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Note

Gas–liquid chromatography of amino acids

Variable arginine response

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Certain of the protein amino acids present various characteristic difficulties during their derivatisation and subsequent determination by gas chromatography. Several papers have described techniques to overcome specific problems presented by, for example, arginine[1,2], histidine[3,4] and tryptophan[5]. Furthermore MacKenzie and Tenaschuk in some detailed studies[6,7] of N-heptafluorobutyryl isobutyl ester amino acid derivatives draw attention to some reasons why amino acids such as arginine, histidine and tryptophan may give rise to difficulties.

Successful acylation of the butyl ester of arginine has long been recognised[1] to be dependent upon high temperature, requiring for example, a treatment at 150°C for 10 min to accomplish complete conversion. An important requirement is to ensure that there is sufficient acylating reagent present at this temperature to prevent complete vaporisation. An additional precaution is to ensure that acylation tubes are immersed in the oil bath to the level of liquid in the tube thus allowing an efficient refluxing of the reagent[8].

Reference has also been made[7,9] to the importance of using high purity heptafluorobutyric anhydride (HFBA); failure to do so has been reported to lead to a poor response for arginine though no detailed explanation is provided to account for this observation. In this paper we report on the deleterious effects on the arginine response of the presence of small amounts of heptafluorobutyric acid (HFB-acid) during acylation of the iso-butyl ester of arginine.

MATERIALS AND METHODS

Crystalline amino acids were obtained from Sigma, St. Louis, MO, U.S.A. HFBA, stated to be greater than 99% pure, was obtained from Fluka, Buchs, Switzerland. Dichloromethane (HPLC grade) was obtained from Carlo Erba, Milan, Italy.

The standard solution (25 ml) of amino acids contained: alanine, glutamic acid and lysine (20 mg of each); glycine, valine, threonine, leucine, isoleucine, proline, phenylalanine, tyrosine, arginine and histidine (10 mg of each); serine, hydroxyproline, methionine, aspartic acid and tryptophan (2 mg of each).

N-Heptafluorobutyryl isobutyl ester (HBB) derivatives of the amino acids were prepared as previously described[10].

Acylations were carried out using 100-μl aliquots of HFBA (>99%) and the various HFBA–HFB acid mixtures shown in Table I.

TABLE I

VOLUMES OF WATER AND HFBA REQUIRED TO PRODUCE VARIOUS HFBA–HFB ACID MIXTURES

Volume of water added (μl)	*HFBA*			*Volume of HFB acid (μl)*	*Total volume of mixture (μl)*	*HFBA (%)*
	Unreacted volume (μl)	*Volume converted to HFB acid (μl)*	*Total volume (μl)*			
1.74	75	23.8	98.8	25	100	75
0.70	90	9.5	99.5	10	100	90
0.39	95	4.8	99.8	5	100	95

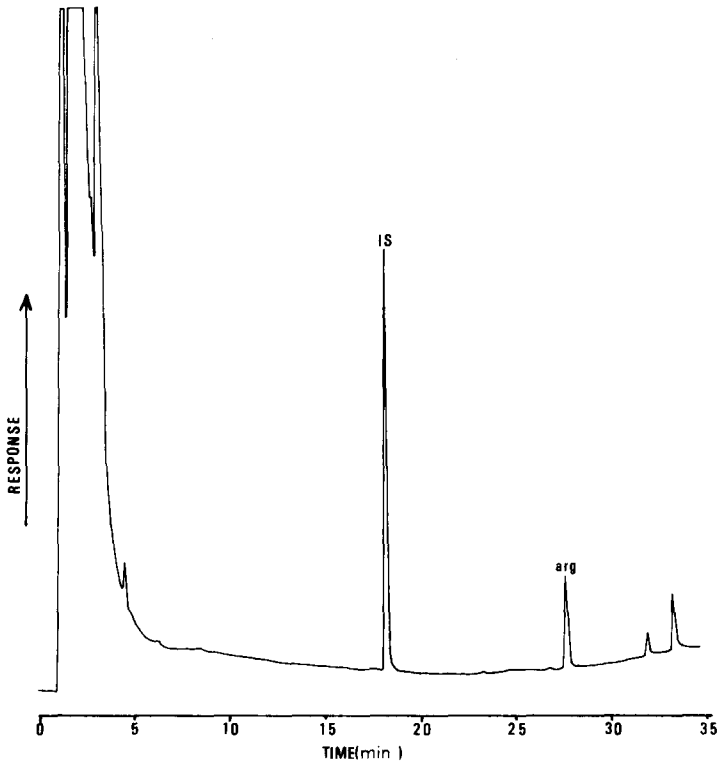

Fig. 1. Chromatogram showing reduced arginine (arg) response and two accompanying spurious peaks in relation to internal standard (IS).

