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AN EXTENSION OF THE "STEROID NUMBER" CONCEPT TO RELATIONSHIPS BETWEEN THE STRUCTURE OF STEROIDS AND THEIR GAS-CHROMATOGRAPHIC RETENTION TIMES OBSERVED WITH SELECTIVE PHASES

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SUMMARY

The "steroid number" concept was described in an earlier paper; this provides a means of correlating the structure of steroids with their retention-time behavior in gas–liquid chromatography. Steroid numbers determined with a non-selective phase (methylsilicone polymer) are independent of small changes in experimental variables (flow rate, amount of phase, temperature) which are difficult to control with high precision in ordinary gas-chromatographic apparatus. The possible extension of this concept to work with selective phases (neopentylglycol succinate polyester and fluoroalkylsilicone polymer) has been explored. The variation of steroid numbers with temperature (over a 20° range) is larger in some instances when a selective phase is used than was observed with methylsilicone polymer. The values are essentially independent of flow rate and amount of phase over the useful range of experimental conditions. Steroid numbers determined with a selective phase may be used to indicate the number, nature, position and stereochemical arrangement of the functional groups which are present.

INTRODUCTION

The "steroid number" concept was advanced by VANDENHEUVEL AND HORNING[1] in order to describe relationships between the structure of steroids and their retention times observed in gas–liquid chromatography. The theoretical foundation for this concept was drawn from the work of MARTIN[2], BATE-SMITH AND WESTALL[3] and BUSH[4]; according to earlier work dealing with the partition-chromatographic separation of steroids (by paper partition chromatography) the following relationship should hold:

$$\log r = \log s + \log f_1 + \ldots + \log f_n$$

Abbreviations: SE-30, methylsilicone polymer; NGS, neopentylglycol succinate polyester; QF-1, fluoroalkylsilicone polymer; Gas Chrom P, a diatomaceous-earth preparation; TMSi, trimethylsilyl; TFA, trifluoroacetate; DMH, *N,N*-dimethylhydrazone.

where r is the relative retention time for a steroid, s is the relative retention time of the parent steroid nucleus and $f_1 \ldots f_n$ are values characteristic of the functional groups which are present. CLAYTON[5] proposed the same relationship, although expressed in a different way, and KNIGHTS AND THOMAS[6] used the expression of BATE-SMITH AND WESTALL[3]:

$$\log r = \log r_N + \Sigma \Delta R_{Mg}$$

to confirm the fact that the gas–liquid chromatographic behavior of steroids was in accord with what would be expected for a partition-chromatographic separation. The chief difficulty in the extension of these elements of earlier theory, developed for paper partition chromatography, to gas–liquid chromatographic work arises from the fact that relative retention times are strongly temperature dependent. Consequently, values of f_n and ΔR_M, are temperature dependent, and are subject to the same element of inexactness in interlaboratory comparisons as relative retention times[1]. By transforming the basic expression relating structure to retention time to the form:

$$SN = S + F_1 + \ldots + F_n$$

where SN is the steroid number, S is the number of C atoms in the steroid nucleus, and $F_1 \ldots F_n$ are values characteristic of the functional groups which are present, and by defining the steroid number in experimental terms as a value determined from retention times relative to two reference steroids rather than one, it was possible to establish a set of steroid-number values which were temperature independent over a relatively wide range of temperature. These values may be used for studying relationships between structure and gas–liquid chromatographic behavior with relatively little dependency on the establishment of highly specific experimental conditions for the separation. The validity of this concept was established by studies with a non-selective phase (SE-30), and values of F_n were determined for many commonly occurring functional groups[1]. At the same time the gas-liquid chromatographic behavior of steroids with selective phases was studied through the use of the T-value procedure of HAAHTI, VANDENHEUVEL AND HORNING[7] rather than by the steroid-number method. The present study was undertaken to determine if the steroid-number concept has validity for work with selective phases as well as for work with non-selective phases. In this event, temperature dependent entities including T, ΔR_{Mg}, and f_n values might well be replaced to good effect with temperature independent F_n values.

