

CHROM. 10,829

TEMPERATURE DEPENDENCE OF METHYLENE-UNIT VALUES OF STEROIDS IN GAS-LIQUID CHROMATOGRAPHY

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(Received December 1st, 1977)

SUMMARY

Methylene-unit values for steroids were found to be dependent on temperature. A procedure for correction to a reference temperature (275°) was investigated and shown to give mean values not significantly different from values determined at the reference temperature although the standard errors of the means were generally significantly higher. The correlation of temperature-coefficient values with steroid structure is discussed in preliminary form.

INTRODUCTION

Calibration with *n*-alkanes makes possible the expression of retention time data from gas-liquid chromatography (GLC) in terms of methylene units (MU)¹ and provides useful information in many series of compounds including derivatives of amino- and other acids¹, carbohydrates² and prostaglandines³. The MU value is on the thermodynamic scale and is analogous to $R_M = \log(1 - R_F)/R_F$ in paper⁴ and thin-layer chromatography. Assignment of increments of R_M , ΔR_M to structural features provides valuable information in the steroid series⁵⁻⁷ among many examples.

The present study was initiated since MU and Δ MU values should also provide information about the structural features of steroids. However, this report is concerned with the temperature dependence of MU values, now observed for the first time, and with a procedure for correcting for this temperature effect so that MU analysis may be carried out.

EXPERIMENTAL

Apparatus

The Pye-Philips 104 and GCD/GLC instruments were used with 2 m × 4 mm I.D. glass tubes and flame-ionization detection (FID). The stationary phase consisted of 2, 2.5 or 3% OV-1 on Chromosorb. The limits of the gas flow-rates (ml/min) were nitrogen 20 to 60, hydrogen 30 to 50 and air 200 to 300. The FID output potential was amplified and recorded on a Servoscribe recording voltmeter.

Methods

MU values were determined by one of the following three methods.

- (1) Isothermal GLC with two or more *n*-alkanes as internal standards.
- (2) Isothermal GLC with external MU calibration by a series of *n*-alkanes (carbons of even numbers from 20 to 28 or 32). In the course of many sample runs the calibration was repeated at approximately hourly intervals.
- (3) With temperature gradient, conditions chosen such that the MU value varied linearly with time over the useful part of the range. These are termed "iso-MU" conditions and were provided by, on the GCD instrument, a starting temperature of 210°, final temperature of 300°, and rise of 4° per min. The MU calibration was run with *n*-alkanes or steroids of accurately known MU values.

Steroids

Reference steroids were of source and purity described elsewhere⁶. Steroid nomenclature in the tables is based on the abbreviated nomenclature of Bush⁷; in brief, O, oestrane; A, androstane; P, pregnane; C, cholestane; ol, hydroxy; olFt, formyloxy; olAc, acetoxy; olPt, propoxy; thiolAc, acetothiol; oxo, ketone; OBU, butoxy.

Calculations

Programmes for the least-squares fit of linear and cubic regression equations were written and specially adapted for the present application using the Hewlett Packard 9810A desk-top programmable calculator.

Retention times (*T*) from isothermal experiments were converted to MU values using the relationship $MU = B \cdot \log T + A$ where *B* and *A* are constants. Internal (method 1) or external (method 2) calibration with *n*-alkanes and fit of least-squares regression line gave values for *B* and *A*. These were used to interpolate MU values for steroids.

Method 3 required interpolation using a similar linear relationship or the cubic relationship $MU = FT^3 + ET^2 + DT + C$.

RESULTS AND DISCUSSION

MU values obtained for steroids under isothermal conditions using methods 1 and 2 were consistently the same; significance was tested using *t* values. There was no trend of change of MU value over three years or abrupt change with change of column, GLC instrument or operator. No distinction will be made between results obtained by these methods in the following discussion. The general homogeneity of the data is illustrated by the S.E.M. values in Table III.

The temperature coefficient and steroid structural features

The steroid MU values showed a consistent variation with temperature as illustrated in Fig. 1. Linear interpolation provided values (Table I) for a temperature coefficient ($\Delta MU/^\circ C$) for each of the steroid derivatives studied. Similar studies with *n*-alkanols and esters of *n*-alkanoic acids showed no temperature effect, substantiating the original observations¹.