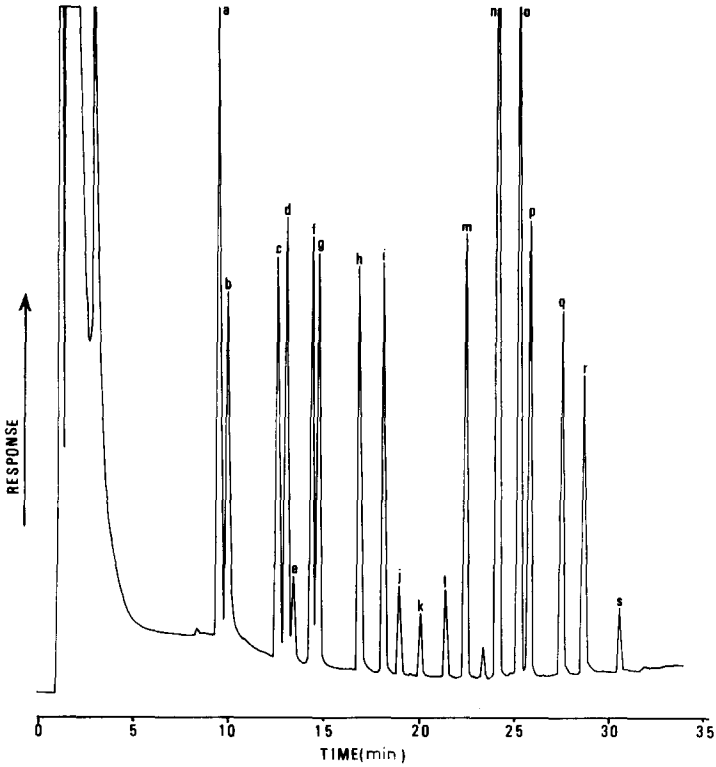

Fig. 2. Chromatogram showing HBB amino acid derivatives prepared from acylation using >99% pure HFBA. Peak identification: a = alanine; b = glycine; c = valine; d = threonine; e = serine; f = leucine; g = isoleucine; h = proline; i = pipecolic acid (internal standard); j = hydroxyproline; k = methionine; l = aspartic acid; m = phenylalanine; n = glutamic acid; o = lysine; p = tyrosine; q = arginine; r = histidine; s = tryptophan.

Following rapid exothermic reaction, the resulting mixture was used directly in the acylation step. Gas–liquid chromatography was carried out as described elsewhere[10] except the oven temperature programme was modified as follows: initial temperature 100°C with post injection hold for 5 min, followed by a heating rate of 5°C/min to 240°C with a 1-min hold followed by a ballistic increase to 295°C with a 6-min hold. Injection port temperature was 210°C.

RESULTS AND DISCUSSION

During the course of an investigation into unacceptable variability of relative responses for arginine following derivatisation of aliquots of a standard mixture incorporating 10-μg quantities of the amino acid, it became apparent that the integrity of HFBA was in some way implicated. It was further suspected that the presence of HFB acid immediately prior to acylation was responsible for the variability though it's origin was not clear. A series of experiments was designed to test whether this contaminant resulted in diminished arginine response and, if so, at what level the effect became significant.

In a preliminary experiment a 75:25 mixture of HFBA–HFB acid was used as acylating reagent and this brought about a massive reduction in level of the arginine response (Fig. 1). It may be further observed that this reduction is accompanied by an increase in size of the two later eluting peaks not normally present in chromatograms arising from mixtures acylated with pure (>99%) HFBA (Fig. 2). These peaks could be attributed to partially acylated arginine derivatives but only a qualitative inverse relationship between their combined areas and that of fully acylated arginine was observed. No further evidence of their identity was sought. In a second experiment, the effects of 5 and 10% levels of contaminating HFB acid in HFBA were investigated. The results (Table II) clearly show that even 5% contamination leads to a 32% increase in the arginine response factor (*i.e.* decrease in the arginine response), and higher contamination levels progressively increase its variability.

TABLE II

EFFECT OF DIFFERENT HFB ACID LEVELS IN HFBA ON THE ARGININE RESPONSE FACTOR

Level of contaminating HFB acid (%)	*Mean relative response factor for arginine**	*Standard deviation*	*Relative standard deviation (%)*
10	2.31	1.111	48.2
5	1.44	0.219	15.2
<1	1.09	0.046	4.2

* Mean of nine determinations from two experiments.

Whilst HFB acid may be an inherent impurity in the HFBA, it may nevertheless arise, when water formed during the esterification step and/or isobutanol remaining in the azeotroped residue react on addition of HFBA to yield the acid. Thus, removal of excess esterification reagent and the water generated during formation of the isobutyl esters must be complete as small volumes (0.39 μl; Table I) of water remaining in the mixture to be acylated will bring about inhibition in the acylation of arginine.

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