*Journal of Chromatography*, 445 (1988) 251–257
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 563

## Note

# Preparation and chromatographic analysis of poly(3-hydroxybutyrate) hydrolysis products

DOUGLAS W. McLELLAN* and PETER J. HALLING*
*Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow G1 1XW (U.K.)*
(First received December 28th, 1987; revised manuscript received March 9th, 1988)

Possible hydrolysis products of poly(3-hydroxybutyrate) (PHB) include the monomer, oligomers and derivatives of these that have been further modified in side reactions. Hauttecoeur *et al.*[1] reported that oligomers (2–7 units long) (with a methyl ester of the COOH-terminus) could be isolated in low yield after mild alkaline hydrolysis[2] of PHB. Oligomeric species that had been dehydrated at the OH-terminus to give a C=C double bond were also present: crotonic acid, formed by similar dehydration of monomeric 3-hydroxybutyrate, is well known as a side product of total hydrolysis. Coulombe *et al.*[3] described an high-performance liquid chromatographic (HPLC) method for analysis of PHB oligomers, which was applied to both longer oligomers from alkaline hydrolysis and short ones produced by boron trifluoride–methanol treatment. We now report further studies on the value of the HPLC method in the analysis of PHB hydrolysis products, and of mild alkaline hydrolysis in the preparation of oligomers.

## EXPERIMENTAL

### *Materials*

Extracted PHB was a gift from ICI Biological Products Business Research Group (Billingham, U.K.). Sodium 3-hydroxybutyrate, free 3-hydroxybutyric acid, its methyl ester and crotonic acid were all purchased from Sigma (Poole, U.K.).

### *Alkaline hydrolysis of PHB*

This method of partial hydrolysis was based on that described by Hauttecoeur *et al.*[2]. Extracted PHB was dissolved in chloroform at 20 g/l by refluxing. This solution was diluted with nine times its volume of methanol and the precipitated gel was filtered, washed with methanol and stored as a suspension in methanol. A suspension containing 10 g/l PHB (dry basis) was mixed with an equal volume of 2 *M* sodium hydroxide in either methanol or water, and stirred at −10°C. Samples were removed after 30, 60 and 120 min, neutralised with concentrated hydrochloric acid,

* Present address: Department of Chemical Engineering, Teeside Polytechnic, Middlesborough TS1 3BA, U.K.

0021-9673/88/$03.50 

filtered through Whatman GF/C, dried by vacuum rotary evaporation at 30°C, dissolved in acetonitrile and analysed.

*Preparation of 3-hydroxybutyric acid*

An amount of 5.2 g of sodium 3-hydroxybutyrate was dissolved in 4 ml 10 *M* hydrochloric acid at 25°C. The resulting slurry was extracted with diethyl ether, to give an approximately 0.2 *M* solution of the free acid. This was stored at −10°C, and was quite stable; after six months, analysis by the HPLC method described below showed crotonic acid as the only decomposition product, but as less than 0.1% of the total material. The free acid was obtained when required by vacuum evaporation of this stock solution, and was extremely hygroscopic.

*HPLC analysis*

This was carried out using a Gilson gradient system 41, incorporating twin 303 pumps and an Holochrome UV detector. Peaks were recorded and integrated on a Shimadzu Chromatopac C-R2A. A 25 cm × 4.6 mm I.D. stainless-steel column packed with Microsorb $C_{18}$ reversed phase was used, and was run isocratically with a mobile phase of 40% 8 m*M* orthophosphoric acid in water and 60% far UV-grade acetonitrile, with detection at 205 nm. Samples were dissolved in the same mixture prior to injection of 20 μl.

*Gas–liquid chromatographic (GLC) analysis*

GLC was performed on a Pye-Unicam GCD instrument with flame ionisation detector. A 6 ft. (1.82 m) × $\frac{1}{8}$ in. (3.2 mm) I.D. glass column was packed with 3% OV-1 on Chromosorb W HP. Chromatograms were run at 90°C for 1 min, then programmed to 180°C at 16°/min. The detector signal was recorded on a BBC model B microcomputer, which was also used to calculate peak areas.

Samples of about 10 mg dry material were dissolved in 200 μl acetonitrile, then silylated by adding 200 μl bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and heating at 50°C for 10 min.

