Isotope effects in gas–liquid chromatography of steroids

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SUMMARY When gas–liquid chromatography was performed with steroids containing H^3 and C^{14}, the H^3/C^{14} ratio of the effluent was higher at the beginning and at the tail of the steroid peak. The H^3/C^{14} ratio of the entire peak agreed with that determined by paper and thin-layer chromatography. Thus, gas–liquid chromatography on polar and nonpolar phases commonly used for chromatography of steroids apparently has sufficient resolving power to partially separate isotopic species whose molecular weights differ by 2% or less.

KEY WORDS gas–liquid chromatography · isotope separations · isotope ratios · steroids · doubly labeled steroid derivatives · isotopic species · resolving power

SEVERAL APPLICATIONS of the combined use of radiolabeled compounds and gas–liquid chromatography (GLC) in biological research have been reported (1–3). In these studies, it has been assumed that labeled and unlabeled molecules behave identically. We have observed a nonuniform distribution of isotope ratios within fractions of the effluent peak of steroid derivatives. This may be without significance in many studies, but is important when double isotope derivative techniques are used with GLC, since a classical criterion of radiohomogeneity of the final steroid derivative is the constancy of the isotope ratios throughout the chromatographic peak.

METHODS

Testosterone-4-C^{14} and dehydroepiandrosterone-4-C^{14} (New England Nuclear Corp., Boston, Mass.), after purification by paper and thin-layer chromatography, were acetylated with acetic anhydride-1-H^3. Similarly, testosterone-1,2-H^3 and dehydroepiandrosterone-7α-H^3 were acetylated with acetic anhydride-1-C^{14}. After addition of 100 μg of unlabeled steroid acetate, each sample was purified to a constant H^3/C^{14} ratio by paper chromatography in ligroin–methanol 100:100 (v/v), followed by chromatography in decalin–methanol–water 100:50:50 (v/v) and silica gel thin-layer chromatography in benzene–ethyl acetate 80:20 (v/v). GLC was performed by means of a Glowall instrument using glass coiled columns, 6 ft × 3.4 mm, and an argon ionization detector (radium source). The columns, packed with either 1% neopentyl glycol succinate, 2% XE-60, or 2.5% SE-30 on Gas Chrom P were operated at temperatures ranging between 200 and 240°, and had 2000–3000 theoretical plates for cholestane. The column effluents were collected on p-terphenyl coated with 5% DC-550 (4). Quenching was not observed for either H^3 or C^{14}. Radioactivity was measured in a Packard Tri-Carb Scintillation Spectrometer operating at 18.4% efficiency for H^3 and 45% efficiency for C^{14} and using discrimination and gain settings such that less than 0.4% of the H^3 was counted in the carbon channel. Sufficient counts were accumulated so that the 2σ errors of the ratios were less than 5%.

RESULTS

The H^3/C^{14} ratio of the doubly labeled steroid was unchanged by GLC when the ratio of the entire effluent peak was measured (Table 1). However, when fractions were collected every 20 sec throughout the steroid peak, then the H^3/C^{14} ratio varied among the fractions as in Fig. 1. In all studies (Table 1), the observed H^3/C^{14} ratios of counts were higher in the early fractions of the peak. The H^3/C^{14} ratio progressively decreased to values lower than the ratio obtained before GLC. Often, the ratio increased again in the tail of the peak. Summation of H^3 and C^{14} counts in the fractions comprising the peak resulted in the same H^3/C^{14} ratio as that obtained before GLC and that of the entire GLC peak.

The steroid acetates were then converted to their dimethylhydrazones (5), thus altering their characteristics on GLC, but not affecting the H^3/C^{14} ratios. Similar fractionation of the dimethylhydrazone peak again resulted in differences in H^3/C^{14} ratios throughout the peak (Table 1). A statistical analysis was made of the H^3/C^{14} ratios obtained in the various fractions of the steroid peak to determine if there was a nonrandom relationship with time. The analysis, based on the ranks of the ratios, indicated the existence of real trends. Replacing the observations by their ranks overcomes the problem that the ratios differ widely in precision. At the bottom of Tables 1 and 2 are listed the probabilities of obtaining the sequence in the absence of a time trend

TABLE 1 H^3/C^{14} RATIOS OF STEROID ACETATES BEFORE AND AFTER GLC

	Testosterone-4-C^{14} Acetate-1-H^3		Dehydroepiandrosterone-4-C^{14} Acetate-1-H^3		Dehydroepiandrosterone-4-C^{14} Acetate-1-H^3		Testosterone-4-C^{14} Acetate-1-H^3
		*DMH**		*DMH**		*DMH**	
Before GLC	0.70	0.70	0.26	0.26	0.34	0.34	8.6
GLC entire peak	0.71 (SE-30) 0.70 (NGS)	0.73 (SE-30)	0.26 (SE-30)	0.26 (SE-30)	0.34 (SE-30)	0.34 (XE-60)	8.6 (XE-60)
Fractionated every 20 sec	(SE-30)	(SE-30)	(SE-30)	(SE-30)	(SE-30)	(XE-60)	(XE-60)
	1.15	0.75	0.42	0.39	0.73	0.38	11.7
					0.44		
	0.88	0.77	0.31	0.33	0.37	0.33	10.5
	0.83	0.75	0.25	0.29	0.32	0.30 †	9.7
	0.75	0.73	0.21	0.23	0.30 †	0.37	9.2
	0.64	0.66	0.21 †	0.22	0.55	0.53	8.9
	0.57	0.63	0.26	0.24	0.85	0.72	8.9
	0.59 †	0.62 †			0.97		8.1
	1.00	0.71					7.4
	0.92						7.5
							7.8 †
							11.5
Weighted average	0.70	0.70	0.26	0.27	0.31	0.34	8.6
Probability	0.03	0.02	0.07	0.08	0.03	0.05	0.03

* Dimethylhydrazone.
† The difference between the indicated ratios is significant at $P < 0.05$.

