CARBOBENZOXY DERIVATIVES OF AMINO ACIDS AND PEPTIDES: INSTANT THIN-LAYER CHROMATOGRAPHY AS HYDROBROMIDES*

MILTON L. MASKALERIS, ELISABETH S. SEVENDAL AND ANDRE C. KIBRICK**
Institute for Muscle Disease, Inc., New York, N.Y. (U.S.A.)
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INTRODUCTION

In the course of radioactive synthesis of glycylprolylhydroxyproline, a substance which has been isolated from human urine and which may be a factor in the metabolism of collagen, it became necessary to devise a system for determining the purity of the ^{14}C-labelled N-carbobenzoxy derivatives. It was difficult to use conventional methods because of the small amounts of material employed for the radioactive synthesis and because of the inability of some of the more common procedures to effect adequate separation of our constituents. To have pure starting materials for subsequent steps of the synthesis, it was necessary to ascertain the purity of the ^{14}C intermediates (in one instance carbobenzoxyglycine and carbobenzoxyglycylproline). The melting points of the respective synthetic products, 120° *vs.* 155°, should have been sufficient; however, it was difficult to be certain of the melting point of a single crystal with the conventional Mel-Temp apparatus***, although we think we were successful. Another possible solution to the problem was the use of infrared spectroscopy on a microscale. This was ruled out because microequipment for our spectrophotometer was not available and especially because of possible contamination of the equipment with radioactivity. Other comparatively expensive instrumental techniques such as mass spectrometry were also ruled out since such apparatus was not available.

Separation from relative solubility of the crystals offered another possibility, although a measurement of purity such as melting points was still necessary. It was found that complete separation of carbobenzoxyglycine from carbobenzoxyglycylproline was possible by extraction with anhydrous ether when the crystals of either one of these substances were of certain dimensions; that is particle size appeared to be the governing factor. When the carbobenzoxyglycylproline crystals were large enough, a given amount of solvent would remove only the carbobenzoxyglycine.

Preliminary experiments with column chromatography on silica gel indicated that this may be suitable for separating the components. With each pure substance, it appeared that complete removal of the carbobenzoxyglycine from the column was possible before carbobenzoxyglycylproline was eluted. One experiment was carried

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*** Laboratory Devices, Cambridge, Mass., U.S.A.

out on a mixture of equal parts of carbobenzoxyglycine and carbobenzoxyglycylproline with increasing amounts of acetone and decreasing amounts of methylene chloride. Carbobenzoxyglycine was eluted with 11 % acetone, whereas carbobenzoxyglycylproline was eluted with 16 % acetone. The lowered melting point and extended range of the 16–22 % acetone fraction attested to the presence of two compounds. It is probable that utilization of 11 % acetone for a longer time would have provided essentially complete separation.

Paper and thin-layer chromatography nevertheless seemed to be a more convenient solution to the problem. Several workers have published methods for the chromatography of N-carbobenzoxy compounds on both paper and thin layers[1–5]. None that we tried with our compounds proved successful. Preliminary experiments with carbobenzoxyglycine and carbobenzoxyglycylproline on Whatman 1 and 3 MM paper were run in the following solvent systems: *s*-collidine–$H_2O(NH_3)$ (125:44); *n*-BuOH–HOAc–H_2O (4:1:2); EtOH–H_2O (7:3); EtOH–H_2O (1:1); acetone–methylene chloride (11:89); acetone–methylene chloride (2:98). Detection was with Cl_2–starch–KI[6]. In all cases where spots were obtained the R_F's were essentially the same for carbobenzoxyglycine and carbobenzoxyglycylproline. In some of these studies with paper, complications resulted because of reaction of the chlorine with developing systems, such as *s*-collidine, which could only be removed adequately from Whatman 3 MM paper by aeration for approximately three days. In other experiments phenol interfered with the chlorine method of detection.