## RESULTS AND DISCUSSION

*HPLC and GLC methods for the analysis of PHB hydrolysis products*

We have made extensive use of a reversed-phase HPLC method slightly modified from that described by Coulombe *et al.*[3] for the analysis of similar materials. The method certainly resolves and detects many components present in hydrolysed samples, but we feel it has a major limitation. With detection by UV adsorption at 205 nm, response to different components varies widely. For example crotonic acid (a common component in hydrolysis products that is formed by dehydration of 3-hydroxybutyric acid) absorbs over 400 times more strongly than the monomer itself, because of the C=C double bond present (Table I). Hence when a sample contains many unknown constituents, such as the products of mild alkaline hydrolysis discussed below, the HPLC trace can give a very misleading indication of which are the major species present, in the absence of UV absorption data.

Accordingly, we have developed a GLC analysis method for these same mixtures. Dried samples were silylated with BSTFA, which normally forms both silyl

TABLE I

BEHAVIOUR OF AVAILABLE AUTHENTIC STANDARDS IN HPLC AND GLC ANALYSIS METHODS

| *Compound* | *HPLC* | | *GLC* | |
|---|---|---|---|---|
| | *Retention time (min)* | *Relative response (molar basis)* | *Retention time (min)* | *Relative response (molar basis)* |
| 3-Hydroxybutyric acid | 2.7 | 100 | 4.0 | 100 |
| Crotonic acid | 3.3 | 41 000 | 1.7 | 110 |
| Methyl 3-hydroxybutyrate | 3.1 | 109 | 2.3 | 132 |

ethers of OH groups and silyl esters of COOH groups[4]. The method gives good clean chromatograms (Figs. 1 and 2). Because no significant peaks attributable to 3-hydroxybutyrate oligomers were found in any samples by this method, we considered the possibility that the ester links in these were cleaved under the silylation conditions. However authentic methyl 3-hydroxybutyrate ester analysed by our method gave a single peak distinct from that of the free acid derivative (Table I), showing that its ester bond was totally unaffected.

Table I shows the retention times and relative response factors in both HPLC and GLC for the three standards available to us: 3-hydroxybutyric acid monomer, its methyl ester and crotonic acid. The HPLC retention times drifted somewhat,

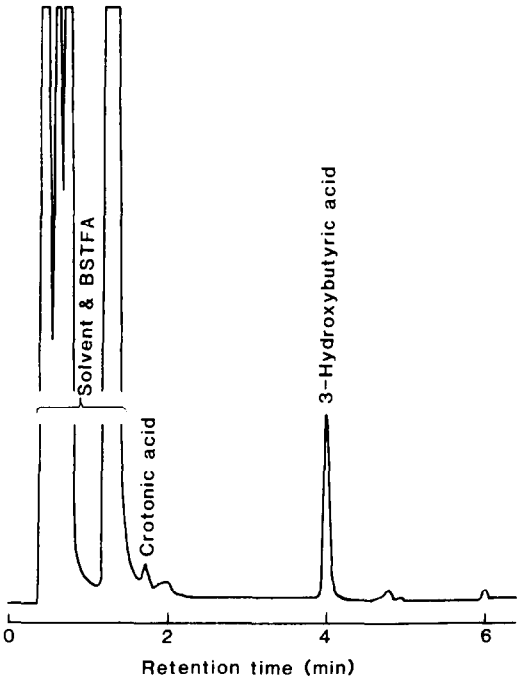


Fig. 1. GLC trace of product from mild alkaline hydrolysis of PHB in methanol–water (50:50). Same preparation as used for Fig. 4.

