Journal of Chromatography, 169 (1979) 93–100
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,371

PROGRAMMED-TEMPERATURE GAS CHROMATOGRAPHY ON MIXED PHASES

SEPARATION OF STEROID DERIVATIVES ON BINARY MIXTURES

P. HURTER
Department of Chemical Pathology, University of Pretoria, Pretoria 0002 (Republic of South Africa)
and
T. VAN REE
Department of Chemistry, University of Pretoria, Pretoria 0002 (Republic of South Africa)
(First received May 26th, 1978; revised manuscript received August 9th, 1978)

SUMMARY

The use of various programmed-temperature gas chromatography (PTGC) retention indices for drawing window plots is described. The separation of steroid derivatives by PTGC is improved by the use of a binary phase mixture which has been selected by means of a window plot. A novel, highly efficient method for packing columns is also described.

INTRODUCTION

At present, gas chromatographic (GC) methods for the analysis of human urinary steroids depend mainly upon the use of packed glass columns giving heights equivalent to a theoretical plate (HETPs) of 0.5 to 0.6 mm, or open-tubular capillary columns also giving HETPs of approximately 0.6 mm; however, mixtures of stationary phases should be able to provide higher efficiencies and, therefore, better separation of these highly complex mixtures.

In a recent series of papers[1–3] it was shown that a simple equation describes the gas–liquid chromatographic retention behaviour of solutes on mixed stationary phases:

$$K_R = \sum_i \Phi_i K_i \qquad (1)$$

or

$$K_R = \sum_i m_i K_i \qquad (2)$$

with

$$\Sigma\Phi = \sum_i m_i = 1$$

where K_R = the infinite dilution partition coefficient in the mixture; K_i = the infinite dilution partition coefficient in phase i; Φ_i = the volume fraction of phase i in the mixture; m_i = the mass fraction of phase i in the mixture.

Plots of α, the relative volatility, *versus* Φ or m (for binary mixtures) result in a set of partially overlapping inverted triangles; regions where no overlap occurs are called windows, with the highest window (*i.e.* the one with the highest α value) giving the optimum value for Φ or m. These window plots are akin to the window plot obtained eighteen years ago by Singliar *et al.*[4].

This method would be very useful indeed if it could be extended to programmed-temperature gas chromatography (PTGC). A major disadvantage of temperature programming is the inability to apply isothermal retention measurements such as relative retention time, etc. Also, the determination of as many operational parameters as possible should be eliminated.

The retention index system introduced by Kováts[5] is based on the n-alkane series, using the alkanes eluting before and after the compound under investigation as reference points, with the retention index for each alkane

$$I_n = 100n \tag{3}$$

where n = number of carbon atoms.

The retention index, I, of a compound is then given by

$$I = 100\, i \frac{\log V_g(x) - \log V_g(n)}{\log V_g(n+i) - \log V_g(n)} + 100\, n \tag{4}$$

where i = the difference in chain length between the two reference alkanes; V_g = the nett retention volume; x = the component under consideration; n and $n+i$ = the two reference alkanes with chain lengths n and $n+i$ carbon atoms, respectively.

As I is linearly dependent on temperature, eqn. 4 has been rewritten[6] for PTGC as:

$$I = 100\, i \frac{T_R - T_R(n)}{T_R(n+i) - T_R(n)} + 100\, n \tag{5}$$

where T_R = the retention temperature, and it was found that this index is identical to the isothermal Kováts index as determined using eqn. 4.

In the steroid field the use of methylene units (MU values) has been widely accepted and MU values for numerous steroid derivatives on various stationary phases have been published[7,8]. This index can be written in the same form as eqns. 4 and 5:

$$\mathrm{MU} = i \frac{T_R - T_R(n)}{T_R(n+i) - T_R(n)} + n \tag{6}$$

A more easily determined retention parameter which is frequently encountered in linear TPGC is T_R, which unfortunately varies with heating rate and carrier gas flow-rate. However, the difference in retention temperature between the compound under consideration and a standard

$$T_{\mathrm{diff.}} = T_s - T_E \tag{7}$$

is remarkably constant[6].

