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GAS–LIQUID CHROMATOGRAPHY OF RADIOACTIVE AMINO ACIDS AS THEIR TRIFLUOROACETYL ESTER DERIVATIVES

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SUMMARY

A method is described for the gas chromatography of radioactive amino acids as their trifluoroacetylated ester derivatives. Comparative studies with the methyl, *n*-butyl and *n*-pentyl esters have been made. The absolute molar response of the flame ionization detector has been calculated for some of the derivatives.

INTRODUCTION

Using the flame ionization detector GEHRKE AND STALLING[1] analyse quantitatively twenty amino acids found in proteins by gas–liquid chromatography. Although this method is sensitive, quantitation relies on the preparation of pure standards which are used to determine relative molar responses. WATERFIELD AND DEL FAVERO[2] have described a rapid method for the purification of trifluoroacetyl (TFA) amino acid esters using liquid chromatography on silicic acid columns. Although after purification by this method the amino acid derivatives give a single peak on gas–liquid chromatography, it is not known if any of the amino acid derivatives are partially destroyed during gas–liquid chromatography resulting in the inaccurate determination of molar response factors.

In the present paper, the authors have used radioactive amino acids to investigate the synthesis of TFA amino acid esters and the possible breakdown of these derivatives during gas–liquid chromatography and to determine absolute response factors for the TFA amino acid esters using the flame ionization detector.

MATERIALS AND METHODS

Amino acids

Amino acids were supplied by the Radiochemical Centre, Amersham, Great Britain. U-^{14}C-L-aspartic acid, 6.1 mC/mmole; U-^{14}C-L-glutamic acid, 276 mC/mmole;

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U-^{14}C-glycine, 8.1 mC/mmole; 2-^{14}C-DL-hydroxyproline, 4.76 mC/mmole; U-^{14}C-DL-isoleucine, 150 mC/mmole; U-^{14}C-DL-leucine, 150 mC/mmole; L-3-phenyl-(U-^{14}C-alanine), 504 mC/mmole; U-^{14}C-L-serine, 160 mC/mmole; U-^{14}C-L-threonine, 208 mC/mmole; U-^{14}C-L-valine, 267 mC/mmole.

The radioactive amino acid (0.05 mC) was added to 25 mg non-radioactive amino acid and made up to 25 ml with water. This was stored at −20°. The specific activity of the starting solution was determined by adding 5 μl to 15 ml scintillation fluid (900 ml dioxane, 100 ml toluene, 50 ml ethanol, 60 g naphthalene, 4 g 2,5-diphenyloxazole (PPO) and 0.2 g 1,4-bis-2-[5-phenyloxazolyl]-benzene (POPOP)) in a counting vial. For all other counting the scintillation fluid consisted of 1 l toluene containing 4 g PPO and 0.1 g POPOP. The radioactivity (4,000–28,000 d.p.m.) was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314 (Packard Instrument Co. Inc., Box 428, La Grange, Ill., U.S.A.).

Preparation of TFA amino acid methyl, n-butyl and n-pentyl esters

Amino acids were esterified in dry alcoholic HCl and the esters were trifluoroacetylated by treatment with trifluoroacetic anhydride. Methyl esters were prepared by adding 2 ml redistilled dry methanol to 2 mg amino acid in a B14 test tube and dry HCl gas was bubbled continuously through the reaction mixture at 70° for 30 min. The alcohol was removed on a rotary evaporator using first a water pump and then an oil pump. The *n*-pentyl esters were similarly prepared with the reaction mixtures maintained at 108° for 30 min. The *n*-butyl esters were prepared by the interesterification procedure of GEHRKE AND STALLING[1]. The method of trifluoroacetylation was the same for all samples. To the dried ester hydrochloride residues in the test tube, 0.2 ml trifluoroacetic anhydride–dichloromethane mixture (1:4, v/v) was added and the stoppered tube was allowed to stand at room temperature for 30 min. The samples were then either made up to 10 ml in a volumetric flask with dichloromethane or the TFA amino acid ester derivatives were purified by silicic acid column chromatography[2]. Samples were stored at −20°.

Gas–liquid chromatography

A gas chromatograph Pye Series 104, Model 24, fitted with two flame ionization detectors (W.G. Pye Ltd., Cambridge, Great Britain) was used in conjunction with a Speedomax W, 1 mV recorder (Leeds and Northrup Ltd., Birmingham, Great Britain). Argon was used as carrier gas. With carrier gas flow rate 30 ml/min optimum response was obtained with air flow 700 ml/min and hydrogen 45 ml/min. Two glass columns 3.5 m × 2.5 mm internal diameter were packed with Celite 560, 72–80 mesh (acid and base washed and deactivated with dimethyldichlorosilane) and coated with 2.5% w/w of stationary phase. The stationary phase was a mixture of XE-60–QF-1–MS 200, 100 cS in the proportions 46:27:27 (w/w) respectively, developed for separating TFA amino acid methyl esters[3]. On-column injection without flash heater was made with a 10 μl syringe. The outlet of the column was connected to a stream splitter consisting of a T-piece with a 38.0 cm length of stainless steel capillary tubing (I.D. 0.3 mm) leading to the detector and a 13.5 cm length leading through a heated outlet in the oven wall to a Packard Gas Chromatograph Fraction Collector Model 850. The capillary tubing was used to increase the outlet pressure of the gas to the fraction collector thus eliminating split ratio changes during collection. The split ratio was calculated from