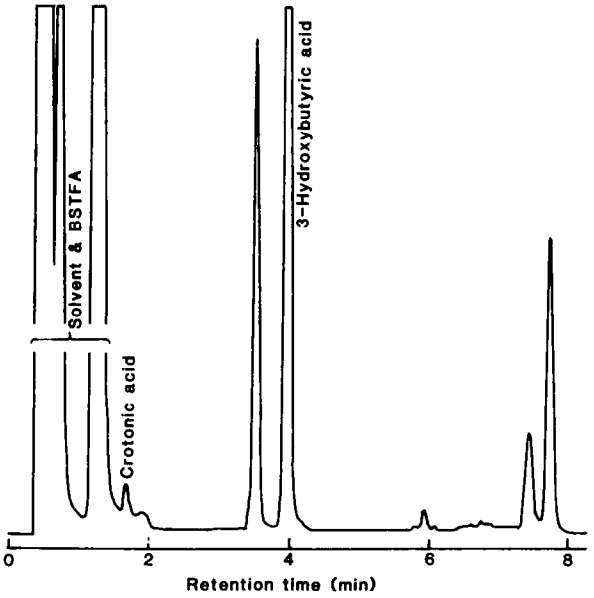


Fig. 2. GLC trace of free 3-hydroxybutyric acid (Sigma).

probably because the column temperature was not controlled: therefore peak identifications were made only by comparing successive injections of sample and standards.

*Mild alkaline hydrolysis of PHB*

Hauttecoeur *et al.*[2] reported the preparation of partial hydrolysis products from PHB by treatment of a polymer gel with sodium hydroxide in methanol at sub-zero temperatures. Hauttecoeur *et al.*[1] showed that short oligomers were present in one fraction obtained by this method. We have analysed a similar fraction by HPLC, and obtained a regular series of peaks suggestive of a mixture of 3-hydroxybutyrate oligomers (Fig. 3); an analogous pattern of peaks was found by Coulombe *et al.*[3] using essentially the same HPLC method on samples containing oligomers of rather larger size range. Even our most extensively hydrolysed samples did not give a significant peak of 3-hydroxybutyrate, through a large peak was identified as crotonic acid (Fig. 3). A slight shoulder on the leading edge of this peak might be methyl 3-hydroxybutyrate, but it is too indistinct to draw any firm conclusion.

We have also carried out similar mild hydrolysis reactions in methanol–water (50:50). The resulting HPLC traces (Fig. 4) were similar, except that a peak of 3-hydroxybutyrate was now clearly identifiable. The retention times of the "oligomer" peaks may also have changed, though we have not made direct comparisons here: an analagous replacement of methyl esters by free acids might be expected.

However, we have also analysed the samples from methanol–water hydrolysis by the GLC method described. This revealed just one significant component, 3-hydroxybutyrate monomer itself, with no evidence for a regular series of peaks that

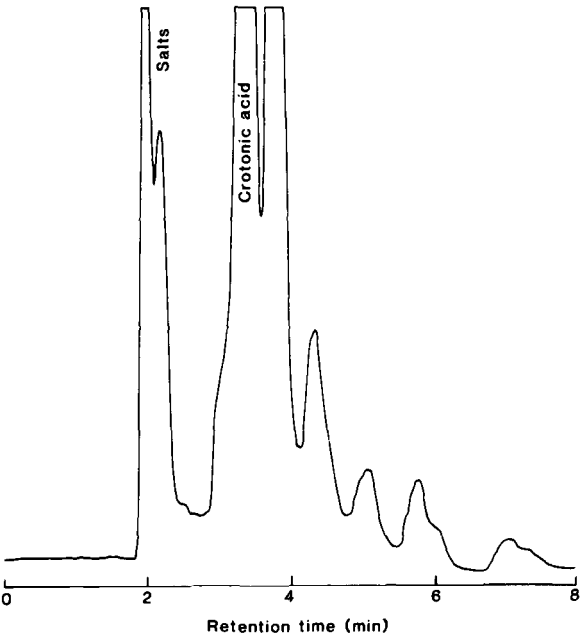


Fig. 3. HPLC trace of products from mild alkaline hydrolysis of PHB in methanol.

