CHROM. 10,722

PREDICTION OF THE RELATIVE MOLAR FLAME IONIZATION RESPONSE FOR STEROIDS

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SUMMARY

The molar flame ionization detection response of steroids is linearly related with both proportional and constant factors to the effective carbon number. The latter is the number of carbon atoms per molecule less half the number of oxygens. The 30 steroid derivatives employed possessed from 18 to 31 carbon atoms and from 0 to 4 oxygen atoms. Chlorine and sulphur were without effect.

INTRODUCTION

The gas–liquid chromatographic (GLC) determination of a series of substances using flame ionization detection (FID) requires values for the relative flame ionization detector response for each substance. For natural products such as the urinary steroids it may not be possible to prepare in pure form every reference substance required for accurate determination of FID response and an alternative procedure becomes valuable.

Perkins *et al.*[1] established that the molar FID response (F_M) of long-chain paraffin hydrocarbons, alcohols and carboxylic esters was linearly related to the effective carbon number, C_e, which they defined as the number of carbon atoms in each molecule corrected for the effect of other atoms. Hydrogen had no effect in the hydrogen flame and two oxygen atoms eliminated the effect of one carbon atom. The present study was initiated to confirm that the effective carbon number procedure applied in the steroid series.

EXPERIMENTAL

Methods

The GCD gas chromatograph (Pye-Unicam, Cambridge, Great Britain) was used at constant temperature and constant gas phase flow-rate (nitrogen, 40 ml/min). The hydrogen flame (50 ml/min from a GEC generator) was fed by air (300 ml/min). 3% OV-1 stationary phase on Chromosorb was used in 2.1 m × 4 mm I.D. glass tubes. Output potential was recorded on a Servoscribe instrument with voltage sensitivity adjusted to set the peak output between 50 and 100% of chart width.

Steroids were injected in solution in pyridine or ethanol (1 μl samples) from GCE syringes. Solutions were prepared by weight and included 5α-cholestan-3α-butoxide as internal standard. This procedure made possible arbitrary dilutions which helped to ensure that observed FID responses were not limited by solubility.

Steroids showing signs of contaminants were replaced by other samples or were recrystalized.

Steroids

The reference steroids were (1) commercial samples, (2) from the Medical Research Council reference steroid collection (Professor W. Klyne) or (3) prepared in this laboratory.

Purity was assessed using, where relevant, the following criteria: (1) melting point, (2) ultraviolet and infrared spectra, (3) fragmentation pattern in the mass spectrometer, (4) that the methylene unit (*MU*) value[2] was as previously observed or as calculated from increment values to within 0.04 *MU* (2 × standard error of the mean (S.E.M.) replicated observations) and (5) GLC of the substance by itself demonstrated no inhomogeneity or decomposition peaks with suitable sensitivity adjustment (0.2% was used as upper limit of tolerated contaminant).

RESULTS AND CALCULATIONS

The molar FID values are presented in ratio form with respect to 5α-cholestan-3α-butoxide (Table I).

Temperature effect

The observed relative molar FID responses did not vary with changing temperature in the range 270–305°.

Molar FID ratios

The molar FID response is measured as A_x/Q_x where A_x is the elution peak area and Q_x is the molar quantity of substance x. Using the retention time, R_x, and peak height, H_x, product as proportional to A_x and including proportionality and constant factors it is expected that:

$$R_x H_x/Q_x = FC_{ex} + G$$

where C_{ex} is the effective carbon number[1] of substance x.

The original investigations[1] were carried out by direct injection into the flame and thus G was zero. The present use of GLC with FID involves extra instrument and column characteristics and the effects of these are included in F and G and are also partially compensated by incorporation of the internal standard (designated s) in the determination run.