Another simple parameter, the relative elution temperature

$$T_{RE} = T_R/T_s \quad (8)$$

is very reproducible, and independent of carrier gas flow-rate, temperature-programme rate, initial temperature, and per cent stationary phase.

In order to design more effective stationary phases for PTGC of steroid derivatives, four parameters (MU, T_R, $T_{diff.}$ and T_{RE}) were evaluated as replacements for K_i in eqn. 1 or 2. The relative volatility, α, which is a measure of the separation of any pair of solutes, 1 and 2, is now redefined as

$$\alpha = X_1/X_2,\ X_1 \text{ always} > X_2 \quad (9)$$

where X = the parameter under consideration.

The plot of α against m yields a series of "windows" with the window with maximum α defining the optimum mixture composition to use.

A further innovation described here is the dynamic coating method originally developed for capillary columns, applied to packed columns. This is a technique where columns are first packed with support material, followed by dynamic coating with stationary phase, analogous to the method[11] for coating capillary columns; this technique combined with the use of mixed stationary phases, improves the efficiency of our packed columns to an HETP of less than 0.3 mm.

EXPERIMENTAL

Packing of columns

A 3.6-m glass column of 6.3 mm O.D. and 2.5 mm I.D., was packed with sieved acid-washed, silanized (dimethyldichlorosilane), 80–100 mesh Gas-Chrom P. The liquid phase was deposited on the support according to the dynamic coating method[11]. In a typical procedure a 3.6-m column containing support was wetted by forcing 5 ml isooctane through the column, using nitrogen at a pressure of 134 kPa. This was followed immediately by 20 ml of a solution of 1% SE-30 in isooctane. The nitrogen flow was maintained for 20 h at room temperature, whereupon the column was conditioned using standard procedures. The column performance was evaluated by means of the optimum resolution obtainable for a mixture of 5α- and 5β-cholestane, and the 5β-cholestane peak was used to determine the HETP.

Extraction of steroids and preparation of derivatives

Methoxime–trimethylsilyl ether (MO–TMS) derivatives of human urinary steroids were prepared according to the method of Thenot and Horning[12].

Analytical conditions

The gas chromatograph used in this work was a Perkin-Elmer Model 3920 programmed-temperature gas chromatograph, equipped with a flame-ionization detector. Urinary steroid samples were separated on 3.6-m columns prepared as above. The GC conditions were: injected amount of solution, 1 μl; inlet temperature, 250°; detector temperature, 300°; initial column temperature, 180°; programming rate, 1°/min; column inlet pressure, 180 kPa.

TABLE I

RETENTION PARAMETERS OF STEROID DERIVATIVES ON DIFFERENT STATIONARY PHASES

Determined with a 3.6-m glass column of 2.5 mm I.D., temperature-programmed at 1°/min from 180°. 80–100 mesh Gas-Chrom P was used as support.