TABLE 2 H^3/C^{14} RATIOS OF STEROIDS BEFORE AND AFTER GLC

	Dehydroepiandrosterone-7α-H^3 Acetate-1-C^{14}		Testosterone-1,2-H^3 Acetate-1-C^{14}		Dehydroepiandrosterone-7α-H^3 Acetate-1-C^{14}	Mixture of Testosterone-1,2-H^3 and Testosterone-4-C^{14}
Before GLC	4.1				0.59	7.2
GLC entire peak	4.1 (SE-30)		6.1 (SE-30)		0.63	
Fractionated peak (every 20 sec)	(SE-30)	(NGS)	(SE-30)	(NGS)	(SE-30)	(SE-30)
	5.6	4.9	7.3	7.6	0.86	9.0
	4.9	4.5	6.8	7.4	0.76	8.1
	4.8	4.4	6.6	7.1	0.66	7.3
	4.5	4.3	5.9	6.6	0.66	6.9
	4.4	4.0	6.0	6.3	0.64	6.7
	4.2	4.2 *	6.1 *	5.7 *	0.61	6.7 *
	4.0	4.5	6.4	6.0	0.58	6.9
	3.7 *	4.8		5.8	0.52 *	
	4.2				0.69	
	4.5				0.79	
					1.08	
Weighted average	4.3	4.2	6.5	6.3	0.63	7.3
Probability	0.02	0.03	0.06	0.03	0.01	0.05

* The difference between the indicated ratios is significant at $P < 0.05$.

These probabilities are all small, giving support to a real relation with time.

To determine whether the position of the tritium was critical, similar experiments were performed using the tritium-labeled steroid as the acetate-C^{14}. GLC fractionations were performed on two phases (Table 2). Again, the H^3/C^{14} ratios were higher at the beginning and tail of the steroid peak and decreased in the middle of the peak. These effects were seen on both polar and nonpolar phases. Finally, mixtures of testosterone-1,2-H^3 and testosterone-4-C^{14} were chromatographed on SE-30 without acetylation. As in the previous studies, the H^3/C^{14} ratios varied throughout the peak (Table 2). Bypassing the ionization detector with a stream splitter did not change the isotope effect.

Since the H^3/C^{14} ratios somewhat unexpectedly increased in the tail of the peak, we analyzed these differences independently of the previous statistical analysis. The brackets enclosing ratio pairs in Tables 1 and 2 indicate that the ratios are different ($P < 0.05$), using the equation for the Poisson distribution (6). A possible systematic error of binding of labeled steroid to the column and subsequent elution in another chromatography was ruled out by interpolating chromatography with unlabeled steroid acetates between runs and finding only traces of tritium in the eluates.

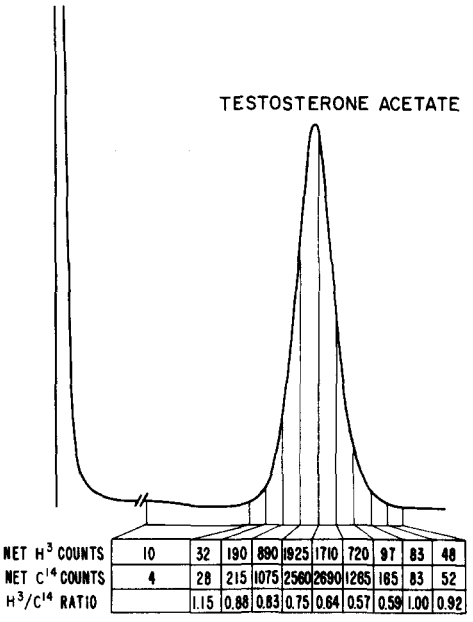

FIG. 1. Counts per minute of H^3 and C^{14} during elution of testosterone-4-C^{14}-acetate-H^3 from SE-30. The fractions were collected at approximately 20 sec intervals.

DISCUSSION

Our studies show that GLC with columns of 2000–3000 theoretical plates containing frequently used polar and nonpolar phases has sufficient resolving power to partially segregate isotopic species that differ in molecular weight by less than 6 parts in 350, or 2%. Thus an important criterion of radiohomogeneity, namely a constant H^3/C^{14} ratio within a steroid peak, cannot be applied when GLC is used in combination with the double isotope derivative technique. However, GLC can serve as a highly discriminative chromatographic system in the measurement of steroids by the double isotope derivative method if the H^3/C^{14} ratio of the entire peak is utilized.

The demonstration that GLC can partially separate species of labeled steroids might have been predicted from previous studies. Wilzbach and Riesz (7) separated cyclohexane from cyclohexane-D_{12} and reported similar separations with the tritium-labeled compounds. Similarly, deuterobenzene and benzene have been separated by GLC using capillary columns (8). Biemann (9) also noted that the hydrogen/deuterium ratio of labeled aniline varied throughout the GLC peak, in a study of similar design.

The quadratic nature of the H^3/C^{14} ratios throughout the steroid peak remains unexplained. On the basis of previous studies and the predicted increase in vapor pressure, the H^3-rich molecule would be expected to elute early. We are unable to explain satisfactorily the appearance in many studies of higher H^3/C^{14} ratios in the tail of the peak. It indicates, however, that there must be at least two processes contributing to this effect. Transient absorption and resorption of H^3-rich molecules due to interaction with the phase is one possible explanation for the late elution of H^3-rich molecules.

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