[1]. Different eluting solvents (methanol, acetonitrile, dioxane) gave useful selective effects that assisted the separation, estimation and identification of closely related groups of adrenal and testicular steroids.

Separation of some components of the complex mixtures of hormones obtained from *in vitro* experiments was, however, close to the limits of resolution (see Fig. 1a). Further experience has shown that small changes in the column packing, including those apparently due to changes in production technique, can significantly influence such separations. To delineate further the factors that influence HPLC of steroids, we have, therefore, evaluated a number of similar C_{18} RP packings. Irrespective of the absolute efficiency of the columns, each packing type was found to exhibit a different pattern of selectivity towards certain groups of steroids, a feature that can be used to advantage to tailor systems for specific separations in biological samples containing these hormones.

MATERIALS AND METHODS

All columns were evaluated using a DuPont Model 830 chromatograph with Model 838 gradient elution module and Model 837 variable-wavelength spectrophotometer, as described previously[1]. Samples of test steroids were injected via a Rheodyne septumless valve, and chromatograms were developed at 45° with an exponential acetonitrile (32–100%)–water gradient of the form $y = x^3$ over 50 min, eluted compounds being detected at 240 nm.

Details of the columns tested are given in Table I. Wettability was determined according to Scott and Kucera[2] and accessible silanol groups by adsorption of methyl red[3]. Other parameters quoted were obtained from the manufacturers. Optimum column efficiencies[4] were determined from samples of cortisone, eluted isocratically with the starting solvent composition (32% acetonitrile), and starting flow-rates were adjusted accordingly. Selectivity (α) was determined as retention ratios $[(t_{r1} - t_0)/(t_{r2} - t_0)]$ for selected pairs of compounds.

TABLE I

CHARACTERISTICS OF OCTADECYLSILANE-BONDED SPHERICAL POROUS MICROPARTICULATE SILICA IN PACKED COLUMNS

Column No.	Packing	Supplier	Length (mm)	I.D. (mm)	Type*	Particle size (μm)	Surface area (m²/g)	Loading (%)	Efficiency (theoretical plates per m)	Wettability (%)**	Methyl red adsorption (%)****
1	Zorbax-ODS	DuPont (Wilmington, Del., U.S.A.)	250	4.6	PP	5	200	10	21,900	29	82.4
2	Spherisorb 5-ODS	Phase-Separations (Queensferry, Great Britain)	100	5	LP	5	230	8	21,000	17	41.6
3	μBondapak-C_{18}	Waters Assoc. (Milford, Mass., U.S.A.)	300	4.6	PP	10	300–350	10	7,800	74	70
4	Nucleosil 5-C_{18}	Macherey-Nagel (Düren, G.F.R.)	100	5	LP	5	500	20	23,000	20	23
5	Hypersil-ODS	Shandon (Runcorn, Great Britain)	100	5	PP***	5	180	10	16,000	12	16

* PP = Prepacked by supplier; LP = laboratory packed.
** Maximum water content in acetonitrile to permit complete wettability.
*** Also tested as LP column.
**** Percentage of methyl red adsorbed from dried benzene, compared with that adsorbed by an equal weight of underivatised silica (5 μm).

RESULTS

A related group of testicular steroids (Table II) was used as a test mixture to evaluate the columns detailed in Table I. Practical reasons dictate the use of gradient elution to separate the wide range of steroid hormones in biological samples[1]. We therefore elected to test the selectivity of column packings under the conditions with which samples from our *in vitro* experiments are analysed.