Steroid	*1% OV-101*				*1% SE-30*				*1% OV-225*				*1% OV-275*			
	MU	T_R (°C)	$T_{diff.}$ (°C)	T_{RE}	*MU*	T_R (°C)	$T_{diff.}$ (°C)	T_{RE}	*MU*	T_R (°C)	$T_{diff.}$ (°C)	T_{RE}	*MU*	T_R (°C)	$T_{diff.}$ (°C)	T_{RE}
Androsterone	25.28	237.6	55.2	0.811	25.22	222.4	57.6	0.794	27.87	200.4	33.6	0.856	28.26	187.1	33.9	0.847
Etiocholanolone	25.40	238.4	54.4	0.814	25.39	223.5	56.5	0.798	28.26	202.4	31.6	0.865	28.81	188.8	32.2	0.854
Dehydroepiandrosterone	25.90	241.6	51.2	0.825	25.82	226.4	53.6	0.809	29.03	206	28.0	0.880	29.23	190.2	30.8	0.861
11-Ketoandrosterone	26.27	244.0	48.8	0.833	26.30	229.2	50.8	0.819	31.05	215.2	18.8	0.920	26.92	182.2	38.8	0.824
11-Ketoetiocholanolone	26.27	244.0	48.8	0.833	26.30	229.2	50.8	0.819	31.30	216.8	17.2	0.926	33.08	202.7	18.3	0.917
11β-Hydroxyandrosterone	27.10	248.9	43.9	0.850	27.04	233.6	46.4	0.834	29.03	206	28.0	0.880	28.81	188.8	32.2	0.854
11β-Hydroxyetiocholanolone	27.23	250.1	42.7	0.854	27.23	234.6	45.4	0.838	29.51	208.4	25.6	0.891	29.23	190.2	30.8	0.861
Pregnanediol	27.80	253.7	39.1	0.866	27.60	237.8	42.2	0.849	29.03	206	28.0	0.880	28.81	188.8	32.2	0.854
Pregnanetriol	28.16	256.0	36.8	0.874	28.00	239.8	40.2	0.856	29.03	206	28.0	0.880	29.47	190.9	30.1	0.864
Tetrahydrocortisone (THE)	29.82	266.6	26.2	0.911	29.65	250.4	29.6	0.894	33.04	225.5	8.5	0.964	33.08	202.7	18.3	0.917
5β-pregnan-3α,21-diol-11,20-dione (THA)	29.90	267.3	25.5	0.913	29.78	251.5	28.5	0.898								
5β-pregnan-3α,11β,21-triol-20-one (THB)	30.09	267.6	25.2	0.914	29.94	252.8	27.2	0.903	32.11	220	14.0	0.941	31.66	198.0	23.0	0.896
allo-THB	30.30	269.4	23.4	0.920	30.11	253.3	26.7	0.905	34.13	231.2	2.8	0.988				
Tetrahydrocortisol (THF)	30.42	270.5	22.3	0.924	30.20	254.0	26.0	0.907	30.59	213.6	20.4	0.913	30.72	194.3	26.7	0.879
allo-THF	30.50	271.2	21.6	0.926	30.36	255.4	24.6	0.912	30.08	210	24.0	0.897	32.42	200.4	20.6	0.907
α-Cortolone	30.71	272.3	20.5	0.930	30.40	255.7	24.3	0.913	33.04	225.5	8.5	0.964	33.82	204.9	16.1	0.927
β-Cortol	31.02	274.3	18.5	0.937	30.70	259.6	20.4	0.927	37.58	218.4	15.6	0.933	33.08	202.7	18.3	0.917
β-Cortolone	31.02	274.3	18.5	0.937	30.70	259.6	20.4	0.927	33.65	228	6.0	0.974	34.69	207.8	13.2	0.940
α-Cortol	31.50	280.1	12.7	0.957	31.22	262.0	18.0	0.936	32.11	220	14.0	0.941	33.52	204.0	17.0	0.923
Cholesterol-*n*-butyrate (internal standard)	33.91	292.8	0	1	33.61	280.0	0	1	34.54	234	0	1	36.89	221.0	0	1

"Window" plots

These plots, as well as all other figures in this report, were generated by means of a 4K Hewlett-Packard Model 9830 programmable calculator with a 32K word tape cassette drive, thermal printer and a Model 9862A plotter. The calculator is programmed in BASIC and plug-in read-only memories (or ROMs) include a string variables ROM and advanced programming ROM.

RESULTS AND DISCUSSION

Table I summarizes the retention data for a series of steroids normally determined in human urine. Two binary mixtures were made and retention data calculated using MU, T_R, $T_{diff.}$ and T_{RE}, respectively, for K in eqn. 1. The experimentally determined retention data for the two mixtures correlated satisfactorily with the calculated data. As can be seen from the correlation coefficients presented in Table II, experimental MU values do not correlate well with calculated values; T_R and $T_{diff.}$ are clearly superior to MU and T_{RE} as parameters.