The temperature-coefficient data were ranked (Table I) and examined for

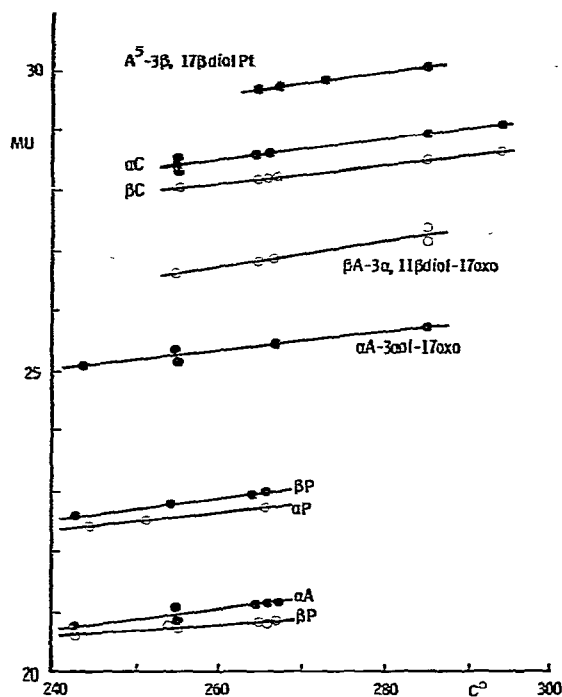


Fig. 1. Dependence of steroid MU values on temperature of observation. Each point was obtained from one GLC run of the steroid in comparison with *n*-alkanes by methods 1 or 2 described in the text. ●, 5 α -epimers; ○, 5 β -epimers. The lines shown are the calculated linear regression lines.

grouping of values in association with structural features (Table II). Within the hydrocarbon group the 5 β -epimer had a lower temperature coefficient than the 5 α -epimer and the alkane side chain was associated with an increased value. Addition of one substituent to the molecule was associated with an increase of temperature coefficient but a second substituent appeared either to raise or lower the value. A search for changes associated with epimer orientation, conformation, position of substitution and nature of substituent did not produce consistent correlations but this will be examined further in due course, using a larger number of epimeric pairs of steroid derivatives.

It is clear from the values in Table II that the nature of the steroid derivative may indicate which value for the temperature coefficient should be used. However, a standard overall value would be more generally useful and the use of the grand mean value would be satisfactory for the mono- and di-substituted steroids, the largest group of natural steroid derivatives. The steroid hydrocarbons would be over-corrected and some of the tri-substituted steroids would be under-corrected but neither to a degree greater than occurring with the outlying members of the difunctional group.

Whatever the effect of substituents on the temperature coefficient may be, it is clear that the rigid structure of the four rings of the steroid skeleton is associated with the deviation from the behaviour expected of the *n*-alkanes, and that the deviation increases with increasing temperature.

TABLE I

VARIATION OF THE TEMPERATURE COEFFICIENT OF THE MU VALUE WITH THE STRUCTURE OF THE STEROID DERIVATIVE

Temperature coefficient ($10^{-2} x$)	Hydrocarbon	Substituents		
		Mono-	Di-	Tri-
1.19			α P-3one-20oxo	
1.24				β A-3 α olPt-11 β ol-17oxo
1.39			A ⁵ -3 β ol-17oxo	
1.41	β A			
1.41			A ⁵ -3 β ol-17 β ol	
1.42			α A-3 α olFt-17oxo	
1.43			α A-3 α ol-17oxo	
1.45			A ⁵ -3 β olAc-17oxo	
1.51	β P			
1.51	β C			
1.52			A ⁵ -3 β olPt-17 β olPt	
1.58			β A-3 α olPt-17oxo	
1.64			A ⁵ -3 β olPt-17oxo	
1.65			P ⁵ -3 β olPt-20 α olPt	
1.74	α A			
1.76			A ⁴ -3one-17 β olFt	
1.77			P ⁵ -3 β ol-20oxo	
1.81	α P			
1.81	α C			
1.81			β A-3 α olAc-17oxo	
1.84			β A-3 α ol-17oxo	
1.85			β P-3 α olPt-20 α olPt	
1.91		α P-20oxo		
1.91		C ⁴ -3oxo		
1.94		α A-3oxo		
1.94			β P-3 α ol-20 α ol	
1.97				β A-3 α ol-11 α ol-17oxo
1.99		C ⁵ -3 β ol		
2.00		β P-3 β ol		
2.01			β P-3,11,17trioxo	
2.02		α P-3oxo		
2.02			A ⁵ -3 β olFt-17oxo	
2.03			P ⁵ -3one-20oxo	
2.03			β P-3 α olAc-20 α olAc	
2.05				β A-3 α ol-11 β ol-17oxo
2.07		A ⁵ -3 β ol		
2.07		C ⁵ -3 β olFt		
2.10			β P-3 α olFt-20 α olFt	
2.12				β A-3 α ol-11,17dioxo
2.12				A ⁴ -3oxo-6 β ol-17 β ol
2.13			α A-3oxo-17 β ol	
2.14			β P-3 α ol-20 β ol	
2.21			A ⁴ -3,17dioxo	
2.21			A ⁴ -3oxo-17 β ol	
2.28		C ⁵ -3 β ol		
2.56			β A-3oxo-17 β ol	
2.86				β A-3 α olFt-11 β ol-17oxo
3.31			β P-3,20dioxo	
3.52			17 α MeA ⁴ -3oxo-17 β ol	