The relative molar FID value, $_rF_M$, as measured is:

$$\frac{R_x H_x Q_s}{Q_x R_s H_s} = \frac{FC_{ex} + G}{FC_{es} + G} = JC_{ex} + K$$

TABLE I

RELATIVE MOLAR FID RESPONSE VALUES

Multiple values indicate replication in different batches. Each value is mean of three or more determinations within the batch.

Based on the abbreviated steroid terminology of Bush[3]; O = oestrane; A = androstane; P = pregnane; G = cholane, C = cholestane; olAc = acetoxy; olPt = propoxy; thiolAc = acetothiol; OPr = propoxide; OBu = butoxide; oicMeEst = carboxylic acid methyl ester; Etkal = ethylene ketal. C_e = Effective carbon number[1] = number of carbon atoms in the molecules less half the number of oxygen atoms.

Steroid	C_e	Molar FID response relative to internal standard (5α-cholestan-3α-butoxide) and GLC temperature
O-3ol-17one	17	0.278, 270; 0.282, 286
O-3ol-17αol	17	0.274, 286
αA-3βol-17one	18	0.368, 270; 0.351, 296
A⁴-3one-17one	18	0.352, 270; 0.352, 296
αA-3one	18 5	0 409, 270
αA-17one	18.5	0.343, 270
αA-3βCl-17one	18.5	0.365, 270; 0.361, 286
A⁵-3βCl-17one	18.5	0.382, 0.386 and 0.395
αA-3αthiol-17one	18.5	0.376, 261; 0.381, 270
αA	19	0.381, 270; 0.375, 276
		0.368 and 0.366, 286
		0.368 and 0.364, 296
p⁵-3βol-20one	20	0.425, 270; 0.387, 296
p⁴-3one-20one	20	0.425, 270; 0.412, 296
αA-3αthiolAc-17one	20	0.436, 270; 0.449, 285
A⁵-3βthiolAc-17one	20	0.450, 270; 0.458, 285
αA-3αolPt-17one	20.5	0.509, 296
A⁵-3βolPt-17one	20.5	0.474, 305
αP	21	0.515 and 0.490, 270; 0.489, 276
		0.483 and 0.478, 286; 0.482 and 0.476, 296
βP	21	0.460, 270
αA¹-3,17diEtkal	21	0.501, 261; 0.507, 285
βA-3α,17β-diolPt	23	0.580, 296, 0.668, 305
αG-3αol-24oiMeEst	23.5	0.649 and 0.655, 286
G⁵-3βol-24oicMeEst	23.5	0.635 and 0.647, 286
βP-3α,20αdiolPt	25	0.693, 270
αC-3βol	26.5	0.811, 270; 0.807, 286
αC-3one	26.5	0.828, 270
αC	27	0.884 and 0.852, 270; 0.873, 276
		0.884 and 0.867, 286; 0.889 and 0.870, 296
βC	27	0.877, 270
αC-3βolAc	28	0.902 and 0.893, 286
αC-3βOPr	29	0.957, 296
αC-3α-OPr	29.5	0.982, 0.987 and 0.991, 286
αC-3α-OBu	30.5	1*

* Reference value and internal standard.

J and K are constants but cannot be related to F and G because algebraic manipulation shows that $(1 - K)/J = C_{es}$.

Three variable linear regression applied to the values in Table I gave:

$$_rF_M = 0.0571 \, (C_n - 0.499.O_n) - 0.697$$

where C_n and O_n are the number of carbon and oxygen atoms respectively in each molecule.

The value in parentheses is the C_e value and confirms that, within a reasonable error,

$$C_e = C_n - 0.5.O_n$$

To further illustrate the value of using this relationship the $_rF_M$ and C_e relationship is illustrated graphically in Fig. 1.

Fig. 1. Relationship of the relative molar FID response, $_rF_M$, and the effective carbon number value, C_e, for 30 steroid derivatives. The two-variable linear regression line shown has the equation

$$_rF_M = 0.0578C_e - 0.710$$

$_rF_M$ is expressed relative to α-cholestane-3α-butoxide internal standard and C_e is the number of carbon atoms per molecule less half the number of oxygen atoms. ×, Single points; ⊙ two points coinciding; ●, three points coinciding.