However, there is no difference in the results when window plots are drawn using MU values, T_R, $T_{diff.}$ or T_{RE}. The window plot obtained for T_R is shown in Fig. 1. The window plots all indicate an optimum composition of 37% OV-225 and 63%

TABLE II

CORRELATION BETWEEN CALCULATED AND EXPERIMENTALLY DETERMINED RETENTION PARAMETERS — CORRELATION COEFFICIENTS

Parameter	*0.65% OV-101–0.35% SE-30*	*0.63% OV-275–0.37% OV-225*
MU	0.9970	0.9155
T_R	0.9995	0.9784
$T_{diff.}$	0.9989	0.9660
T_{RE}	0.9995	0.9453

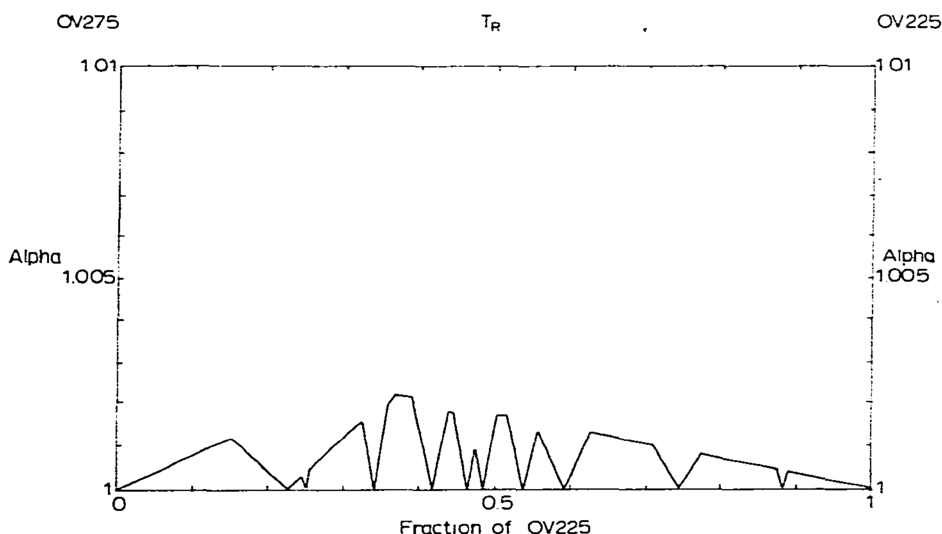

Fig. 1. Window plot for mixtures of 1% OV-225 and 1% OV-275, using T_R values.