As a consequence of the difficulties encountered in the separation on paper, we considered the advantages of the recently introduced Gelman Type S Instant Thin-Layer Chromatography plates (ITLC)[7]. These supports feel and handle like paper, are constructed of potassium silicate and glass fibers, and resemble standard silica gel preparations except that they are more alkaline (pH 10.5). In 1959, NEUMANN, LEVIN, BERGER, AND KATCHALSKI described the detection of N-carbobenzoxy derivatives with gaseous hydrogen bromide and ninhydrin after chromatography on Whatman No. 1 paper with *n*-BuOH–HOAc–H_2O (25:6:25) and *n*-PrOH–H_2O–conc. NH_4OH (100:50:1). In one experiment with the ITLC plates in EtOH–H_2O (7:3) and with this method of detection we did not separate carbobenzoxyglycine and carbobenzoxyglycylproline. It seemed to us that treatment with hydrogen bromide gas before chromatography would decarbobenzoxylate the compounds to hydrobromides directly on the plates and might lead to better separation. This was tried in another experiment with the solvent system *n*-BuOH–HOAc–H_2O (4:1:2), and with 0.3 % ninhydrin in water-saturated *n*-butanol for detection. Two definite spots resulted, which seemed to confirm our prediction. The phenol-water system appeared to be a better one to try with this technique since it yields scattered R_F's with many amino acids and peptides. A method based on these principles, which has proved most helpful in our synthesis, is described in this paper.

EXPERIMENTAL*

Standard method

Preparation of ITLC plates. A plate 10 × 20 cm proved most suitable for the method; we therefore cut the 20 × 20 cm commercial plates in half. Two spots (about 1/4 inch in diameter) of 20 μg of material are placed approximately 2 inches

apart and 1 inch from the bottom. One of these is the substance to be tested; the other is carbobenzoxyglycine, which is included in each determination as a reference. It is convenient to use 0.01 ml of solutions containing 2 mg/ml in acetone.

Treatment with HBr. Treatment with hydrogen bromide gas is performed in a well-vented fume hood. Two plates are placed in a 250 mm glass desiccator with sleeve valve in the bottom of which there is a small inverted glass funnel to keep the plates separated, with all surfaces exposed to the gas. The desiccator cover and the sleeve valve are well greased with silicone grease. The cover is adjusted so that there is an opening of about 1 mm for excess gas to escape during filling, rubber tubing from a small tank (lecture bottle) is attached to the inlet tube, and gaseous hydrogen bromide is rapidly introduced for 20 sec. The tank valve, desiccator cover, and desiccator valve are then closed immediately. To prevent any considerable leakage of gas, the rubber tubing is removed from the desiccator, and the inlet tube is stuffed with a plug of Kimwipe**. The plates are kept in the gas for 17 min, during which time they tend to curl up and accumulate orange spots. The desiccator cover is then drawn about one-third open to allow rapid escape of gas.

Washing with ether. After 10 min each plate is placed in separate shallow glass dishes 300 × 85 mm, and 300 ml of fresh anhydrous ether are added to each. The plates are washed with the ether for 5 min by carefully rocking the dishes 3 or 4 times/min. They are removed to dry beakers, and the ether is allowed to evaporate for 10 min. The waste ether wash contains considerable orange color. After evaporation, the beakers containing the plates are placed in an oven, and drying is continued for 15 min with circulating air at room temperature.

Chromatography. Rectangular glass museum jars, 250 × 250 × 155 mm, with covers that are well greased with silicone grease are used for ascending chromatography. Phenol–water (4:1, w/w), 177.5 ml, usually prepared by adding 20 ml of