The aim of these studies is to provide an effective way of correlating gas-chromatographic behavior with structure. The procedures are not regarded as constituting a proof of structure, but they provide a way of reaching tentative structural conclusions for substances available in microgram amounts and possibly in a mixture.

EXPERIMENTAL PROCEDURES

A Barber–Coleman model 10 gas chromatograph, equipped with 6 ft × 4 mm glass U-tubes and an argon-ionization detection system, was used for the determination of relative retention times. The "flash heating" zone was maintained at 50° above the column temperature, and the detection cell was maintained at 240°. Pressures of 15–20 lb/in^2 were used; the flow rate or retention time for a reference substance

TABLE I

STEROID NUMBERS FOR REPRESENTATIVE STEROIDS DETERMINED AT 222° WITH NGS AND QF-1 LIQUID PHASES

Conditions: 6 ft × 4 mm glass U-tube; 222°; 40 ml/min argon flow; liquid phase 1% NGS or 2% QF-1 on 100–120 mesh silanized Gas Chrom P.

Steroid	*Steroid number*	
	NGS	*QF-1*
Androstane-3β,17β-diol	33.9	28.6
Androstane-3α,17β-diol	33.3	28.3
Androstane-3β-ol-17-one	33.5	30.6
Androstane-3α-ol-17-one	32.9	30.3
Androstane-3,17-dione	34.0	33.9
4-Androstene-3,17-dione	35.8	35.6
4-Androstene-17β-ol-3-one	36.1	33.3
4-Androstene-17α-ol-3-one	35.8	33.2
4-Androstene-3,11,17-trione	39.0	37.6
4-Androstene-11β-ol-3,17-dione	41.7	38.1
Estrone	38.6	30.9
5α-Pregnane-3β,20β-diol	34.9	30.2
5α-Pregnane-3β,20α-diol	35.6	30.6
5α-Pregnane-3β-ol-20-one	35.0	32.1
5α-Pregnane-20β-ol-3-one	35.3	33.1
5α-Pregnane-3,20-dione	35.3	35.0
5α-Pregnane-20β-TMSi ether-3-one	31.2	32.0
5α-Pregnane-3β-ol-20-one DMH	33.8	29.9
Pregnane-3β,20β-diol	34.0	29.3
Pregnane-3β,20α-diol	34.6	29.7
Pregnane-3α,20β-diol	34.5	29.8
Pregnane-3β-ol-20-one	33.9	31.2
Pregnane-3α-ol-20-one	34.5	31.6
Pregnane-3,20-dione	35.0	34.7
4-Pregnene-3,20-dione	37.2	36.8
Cholestanol	34.7	32.2
Epicholestanol	34.2	31.7
Coprostanol	33.9	31.3
Epicoprostanol	34.2	31.8
Cholesterol	35.1	31.9
Cholestanyl TMSi ether	30.1	30.4
Epicholestanyl TMSi ether	28.3	29.1
Coprostanyl TMSi ether	28.3	29.3
Epicoprostanyl TMSi ether	29.3	29.2
Cholesteryl TMSi ether	30.1	30.3
Cholestanyl methyl ether	31.2	30.5
Cholesteryl methyl ether	31.4	30.2
Cholestanyl TFA	30.4	32.0
Cholesteryl TFA	30.2	31.5
Cholestanyl acetate	34.3	34.1
Cholesteryl acetate	34.5	34.0
Cholestane-3-one	35.0	35.1
Cholestane-3-one DMH	33.6	32.2
4-Cholestene-3-one	36.9	37.0
4-Cholestene-3-one DMH	34.9	32.9
Cholesteryl valerate*	37.5	37.0
Cholesteryl heptanoate*	39.8	39.2

* Reference substances for determination of steroid numbers.

TABLE II

STEROID-NUMBER CONTRIBUTIONS FOR REPRESENTATIVE FUNCTIONAL GROUPS DETERMINED WITH NGS AND QF-1 LIQUID PHASES

The steroid-number contributions were determined at 222°; for conditions see Table I.