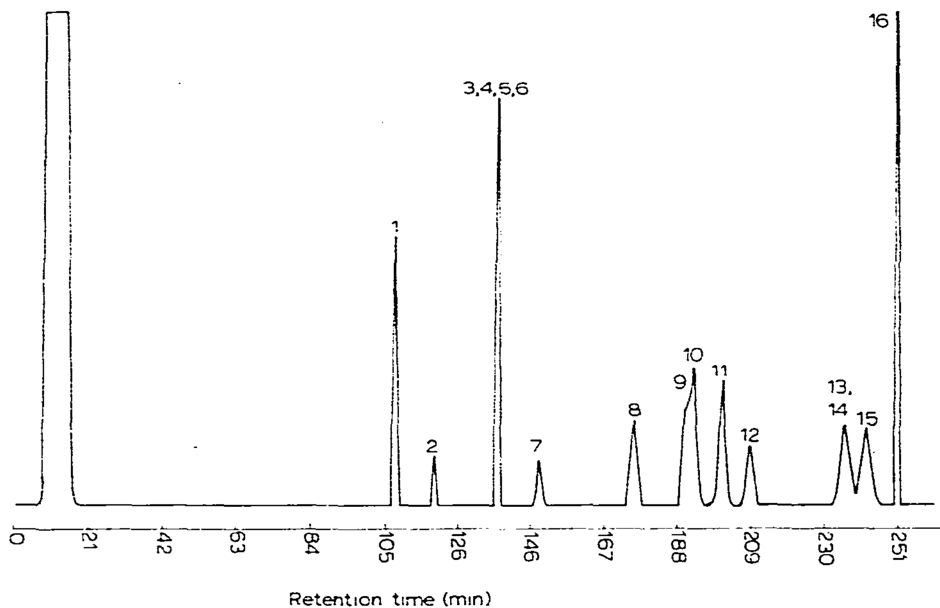

Fig. 2. Separation of MO–TMS derivatives of steroid metabolites on 1% OV-225. 1 = Androsterone, 2 = etiocholanolone, 3 = dehydroepiandrosterone, 4 = 11-ketoandrosterone, 5 = 11-ketoetiocholanolone, 6 = 11-β-hydroxyandrosterone, 7 = 11β-hydroxyetiocholanolone, 8 = pregnanediol, 9 = pregnanetriol, 10 = THE, 11 = THB, 12 = THF, 13 = α-cortolone, 14 = β-cortol, 15 = β-cortolone, 16 = cholesterol-n-butyrate (internal standard).

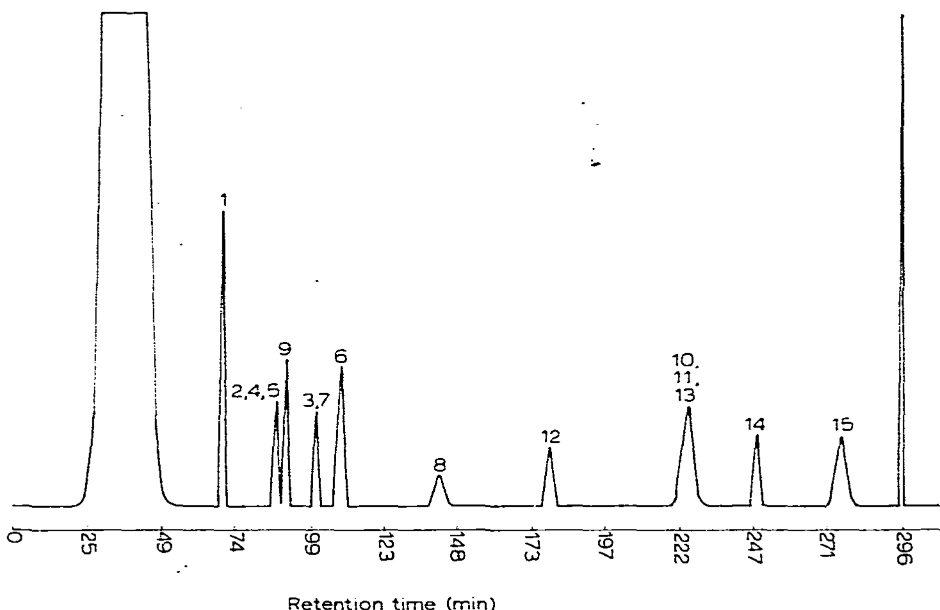

Fig. 3. Separation of MO–TMS derivatives of steroid metabolites on 1% OV-275.

OV-275. Fig. 2 shows a chromatogram of the steroid mixture on OV-225, Fig. 3 shows a chromatogram of the same mixture on OV-275, and Fig. 4 shows the improved resolution obtained with a column with $\Phi_{OV\text{-}275} = 0.63$.

This is not the first application of mixed solvents for GC of steroid mixtures. Pinelli and co-workers[13,14] have described a method wherein thin-layer chromatography is combined with GC on a mixture of 0.66% SE-30 and 0.34% tetramethylcyclobutanediol adipate. The behaviour of mixed stationary phases, even during PTGC, can now easily be calculated, and need not necessarily be restricted to binary mixtures. Furthermore, this elegant concept should also be applicable to capillary columns, making possible separations on shorter columns with all the advantages they offer, especially in the field of steroid profiling.