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* *Materials.* (1) Gelman Instant Thin-Layer Chromatography Type S plates (ITLC), Gelman Instrument Co., Ann. Arbor, Mich. (2) Liquefied phenol, 90.7%, certified reagent, Fisher. (3) Anhydrous ether, ammonium hydroxide, and pyridine, analytical reagents, Mallinckrodt. (4) Anhydrous hydrogen bromide gas, Matheson. (5) 1-Butanol, acetic acid, and *tert.*-butanol, certified reagents, Fisher. (6) Ninhydrin, Dougherty Chemical Co., N.Y., recrystallized once from hot water. (7) Glycine (HGlyOH), A grade, Calbiochem. (8) L-Proline (L-HProOH), L-hydroxyproline (L-HyproOH), carbobenzoxyglycyl-L-leucine (ZGly-L-LeuOH), carbobenzoxyglycyl-L-tryptophan (ZGly-L-TryOH), carbobenzoxy-L-prolyl-L-leucylglycine (Z-L-Pro-L-LeuGlyOH), and carbobenzoxyglycylsarcosine (ZGlySarcosine), M.A., Mann Research Laboratories, Inc., N.Y. (9) Carbobenzoxy-L-proline (Z-L-ProOH), carbobenzoxy-L-hydroxyproline (Z-L-HyproOH), carbobenzoxy-L-tryptophan (Z-L-TryOH), carbobenzoxyglycyl-L-serine (ZGly-L-SerOH), carbobenzoxy-L-methionine (Z-L-MetOH), and dicarbobenzoxy-L-cystine [diZ-L-$(CySOH)_2$], C.P., Mann Research Laboratories, Inc., N.Y. (10) Carbobenzoxy-L-alanine (Z-L-AlaOH), carbobenzoxy-L-phenylalanine (Z-L-PheOH), carbobenzoxy-L-leucine (Z-L-LeuOH), carbobenzoxyglycyl-L-phenylalanine (ZGly-L-PheOH), and carbobenzoxy-L-leucylglycine (Z-L-LeuGlyOH), Cyclo Chemical Corp. (11) L-Tryptophan (L-HTryOH), General Biochemicals, Inc. (12) Carbobenzoxyglycine (ZGlyOH), m.p. 118°–120°, prepared according to procedure described by GREENSTEIN AND WINITZ[8]. (13) Carbobenzoxyglycyl-L-proline (ZGly-L-ProOH), m.p. 152°–156°, prepared according to RYDON AND SMITH[9]. (14) Carbobenzoxyglycyl-L-prolyl-L-hydroxyproline benzyl ester (ZGly-L-Pro-L-HyproOBz)-I, purchased commercially. (15) Carbobenzoxyglycyl-L-prolyl-L-hydroxyproline benzyl ester (ZGly-L-Pro-L-HyproOBz)-II, synthesized according to POROSHIN *et al.*[10] (16) Glycyl-L-prolyl-L-hydroxyproline benzyl ester hydrobromide (HBr·HGly-L-Pro-L-HyproOBz), synthesized from another sample of ZGly-L-Pro-L-HyproOBz by the usual procedure for decarbobenzoxylation with dry hyrogen bromide gas in liquid acetic acid.

** Kimberly Clark Corp., Neenah, Wisc., U.S.A.

water to 157.5 ml of liquefied phenol and shaking, is poured into the jars. A 50-ml beaker containing 1 ml of 2 *N* ammonia and 29 ml of water is placed in the bottom, and one prepared plate is adjusted so that the top is inclined and rests on the wall. The jar is covered immediately, and chromatography is allowed to proceed at room temperature until the solvent front has travelled at least 10 cm. After development, the plate is removed and inverted, and the solvent front is marked with pencil. It is then transferred to a dry beaker and placed in the oven for drying overnight at room temperature with circulating air (usually 16 h).

After drying, the plate is sprayed with 0.3 % ninhydrin in water-saturated 1-butanol, prepared by shaking 100 ml of 1-butanol with 30 ml of water and draining off the bottom layer. The plate is sprayed throughout until the change in color (darkening) indicates complete wetting. After spraying, the plate, resting in a beaker, is brought to 110° in the oven with circulating air. It is then examined for initial coloration, returned to the oven, and kept at 110° for 15 min. This process produces various colors according to the materials tested and leaves the background essentially colorless or white.