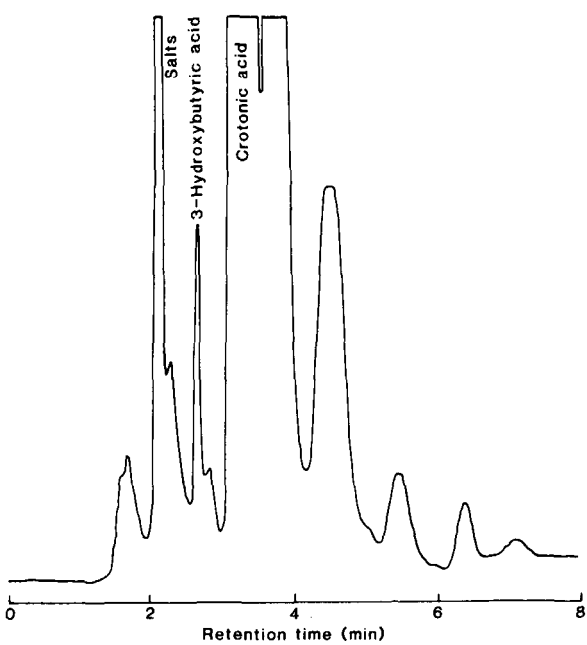


Fig. 4. HPLC trace of products from mild alkaline hydrolysis of PHB in methanol–water (50:50).

might be oligomers (Fig. 1). We conclude that the apparent oligomers demonstrated by HPLC are minor components that have a strong absorption at 205 nm, probably oligomers with a C=C double bond formed by dehydration at the OH-terminus, which are known components of these fractions[1]. Similarily, the major HPLC peaks observed by Coulombe *et al.*[3] may well have been dehydrated oligomers, with the small satellite peaks they sometimes found being the OH-form; this is the reverse of their suggested assignment. We also conclude that the monomer is the only significant small molecule product of chemical hydrolysis under these conditions. This would agree with the statement by Hauttecoeur *et al.*[1] that oligomers are formed in only low (though unquantified) yield.

*Stability of free 3-hydroxybutyric acid*

The Sigma catalogue states that this free acid is unstable, and spontaneously forms "bimolecular esterification products" that are present in their offering. We produced the free acid from sodium 3-hydroxybutyrate by neutralisation with hydrochloric acid and extraction into diethyl ether (see Experimental section). The product gave a single peak on HPLC identical to that from the sodium salt (in the buffered mobile phase used for HPLC both will give predominantly the unionised form). We found this free acid to be relatively stable: either storage for 30 days at 20°C, or heating at 100°C for 2 h, resulted only in the appearance of a small quantity of crotonic acid (<0.1%).

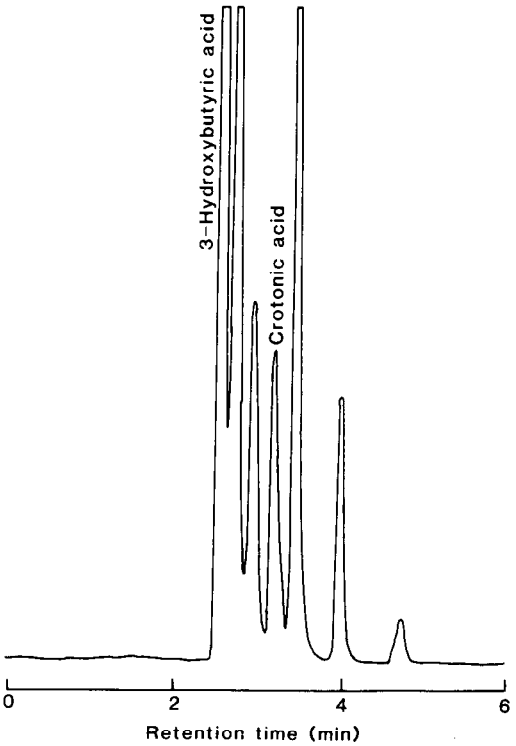


Fig. 5. HPLC trace of free 3-hydroxybutyric acid (Sigma).

Both HPLC and GLC analysis of the Sigma free acid showed several significant peaks (Figs. 2 and 5), two of which were identified as genuine 3-hydroxybutyric acid (50%) and crotonic acid (<0.1%).

ACKNOWLEDGEMENT

We are most grateful to ICI plc. (Biological Products Business) for financial support and for permission to publish these results.

REFERENCES

1 B. Hauttecoeur, M. Jolivet and R. Gavard, *Compt. Rend. Acad. Sci. Paris*, D280 (1975) 2801.
2 B. Hauttecoeur, M. Jolivet and R. Gavard, *Compt. Rend. Acad. Sci. Paris*, D274 (1972) 2729.
3 S. Coulombe, P. Schauwecker, R. H. Marchessault and B. Hauttecoeur, *Macromolecules*, 11 (1978) 279.
4 W. C. Butts, *Anal. Biochem.*, 46 (1972) 187.