Examination of the residuals in the three variable linear regression shows (1) that the error histogram approximates to a normal distribution, (2) that the residuals show no systematic relationship to C_e and (3) that the standard error is 0.0497 on the C_e scale.

DISCUSSION

The systematic understanding of the relationship of molecular structure and FID response has generally been approached empirically although attempts have

been made to understand the processes in the flame and particularly the mechanism for the production of ions but with no useful outcome[4].

Examination of FID response values expressed on a weight basis lead to pronounced curved relationships between response and quantity and lead to the use of "normalization factors" which had to be determined for each substance[5]. The change to molar units lead to linear relationships with number of carbon atoms per molecule[6], but anomalies were recognized for example with short-chain fatty acids[7]. Results were lower than expected on the basis of results from longer chain fatty acids and it was suggested that there were non-effective carbon atoms associated with the carboxyl groups[7,8]. This carbon-deficit was of the order of one for each pair of oxygens in the molecule.

Meanwhile the association of oxygen in the molecule with diminished FID response, showed clearly in comparison of results from a series of paraffin hydrocarbons and from the analogous alcohols and lead[1] to the concept of an effective carbon number which is the number of carbon atoms in the molecule diminished by half the number of oxygens. Here, linearity is achieved by correcting all the molecules and not just the lower members of the homologous series. It is interesting to note that among the early data was the observation of a good fit in linear regression of $_rF_M$ and the number of carbons in the fatty acid moiety for the series of methyl esters[9]. Here this number happens to be the same as the effective carbon number.

The present investigations confirm that the molar FID response is proportional to the effective carbon number in the steroid series. Three variable regressions showed that the effective carbon number of oxygen was -0.5, on rounding off, in confirmation of the earlier observations[1] and as expected in the intramolecular consumption of carbon in CO_2 production. The standard error in the present study, 0.0497, is some 10% of the value of the effective carbon number of oxygen and it is thus worthwhile to correct for the effect of oxygen in calculation of C_e, even when there is only one oxygen present. Another aspect arises in comparison of two variable regressions with and without the oxygen correction when the variance shows a 6.9-fold increase; as the 1% significance level for F is 1.8, the change associated with the oxygen correction is highly significant and errors are reduced.

Perkins et al.[1] suggested that the correction due to the presence of oxygen should vary with different functional groups. There is no evidence to support this proposition in the present study. Chlorine in two examples and sulphur in three showed no significant effect on C_e value.

The present extension of the use of the effective carbon number from simple substances to the complex steroids provides an interpolation procedure for obtaining the relative molar FID response values for steroids in general. The effective carbon number principle should also prove valuable in the GLC determination of other substances when FID is employed.

ACKNOWLEDGEMENT

REFERENCES

1 G. Perkins, R. E. Laramy and L. D. Lively, *Anal. Chem.*, 35 (1963) 360.
2 C. E. Dalgliesh, E. C. Horning, M. G. Horning, K. L. Knox and K. Yarger, *Biochem. J.*, 101 (1966) 792.
3 I. E. Bush, *Chromatography of the Steroids*, Pergamon, Oxford, 1961.
4 A. T. Blades, *J. Chromatogr. Sci.*, 11 (1973) 251.
5 I. Halasz and W. Schneider, *Anal. Chem.*, 33 (1961) 978.
6 L. S. Ettre, *J. Chromatogr.*, 8 (1962) 525.
7 F. J. Kabot and L. S. Ettre, *J. Gas Chromatogr.*, 1, No. 10 (1963) 7.
8 H. Binder and W. Lindner, *J. Chromatogr.*, 77 (1973) 175.
9 L. S. Ettre and F. J. Kabot, *J. Chromatogr.*, 11 (1963) 114.