Method with equilibration

This method is the same as the standard method until chromatography. The only difference is that the walls of the museum jar are neatly lined with Whatman No. 1 filter paper while still dry. The solvent mixture is then poured in and shaken around in the jar to wet the lower portions of the filter paper lining. A beaker of the dilute ammonia is placed in the bottom, and the prepared plate is adjusted in the chamber with its bottom resting on an inverted dish to keep the plate from coming in contact with solvent. The covered jar is allowed to equilibrate in this way for 1 h. Then the cover is opened rapidly, the dish is removed, and the plate is placed in contact with the solvent. After replacing the cover, chromatography is allowed to proceed as usual. The remainder of the method is the same as the standard method.

Method modified for other systems

This is the same as the standard method except that the plates, after chromatography, are dried at room temperature in the circulating oven for only 1 h instead of overnight.

RESULTS AND DISCUSSION

Most of the chromatographic work reported in this study was done without equilibration. In 35 determinations of carbobenzoxyglycine, 11 of carbobenzoxyglycylproline, 6 of carbobenzoxyhydroxyproline, and 7 of carbobenzoxytryptophan without equilibration, the values of R_F were found to vary considerably. However, some that were obtained with equilibration showed more uniformity; this is consistent with the literature for thin layer chromatography[11–13]. Part of Table I shows the results by the standard method for four pairs of amino acids and their corresponding carbobenzoxy derivatives. Each pair was chromatographed in the same tank and at the same time. Each has almost identical values, indicating that the same, and only one compound, probably results on hydrogen bromide treatment of the amino acid and of its respective carbobenzoxy derivative. (Omission of hydrogen bromide before

chromatography of the free amino acids in the phenol–water system on the Gelman Type S plates produces considerable blurring that masks subsequent detection with ninhydrin.) The rest of Table I contains results of individual determinations (two or three are averages of duplicates) of 13 other N-carbobenzoxy-protected amino acids and peptides. Most of the carbobenzoxy compounds flow very nearly as fast as the solvent front in the phenol–water system; *i.e.* their R_F values are about 0.9 or higher.

TABLE I

R_F VALUES OF CARBOBENZOXY COMPOUNDS AND SOME FREE AMINO ACIDS

Compound	R_F[a]	*Solvent front (mm)*	*Development time (min)*
ZGlyOH	0.395	139	120
HGlyOH	0.42	139	120
ZProOH	0.91	144	128
HProOH	0.98	137	128
ZHyproOH	0.89	137	120
HHyproOH	0.84	137	120
ZTryOH	0.97	148	128
HTryOH	0.94	144	128
ZMetOH	0.97	118	205
Di-Z(CySOH)$_2$	0.27	106	205
ZPheOH	0.965	115	210
ZALaOH	0.68	127	210
ZLeuOH	0.97	124	132
ZGlyProOH	0.93[b]	—	—
ZGlyLeuOH	0.93	134	126
ZGlySarcosine	0.89	110	136
ZGlySerOH	0.56	104	136
ZGlyTryOH	0.94	139	125
ZLeuGlyOH	0.96	137	154
ZGlyPheOH	0.97	129	132
ZProLeuGlyOH	0.93	123	236

[a] R_F values without equilibration.
[b] Average for this compound.

The carbobenzoxy compounds probably are quantitatively converted into the hydrobromides by the hydrogen bromide technique, and, therefore, other solvent systems, more suitable for the separation of specific hydrobromides from mixtures of carbobenzoxy compounds, can be substituted for the phenol–water system of the standard method. Table II shows the results of determinations on two samples of N-carbobenzoxyglycyl-L-prolyl-L-hydroxyproline benzyl ester (ZGly-L-Pro-L-HyproOBz) and one of glycyl-L-prolyl-L-hydroxyproline benzyl ester hydrobromide (HBr·HGly-L-Pro-L-HyproOBz) by the standard method and by the method modified for other systems. On the assumption that a second treatment with hydrogen bromide does not alter the ester hydrobromide, all three compounds should have the same R_F in a given solvent system. The values are the same in all five systems used. The presence of additional ninhydrin spots with different R_F's indicates that impurities are present in one of the carbobenzoxy esters and in the hydrobromide.