TABLE II

VALUES OF THE MEAN TEMPERATURE COEFFICIENTS FOR GROUPS OF STEROIDS AND DERIVATIVES

 $DF = \Sigma n - 2$, P = single tailed probability.

Item	Groups of steroid derivatives	n	Mean ($10^{-2} x$)	S.E.M. ($10^{-2} x$)
1	5 α -Hydrocarbons	3	1.79	0.023
2	5 β -Hydrocarbons	3	1.48	0.036
3	5 α - and 5 β -hydrocarbons	6	1.63	0.072
4	Mono-substituted	9	2.02	0.038
5	Di-substituted*	28	1.926	0.100
6	Tri-substituted	7	2.05	0.180
7	All together*	50	1.925	0.063
8	Hydrocarbons + mono- and tri-substituted*	22	1.924	0.069

 t VALUES

Groups	t	DF	P (%)
1 and 2	7.3	4	0.2
3 and 4	5.2	13	0.1

* Histograms showed the data in these groups to be approximately normally distributed.

Correction of MU values to a standard temperature

The temperature to which correction was made (275°), was selected arbitrarily to be approximately in the middle of the range used in the chromatography of urinary steroids and their derivatives. On comparing values determined at 275° with temperature corrected values (Table III) only one among seventeen pairs showed a t value indicating significance for the difference between the means. This was not regarded as nullifying the use of the temperature-correction procedure because (1) it is not unreasonable to accept 1 per 20 as outliers, (2) both the S.E.M. values for this exceptional pair happened to be among the lowest observed and (3) the difference between the means (0.064) was of the order of the higher S.E.M. values.

Use of the temperature-correction procedure for MU values thus appears to be fully justified and variance analysis using all 460 observations shows that the grand S.E.M. is of the order of 0.026. Under particular circumstances, for example when working with steroid hydrocarbons, it might be thought necessary to use a temperature coefficient from a subgroup (Table II) rather than that of all steroids. The correction uses the following equation, in which, at temperature t the MU value is MU_t and the temperature coefficient is θ then the corrected MU value:

$$MU_c = MU_t - \theta(t - 275)$$

An attempt was made to calculate MU values using temperature gradient conditions (method 3) but the result was unsatisfactory because of the high errors in making a correction for the temperature effect with the n -alkane standards. The use of steroids for calibration seemed feasible and was more satisfactory than using n -alkanes but the residuals in linear regression (Table IV) were up to six times higher than the generally acceptable S.E.M. value for repeated MU observation (0.026). Use of cubic regression appeared to reduce the errors but the variance ratio did not