Functional group	*Steroid parent*	*Steroid number (F)*	
		NGS	*QF-1*
Δ^5	cholestanol/cholesterol	0.4	—0.3
Δ^5	cholestanyl methyl ether/ cholesteryl methyl ether	0.2	—0.3
Δ^5	cholestanyl TFA/ cholesteryl TFA	—0.2	—0.5
Δ^5	cholestanyl TMSi/ cholesteryl TMSi	—0.1	—0.1
3-One	cholestane	8.0	8.1
3-One DMH	cholestane	6.6	5.2
3-One-Δ^4	cholestane	9.9	10.0
3-One-Δ^4 DMH	cholestane	7.9	5.9
11-One (4-ene)	androstene	3.2	2.0
17-One	androstane	6.9	6.7
20-One	5α-pregnane	6.3	5.9
3α-Ol(*ax.*)	cholestane	7.2	4.7
3β-Ol(*ax.*)	coprostane	7.2	4.6
3α-Ol(*eq.*)	coprostane	7.5	5.1
3β-Ol(*eq.*)	cholestane	7.7	5.2
3α-Ol(*ax.*)TMSi	cholestane	1.3	2.1
3β-Ol(*ax.*)TMSi	coprostane	1.6	2.6
3α-Ol(*eq.*)TMSi	coprostane	2.6	2.4
3β-Ol(*eq.*)TMSi	cholestane	3.1	3.4
3β-Trifluoro-acetoxy(*eq.*)	cholestane	3.4	5.0
3β-Methoxy(*eq.*)	cholestane	4.2	3.5
3β-Acetoxy(*eq.*)	cholestane	7.3	7.1
11β-Ol(*ax.*)-(4-ene)	androstene	5.9	2.5
17α-Ol(*sec.*)	androstane	7.0	4.2
17β-Ol(*sec.*)	androstane	7.3	4.3
20α-Ol	5α-pregnane	6.9	4.4
20β-Ol	5α-pregnane	6.3	4.0
20β-OlTMSi	5α-pregnane	2.2	2.9
A/B *cis*	5α-pregnane/5β-pregnane	—0.3	—0.3
A/B *cis*	cholestane/coprostane	—0.3	—0.3

is specified in the Tables. Column packings were prepared as described in an earlier paper, and the experimental procedures were also the same as described earlier[1].

The steroid-number value for stigmastane was determined with androstane and cholestane as reference substances, and with an SE-30 (non-selective liquid phase) column; a value of 28.95 was found for the hydrocarbon. Cholestane and stigmastane were then used as reference substances with NGS and QF-1 columns to determine steroid numbers for the following cholesteryl esters: acetate, propionate, butyrate, valerate and heptanoate. The valerate was chosen as an appropriate reference substance for determining relative retention times for the steroids listed in Table I; the heptanoate was used as a second reference substance, and the method of determination of a steroid number with an NGS phase is shown in Fig. 1. The same procedure was used with data obtained with a QF-1 phase to determine the steroid-number values in Table I.

The functional group values in Table II were calculated from the steroid-number values for androstane, 5α- and 5β-pregnane, cholestane and coprostane derivatives listed in Table I.

Derivatives were prepared by published procedures (trifluoroacetoxy derivatives by the method of VANDENHEUVEL, SJÖVALL AND HORNING[8], TMSi ethers by the procedure of LUUKKAINEN, VANDENHEUVEL, HAAHTI AND HORNING[9], and *N,N*-dimethylhydrazones according to VANDENHEUVEL AND HORNING[10].

The experimental data given in the examples were obtained with columns previously used for the determination of steroid-number values.