measurements of gas flow to the detector and fraction collector at the oven temperature for peak elution. Approximately 60% of the effluent gas was collected. Because of the low flow rate of carrier gas to the jet of the detector, the response was reduced and baseline noise on the recorder was much increased. Additional argon therefore was introduced via a capillary tube into the hydrogen gas stream, so that the total argon flow through the detector was maintained at 30 ml/min. The effluent carrier gas to the collector passed through a glass tube 4.75 × 0.75 cm packed with scintillation grade 3-p terphenyl coated with MS 550, 10% w/w (Packard Instrument Co. Inc.). The terphenyl was supported at the lower end by a 0.75 cm length of cigarette filter. During collection the upper end of the tube was pressed against a rubber seal. The tube was rapidly changed for a fresh tube by movement of the collector turntable which held 50 tubes.

To measure the trapped radioactivity, the cigarette filter and packed terphenyl were pushed by means of a glass rod through the glass tube into a counting vial containing 15 ml of scintillation fluid. The counting efficiency of the terphenyl scintillation fluid mixture was 71%. No measurable radioactivity remained in the glass tube. The values were corrected for background and for quenching.

RESULTS AND DISCUSSION

TFA amino acid esters were prepared from radioactive amino acids diluted with suitable amounts of non-radioactive amino acids and purified by liquid chromatography on silicic acid columns. A 7 μl sample of the pure derivative containing a known amount of radioactivity was injected on to the column and a known percentage of the effluent was passed through the splitter to the fraction collector. The effluent was collected during an initial isothermal period and also after the column temperature, was increased to 220° and maintained at this temperature for 20 min.

TABLE I

AMOUNT OF RADIOACTIVITY COLLECTED AFTER GAS CHROMATOGRAPHY OF RADIOACTIVE TFA AMINO ACID ESTERS

Experimental details are given in the text. 7 μl samples were injected. Isothermal conditions were maintained as shown until the peak was eluted. The oven temperature was then raised at 48°/min and held at 220° for 20 min. Trapping of radioactivity was continuous. All results are for separate experiments and are expressed as a percentage of the amount injected on to the column.

Argon passing to collector (ml/min)	*Derivative*		
	TFA valine methyl ester (initial temperature 100°)	*TFA glycine n-butyl ester (initial temperature 125°)*	*TFA aspartic acid di-n-butyl ester (initial temperature 179°)*
14.3	100	99	101
	100	100	101
	100	100	100
21.4	91	90	95
	90	92	96
	90	90	96
31.5	80	87	90
	80	87	88
	81	86	88

Table I shows the effect of carrier gas flow rate on the efficiency of trapping of the radioactive compounds. Thus at 14.3 ml/min 100% of the radioactivity was recovered for the TFA valine methyl ester but at 31.5 ml/min only 80% was obtained.

Although the radioactivity injected as the TFA amino acid ester could be recovered quantitatively at low gas flow it was found that not all the radioactivity corresponded to a single derivative peak seen on the recorder chart. When the radioactivity was collected in three separate tubes representing fractions obtained prior to the peak, during peak elution and after the peak when the oven temperature was raised to 220°, it was found that small but consistent amounts of radioactivity were eluted both before and after the peak. Table II shows such results for seven TFA amino acids as their methyl, *n*-butyl and *n*-pentyl esters. Hydroxyproline in Table II is the only amino acid consistently to show no elution of radioactive material before the peak. No explanation is offered for the 13.1% radioactivity coming off the column before the TFA phenylalanine methyl ester. It is known that phenylalanine methyl ester is more volatile than the corresponding TFA derivative, but no peak was seen on the recorder chart. All the derivatives show considerable amounts of post-peak radioactivity. The yield of TFA amino acid ester prepared under the conditions described was measured by converting a solution of amino acid of known radioactivity to the derivative. A sample of the final product was analysed by gas–liquid chromato-

TABLE II

GAS CHROMATOGRAPHY OF RADIOACTIVE TFA AMINO ACID ESTERS WITH THE RADIOACTIVITY COLLECTED BEFORE, DURING AND AFTER ELUTION OF THE PEAK

The three fractions which are expressed as a percentage of the total collected are each the average for three separate injections. The instrument details and experimental conditions are given in the text and Table I. Carrier gas flow to the collector was 14.3 ml/min.