TABLE III
COMPARISON OF MU VALUES OBTAINED AT 275° AND AFTER TEMPERATURE CORRECTION

Steroid	At 275°				Corrected to 275° over range 243° to 296°				Statistical values			
	MU	n	S.E.M.	MU	n	S.E.M.	DF	t for difference of mean	P for t (%)	F*	P for F (%)	S.E.M. ratio**
α A	21,445	5	0.012	21,401	22	0.022	25	0.97	>5	79.1	<1	1.84
α A-3 α ol-17oxo	25,790	8	0.024	25,752	17	0.043	23	0.57	>5	16.3	<1	1.78
α A-3 α olPt-17oxo	27,307	7	0.018	27,278	13	0.040	18	0.52	>5	18.4	<1	2.21
α A-3,17dioxo	26,073	5	0.032	26,042	8	0.030	11	0.68	>5	2.46	>5	—
α A-3 α thiol-17oxo	26,647	5	0.018	26,659	36	0.026	39	0.17	>5	134	<1	1.44
α A-3 α thiolAc-17oxo	28,907	4	0.069	28,896	16	0.018	18	0.22	>5	1.3*	>5	—
α A-17oxo	23,510	4	0.018	23,574	29	0.015	31	1.54	>5	45	<1	0.83
β A-3 α olPt-11/ol-17oxo	28,859	5	0.036	28,845	13	0.031	16	0.26	>5	5.75	<1	0.83
β A-3 α 17 β dioxoPt	29,464	4	0.017	29,393	5	0.038	7	1.57	>5	8.44	\approx 2	2.24
β A-3 α ol-17oxo	25,389	7	0.027	25,365	39	0.011	44	0.87	>5	5.57	<1	0.41
A ³ -3 β ,17 β dioxoPt	29,948	4	0.068	29,923	7	0.038	9	0.34	>5	1.1	>5	—
α P	23,370	8	0.076	23,273	17	0.016	23	1.69	>5	4.58*	<1	—
β P-3 α ,20 α dioxoPt	31,271	7	0.036	31,210	15	0.029	20	1.23	>5	3.24	\approx 3	0.80
α C	28,834	10	0.016	28,771	35	0.019	43	1.71	>5	18.96	<1	1.2
α C-3 α -OBu	32,206	25	0.014	32,143	37	0.018	60	2.60	1	3.62	<1	1.3
α C-3 α -thiol	31,846	6	0.076	31,941	14	0.061	18	0.88	>5	3.92	\approx 4	0.80
β C	28,459	4	0.031	28,362	19	0.032	21	1.36	>5	29.18	<1	1.0
Weighted mean			0.031			0.0246						

* F = ratio of variances with corrected divided by uncorrected variance with the exceptions marked when the contrary is shown.

** Corrected value divided by uncorrected value; listed only when the F value was significant.

TABLE IV

MU VALUES OBTAINED UNDER TEMPERATURE-GRADIENT CONDITIONS

Results of four separate determinations are shown. The residuals from the linear-regression calculation should be compared with the generally acceptable S.E. (0.026).

Steroid	Isothermal			Residuals in linear regression; MU units			
	MU	n	S.E.M.	Expt. 1	Expt. 2	Expt. 3	Expt. 4
α A-3 α olPt-17oxo	27.2878	20	0.027	-0.0201	-0.0263	-0.0331	-0.0037
β A-3 α olPt-11 β ol-17oxo	28.8487	18	0.025	+0.0067	+0.0062	+0.0130	-0.0212
β A-3 α ,17 β diolPt	29.4246	9	0.024	+0.0837	+0.0772	+0.0982	+0.0703
β P-3 α ,20 α diolPt	31.4273	19	0.023	-0.1250	-0.1278	-0.1274	-0.1109
α C-3 α OBu	32.1686	62	0.013	+0.0716	+0.0738	+0.0594	+0.0755
S.E.M. at mid-point of regression				0.0952	0.0969	0.0980	0.0856

reach significance ($F = 2.1$; 4,4 DF; $P > 5\%$). It was considered that MU values obtained by method 3 would not be accurate enough for MU analysis although they might be suitable for other applications where accuracy was not so important.

Errors in using the correction procedure

On investigating the errors in results obtained with the correction procedure in comparison with results obtained at 275°, 13 out of 17 showed a significant increase (Table III) although with 1 pair (5 α -pregnane) the errors decreased and in 3 pairs the variance ratio was not large enough to be significant. The ratio of the S.E.M. values varied from 0.8 to 2.24 and thus it is likely that both methods will give the same mean value for the MU_c value if the correction procedure uses 2 or 3 times as many replicates.

MU analysis in the steroid series

The temperature-correction procedure for steroid MU values makes possible a general MU analysis in the steroid series, a procedure analogous to R_M analysis. Furthermore, the temperature coefficient may be used to calculate a temperature change likely to increase the resolution between a steroid and a contaminant which is probably an *n*-alkane derivative. Further aspects of this application and general MU analysis in the steroid series will be discussed elsewhere.

It is suggested above that the partition properties of steroids differ from those of straight-chain hydrocarbons because of the presence of the four rigid rings. This might also apply to other substances with ring systems thus expediting their MU analysis by a temperature-correction procedure similar to that described for the steroids.

ACKNOWLEDGEMENTS

Mr. R. M. Leone determined many of the MU values during the course of other studies and his help is warmly acknowledged.

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