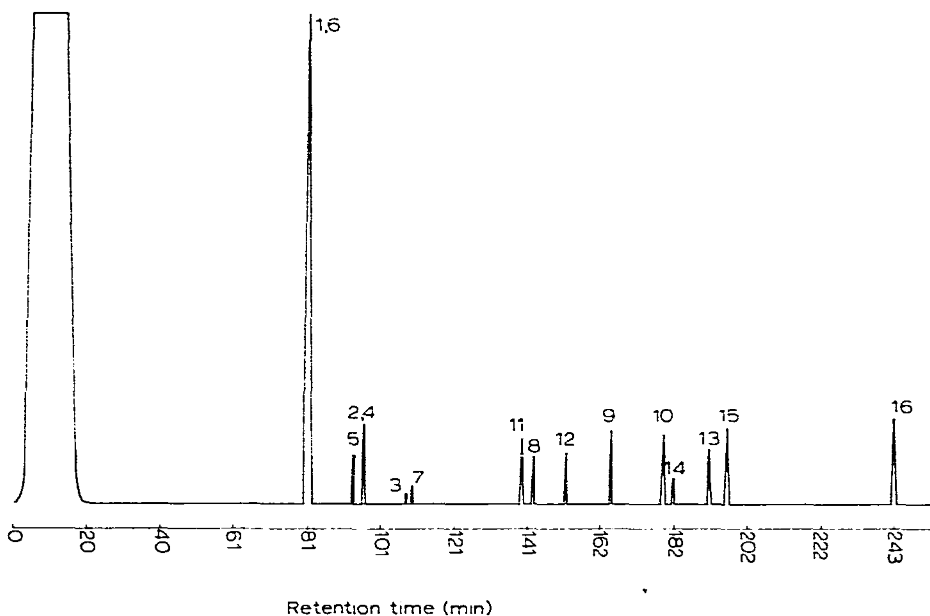

Fig. 4. Separation of MO-TMS derivatives of steroid metabolites on a mixture of 0.57% OV-225 and 0.43% OV-275.

The dynamic coating method offers substantial advantages over conventional techniques, giving HETPs of 0.53 mm (1% SE-30), 0.45 mm (1% OV-275) and less than 0.28 mm for a binary phase column (0.65% OV-101) 0.35% SE-30).

As the stabilities of OV-275 and OV-225 are nearly identical the mixture gave separations that did not change during a two-week period. As a result of the much lower stability of this mixture compared to the OV-101–SE-30 mixture as well as the inability of the OV-275–OV-225 mixture to separate androsterone and 11β-hydroxy androsterone, the more stable mixture is now being used in this laboratory for the routine separation of urinary steroid metabolites.

REFERENCES

1 R. J. Laub and J. H. Purnell, *J. Chromatogr.*, 112 (1975) 71.
2 R. J. Laub and J. H. Purnell, *Anal. Chem.*, 48 (1976) 799.

3 R. J. Laub, J. H. Purnell and P. S. Williams, *J. Chromatogr.*, 134 (1977) 249.
4 M. Singliar, A. Bobák, J. Brida and L. Lukacovic, *Z. Anal. Chem.*, 177 (1960) 161.
5 E. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
6 H. van den Dool and P. D. Kratz, *J. Chromatogr.*, 11 (1963) 463.
7 E. C. Horning, M. G. Horning, N. Ikekawa, E. M. Chambaz, P. I. Jaakonmaki and C. J. W. Brooks, *J. Gas Chrom.*, 5 (1967) 283.
8 J. A. Luyten and G. A. F. M. Rutten, *J. Chromatogr.*, 91 (1974) 393.
9 J. A. Schmit and R. B. Wynne, *J. Gas Chromatogr.*, 4 (1966) 325.
10 R. Watts and R. Dils, *J. Lipid Res.*, (1968) 40.
11 A. L. German and E. C. Horning, *J. Chromatogr. Sci.*, 11 (1973) 76.
12 J.-P. Thenot and E. C. Horning, *Anal. Lett*, 5 (1972) 21.
13 A. Pinelli and P. P. Nair, *J. Chromatogr.*, 43 (1969) 223.
14 A. Pinelli and M. L. Formento, *J. Chromatogr.*, 68 (1972) 67.