TABLE II

RETENTION TIMES (min) OF STEROIDS ON THE COLUMNS DETAILED IN TABLE I

Steroid	Column				
	1	2	3	4	5
17α-Hydroxy-20α-dihydroprogesterone	26	20.4	17.2	16.4	15.6
Testosterone	28.4	22.8	18.5	19.2	16.8
Androstenedione	28.8	24.4	20.4	22	22
17α-Hydroxyprogesterone	29.6	25.2	22.8	24	24.4
20α-Dihydroprogesterone	39	34.4	34	30.4	32.8
Progesterone	40.4	36.4	37.6	34.2	40

Retention times for the test compounds on the different columns are given in Table II. Using optimal starting flow-rates with each column (1.0–1.5 ml/min), the individual test compounds were eluted at similar elapsed times, *i.e.* over the same region of the gradient (see Table II). Thus, although the use of acetonitrile–water gradients at constant pressure results in small flow-rate changes[1], the differences between retention ratios calculated directly from retention times under these conditions (Table III) were far greater ($< 35\%$) than any residual differences between retention times and retention volumes ($< 5\%$). Confirmation of the intrinsic nature of these selective effects, and the ranking order of the columns (Table III) was obtained when steroid pairs were eluted isocratically using the different column packings. Their practical value is, however, only apparent when chromatograms obtained under conditions of gradient elution are compared.

TABLE III

RETENTION RATIOS OF STEROIDS ON THE COLUMNS DETAILED IN TABLE I

Steroid	Column				
	1	2	3	4	5
Androstenedione/testosterone	1.01	1.07	1.10	1.15	1.31
Progesterone/20α-dihydroprogesterone	1.04	1.06	1.11	1.12	1.22
17α-Hydroxyprogesterone/17α-hydroxy-20α-dihydroprogesterone	1.14	1.24	1.33	1.46	1.56

Fig. 1 illustrates the wide range of selectivities observed in the present study of five octadecylsilane-bonded microparticulate spherical porous silica packings. At one extreme, the test compounds, testosterone and androstenedione, were not resolved using Zorbax-ODS (Fig. 1a) in spite of its high efficiency. The selectivity of Hypersil ODS under the same chromatographic conditions was so great that, although separation of androstenedione and testosterone was readily achieved, resolution of the latter
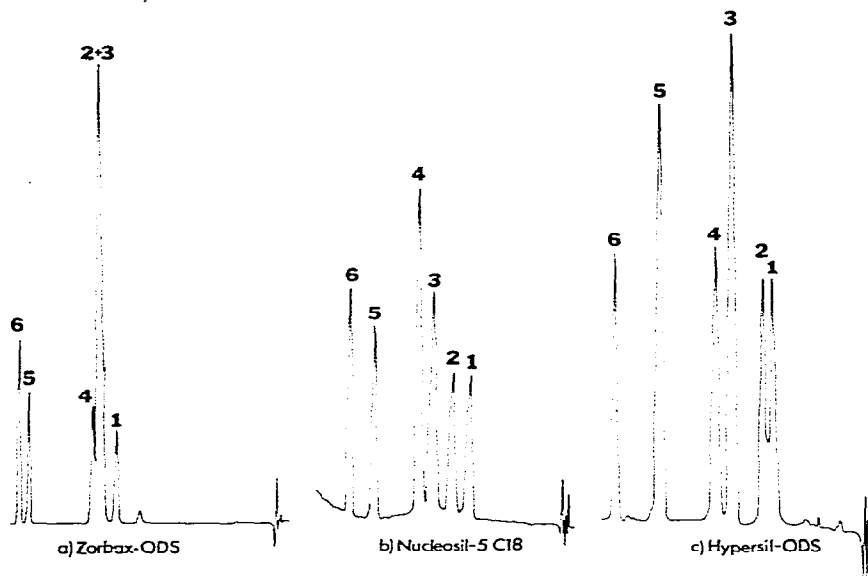
Fig. 1. Selective effects of reversed-phase column packings on steroid hormones. Chromatograms were obtained under identical conditions of gradient elution ($y = x^3$ with 32–100% acetonitrile over 50 min). For details of column packings and retention times see Tables I and II. Note the wide differences in effective separations. 1 = 17α-Hydroxy-20α-dihydroprogesterone; 2 = testosterone; 3 = androstenedione; 4 = 17α-hydroxyprogesterone; 5 = 20α-dihydroprogesterone; 6 = progesterone.

from 17α-hydroxy-20α-dihydroprogesterone was compromised (Fig. 1c). Nucleosil 5-C_{18}, which gave intermediate retention ratios, enabled the complete separation of these test compounds to be achieved (Fig. 1b). Similar differential selective effects were observed with other steroids and when other eluting solvents were used.