TFA amino acid	*Ester*	*Temp. (°C)*	R_T *(min)*	*% Radioactivity collected*			*Yield (%)*
				Pre-peak	*Peak*	*Post-peak*	
Isoleucine	Methyl	118	6.3	0.0	92.6	7.4	92
Leucine		118	7.5	1.0	93.3	5.7	92
Threonine		118	6.5	1.1	94.1	4.8	75
Serine		118	11.4	5.2	89.4	5.4	74
Hydroxyproline		144	10.5	0.0	93.4	6.6	97
Phenylalanine		170	5.3	13.1	73.0	13.9	57
Glutamic acid		170	4.4	5.1	88.4	6.5	75
Isoleucine	*n*-Butyl	128	12.0	0.0	90.8	9.2	87
Leucine		128	15.5	4.7	92.2	3.1	81
Threonine		128	9.3	0.9	93.3	5.8	94
Serine		128	18.0	5.4	89.1	5.5	88
Hydroxyproline		170	7.2	0.0	88.2	11.8	88
Phenylalanine		179	7.5	2.5	89.3	8.2	85
Glutamic acid		179	14.0	2.8	90.4	6.8	84
Isoleucine	*n*-Pentyl	130	21.2	1.1	89.5	9.4	91
Leucine		130	25.0	5.1	91.9	3.0	88
Threonine		130	15.3	1.2	87.5	11.3	84
Serine		141	13.3	7.0	88.6	4.4	87
Hydroxyproline		170	11.0	0.0	89.6	10.4	93
Phenylalanine		183	9.2	4.0	80.0	16.0	69
Glutamic acid		183	21.3	8.1	79.6	12.3	67

graphy and the radioactivity was collected during elution of the peak. The percentage yield was calculated from the radioactivity in the starting solution and in the collected peak. These results are shown in the last column in Table II. For all the amino acids examined derivatization under the conditions described was incomplete and was not systematically influenced by the ester substituent. Experiments indicated that if a derivative was purified by silicic acid column chromatography prior to gas–liquid chromatography pre-peak radioactivity was less than 0.4% and post-peak radioactivity was less than 3.0%. Table III presents results for the TFA glycine and TFA aspartic acid methyl esters. It was not possible within these limits to determine whether the derivatives were incompletely purified or whether some breakdown was occurring during gas chromatography.

TABLE III

AMOUNT OF RADIOACTIVITY ELUTED BEFORE, DURING AND AFTER PEAK ELUTION OF TFA AMINO ACID ESTERS

The derivatives were purified by silicic acid column chromatography before analysis. For experimental conditions see Table II. Results are shown for six separate experiments, with the radioactivity from each 7 μl injection collected in three tubes.

% of total radioactivity collected	*Derivative*					
	TFA glycine n-butyl ester			*TFA aspartic acid di-n-butyl ester*		
	Before	*During*	*After*	*Before*	*During*	*After*
	0.2	97.9	1.9	0.2	97.1	2.7
	0.1	97.7	2.2	0.3	96.9	2.8
	0.2	97.5	2.3	0.2	96.9	2.9

The response of a gas chromatograph detector may be expressed as the quantity of electricity produced by one mole of a given compound[4]. The absolute molar response factor can be determined by injecting known amounts of a pure standard and measuring the current produced by the detector. However, it must be assumed that the standard is pure and that no destruction of the standard occurs during chromatography. Using the gas chromatograph-fraction collector system described here it is possible to determine accurately the molar response factor of radioactive compounds. This method eliminates the problem of injecting an exact and known amount on to the column. The peak area displayed on the recorder chart can be related directly to the amount of radioactivity collected during the period of peak elution. From the specific activity of the original amino acid solution and the split ratio of the emerging carrier gas the exact amount of derivative corresponding to the peak on the recorder chart can be determined. The number of coulombs produced can then be calculated if the amount of current corresponding to full scale deflection on the recorder chart is known for any particular attenuation setting of the amplifier.

Table IV shows the results for the methyl, *n*-butyl and *n*-pentyl esters of 5 TFA amino acids obtained in one experiment. The *n*-pentyl ester derivatives give the highest molar response in coulombs per mole, as expected because of their higher carbon content. They are useful for the study of a limited number of amino acids[5]. However,

TABLE IV

ABSOLUTE MOLAR RESPONSE OF THE FLAME IONIZATION DETECTOR TO TFA AMINO ACID ESTERS

Samples of TFA amino acid esters of known specific activity were chromatographed separately under isothermal conditions. Radioactivity was collected only during elution of the peaks under isothermal conditions. Gas flow to the collector was 14.3 ml/min. Full scale deflection on the recorder corresponded to 10^{-12} A for attenuation setting 1 × 1.

Amino acid	*Molar response (coulombs per mole)*		
	TFA methyl ester	*TFA n-butyl ester*	*TFA n-pentyl ester*
Glutamic acid	0.86	2.70	3.05
Hydroxyproline	1.13	1.54	2.08
Isoleucine	1.37	1.97	2.30
Serine	0.67	1.28	1.51
Threonine	0.89	1.40	1.76

all the protein amino acids have been successfully separated as the *n*-butyl[1] and methyl[3] ester derivatives using temperature programming.

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