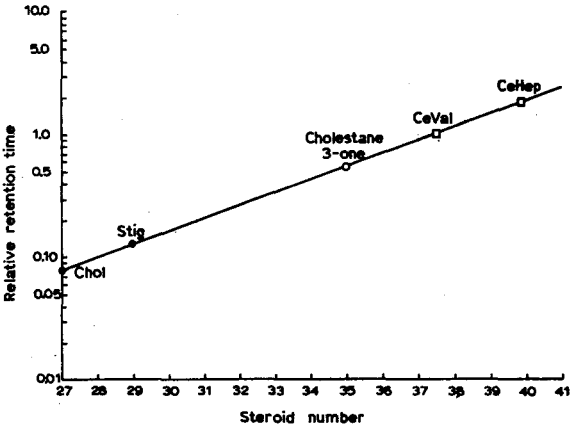

Fig. 1. Steroid-number chart used to determine steroid numbers from relative retention times with a selective phase (1% NGS at 222°). The retention times of cholestane (Chol) and stigmastane (Stig) (whose steroid-number values are 27 and 29, respectively, by definition) relative to cholesteryl valerate (CeVal) are used to determine the straight line. Using this line and the relative retention times of CeVal and cholesteryl heptanoate (CeHep) steroid-number values are assigned to these compounds. These steroid-number values are then used as reference points for the determination of steroid-number values of other steroids. Thus the steroid number of cholestane-3-one, 35.0, can be obtained from its relative (to CeVal) retention time, 0.50, and the reference line.

RESULTS AND DISCUSSION

A method for determining steroid numbers was described earlier[1], and it is obvious that the procedure might be extended to comparable determinations carried out with selective phases. However, in considering this problem it was evident at the outset that a modification of experimental technique would be required before a test of the steroid-number hypothesis could be made for a selective phase. Although androstane (steroid number 19) and cholestane (steroid number 27) were suitable reference substances for use with a non-selective phase, these compounds were not useful for the same purpose when separations were carried out with a selective phase. Conditions for the separation of steroids with two or more functional groups were such that androstane and often cholestane were present in the solvent front. The steroid number for stigmastane was determined experimentally with a non-selective phase (SE-30) and found to be 28.95; this was considered sufficiently close to the expected value of 29.0 to warrant the use of stigmastane as a third reference substance with steroid number 29.0. However, additional reference substances were needed, and after several experimental trials cholesteryl valerate and cholesteryl heptanoate were

chosen as secondary reference standards. These compounds are currently available in high purity, and their retention times with selective phases are such that both monofunctional and polyfunctional steroids can be studied.

Fig. 1, which is analogous to Fig. 1 of the earlier paper[1], shows the procedure for determining a steroid number with a column containing NGS as the stationary phase. The steroid numbers of the secondary reference substances were determined separately and were found to be 37.5 and 39.8, respectively, for cholesteryl valerate and cholesteryl heptanoate. The valerate ester was then used to determine relative retention times at 222° for the heptanoate and the steroid under study. The steroid number for cholestane-3-one, determined as indicated in the Figure, was found to be 35.0. The F_{NGS} value for the 3-one group is therefore 8.0. Table I contains steroid-number values determined for a number of steroids at 222° with NGS (1%) and QF-1 (2%) columns. Steroid-number values determined with NGS and QF-1 for the two reference substances are included in the Table. Values for F_{NGS} and $F_{QF\text{-}1}$ for commonly occurring functional groups are in Table II; these values are derived from the data in Table I.

TABLE III

VARIATION OF STEROID NUMBER WITH FLOW RATE

Column: 1% NGS, 232°. Steroid numbers were determined with cholesteryl valerate and cholesteryl heptanoate as reference substances.

Steroid	*Steroid numbers*		
	Flow rate (ml/min)		
	24	*40*	*60*
Cholestanyl TFA	30.4	30.4	30.4
Androstane-3,17-dione	34.1	34.0	34.1
5α-Pregnane-3β,20β-diol	35.0	34.9	34.9
Estrone	38.5	38.6	38.5

The major issue relating to the usefulness of these steroid number and F values is much the same as that posed earlier for work with a non-selective liquid phase. If minor changes in experimental conditions (amount of phase, flow rate or temperature) were to lead to different steroid number values, the usefulness of these data would be severely restricted since it would be difficult with current equipment to make interlaboratory comparisons of data. Table III shows the effect of relatively large changes in flow rate on steroid numbers determined for several steroids. The relative constancy of the steroid numbers is not unexpected, in view of an earlier finding of the same nature for a non-selective phase. Table IV shows the effect on steroid numbers of a change in the amount of phase. Very little variation would be expected, unless the surface of the phase or the support participate in the chromatographic process. There is in fact very little effect resulting from the doubling of the amount of liquid phase (although the observed retention times are of course widely different when the amount of phase is changed). For convenience the column conditions chosen for steroid-number determinations in Table I were 1% NGS and 2% QF-1. Table V shows the effect on steroid numbers of varying the temperature of the determination. Relative retention times are strongly temperature dependent,

TABLE IV

VARIATION OF STEROID NUMBER WITH AMOUNT OF LIQUID PHASE

Conditions: 222°; 40 ml/min. The steroid numbers of cholesteryl valerate and heptanoate were determined with cholestane and stigmastane as reference substances. The others were determined with cholesteryl valerate and cholesteryl heptanoate as reference substances.