Further experiments, not illustrated here, showed that selectivities were not related to column efficiencies, column diameters, or dependent on whether commercially or laboratory-packed columns of the same material were used. Furthermore, column length variation over the range 100–250 mm made little difference to the effective separations that could be achieved with the same packing —much less, in fact, than the difference between alternative packings of the same length. No significant changes in selectivity were noted during prolonged use of the columns, and in spite of extensive experimentation, we were unable to reproduce the selective effects of the different packings by using a single packing and varying the flow-rates, gradient profiles, temperatures, or starting solvent composition. It may be concluded, therefore, that the selectivities observed here are intrinsic properties of each packing material in spite of similarities in their specifications (Table I). It appears, consequently, that the precise choice of column packing is as important as the choice of eluting solvent system[1] in defining effective systems for the HPLC of steroid hormones.

DISCUSSION

The use of RP materials in liquid chromatography has recently burgeoned to the extent that they now reputedly account for the majority of HPLC separations[5].

The selective effects obtainable with different eluting solvents have been rapidly appreciated, analysed[6], and utilised to facilitate specific separations[1]. In many analytical situations pertaining to biological samples, however, only a limited number of solvents are compatible with both UV detection and the powerful adjunct of gradient elution. Under these circumstances it may be feasible, as in the present case, to utilize selective effects of the individual packings themselves to enhance the resolving power of the method.

In general, the retention of a given solute under constant mobile-phase conditions in HPLC increases both with the chain length and carbon content of the RP packing[2,7]. The mechanisms that govern the selective effects of such materials in respect of related solutes, on the other hand, are not yet clear. With some RP packings and solutes separation ratios correlate with absolute carbon content, but not with either the percentage derivatization or the use of mono- or polymeric carbon moieties[2]. There is, however, no evidence that this is necessarily a general relationship. Increased loading with stationary phase can reduce retention ratios with certain solutes and even cause reversals in retention order[8], presumably as accessible silanol groups are masked and adsorption effects are minimised.

In the present study with chemically bonded reversed-phase packings, marked differences in selectivity were obtained (Table III). For the test compounds in question this selectivity appeared to correlate with the adsorption of methyl red, which is a measure of accessible silanol groups[3], at least in so far as the $5-\mu m$ packings were concerned. Thus, in this instance, the masking of free silanol groups results in enhanced selectivity towards related keto- and hydroxy-steroids. There was no obvious correlation between selectivity and other column packing parameters, such as wettability or percentage loading. However, irrespective of the actual mechanisms, screening the wide range of available high-efficiency RP packings now available offers one potential solution to the problems encountered in separating the components of complex mixtures of biological origin. The simple methyl red adsorption, measured spectrophotometrically, may afford a useful indication of the likely behaviour of individual packings and enable significant changes in their properties from batch to batch to be monitored, in relation to specific separations required.

REFERENCES

1 M. J. O'Hare, E. C. Nice, R. Magee-Brown and H. Bullman, *J. Chromatogr.*, 125 (1976) 357.
2 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 142 (1977) 213.
3 I. Shapiro and I. M. Kolthoff, *J. Amer. Chem. Soc.*, 72 (1950) 776.
4 N. A. Parris, *Instrumental Liquid Chromatography*, Elsevier, Amsterdam, Oxford, New York, 1976.
5 C. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
6 S. R. Bakalyar, R. McIlwrick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
7 K. Karch, I. Sebastian and I. Halász, *J. Chromatogr.*, 122 (1976) 3.
8 B. B. Wheals, *J. Chromatogr.*, 122 (1976) 85.