It should be mentioned that when the technique of hydrogen bromide treatment before chromatography was tried on filter paper (Whatman No. 1, 3 MM, 3 MC), a

TABLE II

CHROMATOGRAPHY OF GLYCYLPROLYL HYDROXYPROLINE BENZYL ESTERS

Compound	*Developing system*	R_F	*Solvent front (mm)*	*Development time (min)*
ZGly-L-Pro-L-HyproOBz (I)	phenol–$H_2O(NH_3)$(4:1)	0.96	149	120
HBr·HGly-L-Pro-L-HyproOBz	phenol–$H_2O(NH_3)$(4:1)	0.97	146	120
ZGly-L-Pro-L-HyproOBz (I)	*n*-BuOH–HOAc–H_2O(4:1:2)	0.71	113	150
		0.345		
HBr·HGly-L-Pro-L-HyproOBz	*n*-BuOH–HOAc–H_2O(4:1:2)	0.71	110	150
ZGly-L-Pro-L-HyproOBz (I)	*n*-BuOH–HOAc–H_2O (4:1:2)	0.66	139	—
		0.29		
HBr·HGly-L-Pro-L-HyproOBz	*n*-BuOH–HOAc–H_2O(4:1:2)	0.66	139	—
ZGly-L-Pro-L-HyproOBz (I)	*n*-BuOH–HOAc–H_2O(4:1:1)	0.69	137	—
HBr·HGly-L-Pro-L-HyproOBz	*n*-BuOH–HOAc–H_2O(4:1:1)	0.70	140	—
ZGly-L-Pro-L-HyproOBz (I)	*t*-BuOH–HOAc–H_2O(4:2:1)	0.90	140	165
HBr·HGly-L-Pro-L-HyproOBz	*t*-BuOH–HOAc–H_2O(4:2:1)	0.82	147	165
		0.53		
ZGly-L-Pro-L-HyproOBz (II)	*n*-BuOH–ϕOH–HOAC–H_2O(25:10:10:10)	0.71	135	142
HBr·HGly-L-Pro-L-HyproOBz	*n*-BuOH–ϕOH–HOAc–H_2O(25:10:10:10)	0.69	130	142
		0.13		
ZGly-L-Pro-L-HyproOBz (II)	*n*-BuOH–ϕOH–HOAC–H_2O(25:10:10:10)	0.68	130	142
HBr·HGly-L-Pro-L-HyproOBz	*n*-BuOH–ϕOH–HOAc–H_2O(25:10:10:10)	0.64	133	142

marked blurring was encountered with all of our solvent systems. This was quite the same as the blurring produced by omission of hydrogen bromide before chromatography in the phenol–water system with the Gelman plates. This effect on filter paper was unexpected, since the conditions of Katchalski *et al.*[5] and ours were apparently similar. Although we were unable to avoid blurring completely with ninhydrin in acetone and pyridine for detection[5] and incorporation of bases with the ether wash after hydrogen bromide treatment, these modifications did yield acceptable chromatograms about half of the time. This difficulty can probably be completely avoided with further study. The technique of hydrogen bromide treatment before chromatography can then be extended to filter paper.

SUMMARY

A method is described for the chromatography of carbobenzoxy derivatives of amino acids and peptides on instant thin-layer chromatography plates as the hydrobromides by treatment with HBr gas on the plates before chromatography. It is shown that the method produces the same and only one compound from each of four pairs of free amino acids and their corresponding carbobenzoxy derivatives. Data are presented for the chromatography of 18 carbobenzoxy derivatives by the method. The results and the method, as well as modifications for equilibration and for solvent systems other than the phenol–water system, are described. The method appears to be suitable for the detection of impurities in this type of compound and therefore is useful in the synthesis of peptides.

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