Steroid	*Steroid number*			
	NGS		*QF-1*	
	1%	*2%*	*1%*	*2%*
Cholesteryl valerate	37.5	37.5	37.1	37.0
Cholesteryl heptanoate	39.8	39.9	39.4	39.2
Cholestanol	34.7	34.8	32.3	32.2
Cholestane-3-one	35.0	35.0	35.1	35.1
Androstane-3,17-dione	34.0	34.1	33.7	33.9

TABLE V

VARIATION OF STEROID NUMBER WITH TEMPERATURE

Column: 1% NGS; 40 ml/min. The steroid numbers were determined with cholesteryl valerate and cholesteryl heptanoate as reference substances.

Steroid	*Steroid number*		
	212°	*222°*	*232°*
Cholesteryl TFA	30.0	30.2	30.3
Coprostanol	33.8	33.9	34.0
Coprostanyl TMSi ether	28.3	28.3	28.3
4-Cholestene-3-one	36.8	36.9	36.9
5α-Pregnane-3β,20β-diol	34.8	34.9	34.9
5α-Pregnane-3,20-dione	35.1	35.3	35.3
5β-Pregnane-3,20-dione	34.9	35.0	35.0

but nevertheless the steroid numbers for these representative steroids are virtually temperature independent over a range of 20°. Small steroid-number changes might well be expected, since it is known that there are small differences in the rate of change of partition coefficients with temperature for compounds with different functional groups. A consequence of this effect is seen in Table V; a TMSi ether shows no change in steroid-number value over a 20° temperature range, while a TFA derivative shows a change of 0.3 over the same temperature range. However, these differences are small, and for practical purposes it may be considered that steroid-number values obtained with selective phases are essentially temperature independent over the useful range of separation temperatures.

The present study is limited to gas–liquid chromatographic behavior observed with NGS and QF-1 liquid phases. These were chosen because they have been used in many steroid separations, and because relative retention times observed with these phases vary with the number, nature, positional and stereochemical arrangement of functional groups. Steroid numbers may be determined readily for derivatives as well as for parent compounds, and these techniques may therefore be used to obtain rather extensive structural information. Two brief examples of the use of these data are given in the following section; in the first the calculated values for

a steroid are compared with the experimental values, and in the second an identification problem is described.

Example 1. Pregnane-3α,20α-diol is an example of a commonly occurring bifunctional steroid. Calculated steroid-number values for the gas–liquid chromatographic behavior of this steroid with NGS and QF-1 liquid phases at 222° may be arrived at from F values in Table II of this paper:

	Steroid number NGS	*Steroid number QF-1*
C_{21} steroid, A/B *trans*	21.0	21.0
3α (*eq.*)-ol	7.5	5.1
20α-ol	6.9	4.4
A/B *cis*	—0.3	—0.3
Calc.	35.1	30.2
Found	35.1	30.2

The relative retention time of pregnane-3α,20α-diol was not used for the determination of these F values, and therefore the summation of values represents in each case a calculation of data derived from other steroids. The experimentally determined steroid-number values for the steroid are shown for comparison with the calculated values. The correspondence is good.

Example 2. Steroid numbers were determined for a compound whose identity was unknown. The values were 34.2 (NGS) and 31.6 (QF-1). The difference in steroid numbers suggested that an hydroxyl group was present (F_{NGS} and $F_{QF\text{-}1}$ values are the same for a ketone group, but are different by about 2.5 for an unhindered secondary hydroxyl group) and the compound was therefore treated with hexamethyldisilazane[9]. A derivative was obtained; the steroid-number values for the TMSi ether were 29.0 with NGS and 30.0 with QF-1.

A steroid-number determination with SE-30 would have determined the molecular size of the original compound, but it was also possible to arrive at a tentative conclusion in another way. The QF-1 values were used for preliminary calculations with the assumption that the original compound was in the C_{19}, C_{21} or C_{27} series.

	C_{19} series:	*C_{21} series:*	*C_{27} series:*
Steroid-number value	31.6	31.6	31.6
Steroid nucleus	—19.0	—21.0	—27.0
	12.6	10.6	4.6
Possible groups	ketone and hydroxyl	ketone and hydroxyl	hydroxyl

To determine if a reactive ketone group was present the steroid was treated with *N,N*-dimethylhydrazine, both with and without added acetic acid[10]. A derivative was formed without the need for an acid catalyst, indicating that a highly reactive ketone group was present (this effect is characteristic of the 3-one group). The steroid-number values were 32.7 (NGS) and 28.7 (QF-1) for the DMH derivative. On the basis of these data, the original compound was considered to have both a ketone and an hydroxyl group, with a 3-one structure as the most likely possibility for the ketone group, and with a steroid nucleus in the C_{19} or C_{21} series.

The QF-1 data were used to draw tentative structural conclusions.

	C_{19} *series*:	C_{21} *series*:
Steroid-number value	31.6	31.6
Steroid nucleus	—19.0	—21.0
3-one	—8.1	—8.1
	4.5	2.5

A value of 4.5 for an hydroxyl group corresponds to what would be expected for a 3-ol(*ax.*), a 17β-ol(*sec.*) or a 20α-ol group. A value of 2.5 is possible only for a highly hindered hydroxyl group. A possible structure was therefore androstane-17β-ol-3-one. The steroid-number values for the original compound and the DMH derivative were calculated for both phases:

Androstane-17β-ol-3-one	*QF-1*:	*NGS*:
Steroid nucleus	19.0	19.0
17β-ol(*sec.*)	4.3	7.3
3-one	8.1	8.0
	31.4	34.3
Found	31.6	34.2
Androstane-17β-ol-3-DMH		
Steroid nucleus	19.0	19.0
17β-ol(*sec.*)	4.3	7.3
3-DMH	5.2	6.6
	28.5	32.9
Found	28.7	32.7

A value was not assigned to a 17β-TMSi ether group in Table II. The values found in this experiment were 2.0 (NGS) and 2.9 (QF-1). These values are not incompatible with a *sec.*-TMSi ether structure.

A tentative identification of the steroid as androstane-17β-ol-3-one was confirmed by gas–liquid chromatographic comparison of the original material and its derivatives with an authentic sample and authentic derivatives with both NGS and QF-1 phases. Complete correspondence was observed. The structural conclusion was confirmed by the supplier of the steroid.

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REFERENCES

1 W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochim. Biophys. Acta*, 64 (1962) 416.
2 A. J. P. MARTIN, *Biochem. Soc. Symp. (Cambridge, Engl.)*, 3 (1949) 4; *Ann. Rev. Biochem.*, 19 (1950) 517.
3 E. C. BATE-SMITH AND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
4 I. E. BUSH, *The Chromatography of Steroids*, Pergamon, Oxford, 1961, Chapters I, II and V.
5 R. B. CLAYTON, *Biochemistry*, 1 (1962) 357.
6 B. A. KNIGHTS AND G. H. THOMAS, *Nature*, 194 (1962) 833.
7 E. O. A. HAAHTI, W. J. A. VANDENHEUVEL AND E. C. HORNING, *Anal. Biochem.*, 2 (1961) 344.
8 W. J. A. VANDENHEUVEL, J. SJÖVALL AND E. C. HORNING, *Biochim. Biophys. Acta*, 48 (1961) 595.
9 T. LUUKKAINEN, W. J. A. VANDENHEUVEL, E. O. A. HAAHTI AND E. C. HORNING, *Biochim. Biophys. Acta*, 52 (1961) 599.
10 W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochim. Biophys. Acta*, 74 (1963) 560.