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Note

Determination of impurity profiles in drugs and related materials

II*. Detection and quantification of a diastereomeric impurity in the peptide RGH-0205 (Arg–Lys–Asp)

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In the determination of the impurity profile of the immunostimulant peptide RGH-0205 (Arg–Lys–Asp)[1], the separation of a major impurity was a difficult task. The usual methods for the separation of impurities in small peptides such as thin-layer chromatography (TLC) and reversed-phase high-performance liquid chromatography (HPLC) using acidic or neutral buffers as eluents failed to separate this impurity. We report here a successful separation using an HPLC eluent of higher pH than usual.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (Waldbronn, F.R.G.) 1090 high-performance liquid chromatograph equipped with an HP-1040 diode-array detector was used. Optical rotations were measured using a Perkin-Elmer (Überlingen, F.R.G.) 241 polarimeter. The NMR spectra were taken using a Varian (Palo Alto, CA, U.S.A.) XL-400 instrument.

Reagents

Water used in HPLC was purified by double distillation followed by passing it through a LiChroprep RP-8 column (Merck, Darmstadt, F.R.G.). Other solvents and reagents were of ACS reagent grade and were purchased from Aldrich (Beerse, Belgium).

HPLC procedure

A 250 × 4 mm I.D. column was used, packed by Bio Separation Technologies (Budapest, Hungary) with LiChrosorb RP-18 (Merck). The eluent was 0.015 *M* disodium hydrogenphosphate solution adjusted to pH 7.0–9.5 by adding hydrochloric acid and sodium hydroxide. The flow-rate was 1 ml/min. The analytical separations were performed at pH 9.0. The concentration of the sample solution was 0.1% in the eluent. The volume injected was 20 μl.

* For Part I, see S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.*, 6 (1988) in press.

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TLC procedure

The solvent systems were (1) *n*-butanol–pyridine–acetic acid–water (9:20:6:11) and (2) *n*-butanol–ethyl acetate–acetic acid–water (1:1:1:1). A solution of 100 μg of RGH-0205 in 20 μl of water was applied to the plate (Kieselgel 60 HPTLC plate 13748; Merck). The length of the run was 10 cm. The spots were revealed with chlorine–tolidine reagent[2].

Investigation of the protected peptide

A 100-mg amount of Boc-Arg(HCl)–Lys(Boc)–Asp(OBu^t)-OBu^t (Bu^t = *tert.* butyl), synthesized as described in ref. 1, was dissolved in 2 ml of trifluoroacetic acid in a small glass-stoppered bottle. The mixture was allowed to stand at room temperature for 60 min, then 200 μl were transferred into a 10-ml volumetric flask, 2 ml of 1 *M* sodium hydroxide solution were added and the content were diluted to volume with water. A 20-μl volume of this solution was injected into the chromatograph.

RESULTS AND DISCUSSION

As can be seen in Table I, measuring the optical protation, TLC using various systems (of which two are shown here) and reversed-phase HPLC using neutral or acidic eluents cannot differentiate between pure RGH-0205 and a batch containing about 10% of an unknown impurity. The presence of the latter was detected by HPLC using a pH as high as 9 for the eluent.

Fig. 1a and b show the chromatograms of batches 1 and 2, respectively, at pH 9. The fact that the impurity peak at $k' = 1.20$ does not appear at all in batch 1, even if the test solution is allowed to stand at this high pH for 24 h before the injection, indicated that this impurity is by no means a chromatographic artifact or an alkaline degradation product of RGH-0205.

The UV spectrum of the impurity obtained with the diode array detector was identical with that of the main component. The amino acid analysis of a sample enriched in impurity to about 35% was also identical with that of batch 1. However, the HPLC analysis of the acidic hydrolysate of the sample (6 *M* hydrochloric acid,

TABLE I

ANALYTICAL DATA OF TWO BATCHES OF RGH-0205

Batch No.	$[\alpha]_D^{20}$*	*TLC*		*HPLC*	
		System 1	*System 2*	*pH 8*	*pH 9*
1	+3.8°	Single spot, $R_F = 0.15$	Single spot, $R_F = 0.20$	Single peak, $k' = 0.76$	$k' = 0.80$
2	+3.6°	Single spot, $R_F = 0.15$	Single spot, $R_F = 0.20$	Single peak, $k' = 0.76$	Main peak $k' = 0.80$, impurity peak (10%), $k' = 1.20$

* Solvent: 10% (v/v) acetic acid.

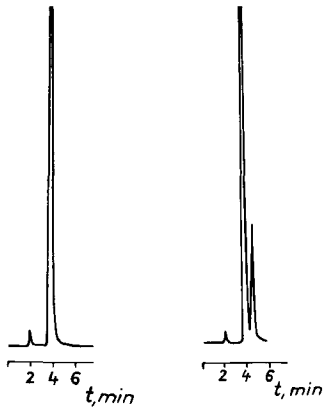

Fig. 1. HPLC of (a) pure and (b) contaminated RGH-0205 at pH 9. For chromatographic conditions, see Experimental.

24 h, 110°C) after enantiomeric derivatization with Marfey's reagent[3] showed the presence of D-aspartic acid. This was supported by a comparison of the 400 MHz ^{1}H NMR spectra of the pure and contaminated samples. In addition to the multiplets of α-CH at 4.403 ppm (dd) and β-CH_2 at 2.695 and 2.575 ppm (dd) of the pure substance, new signals appeared at 4.442 (dd) and at 2.712 and 2.495 ppm (dd), respectively.

On the basis of these findings we concluded that epimerization had taken place during the synthesis, resulting in the formation of Arg–Lys–D-Asp (LLD impurity). This was later proved by retention matching with synthesized Arg–Lys–D-Asp[4].

In possession of the pure LLD derivative it was possible to establish its quantitative determination. The identity of their UV spectra permitted the determination of the percentage of the LLD impurity in RGH-0205 based on area normalization in the chromatogram taken at 215 nm, as shown by the following regression equation derived from model experiments with RGH-0205 samples spiked with LLD impurity:

$$\text{LLD, A (\%)} = 1.02 \times \text{LLD, T (\%)} + 0.03 \quad (r = 0.99)$$

where LLD, A (%) and LLD, T (%) are percentages of the impurity found by area normalization and taken, respectively. The relative standard deviation at the 5% impurity level was 1.6% ($n = 6$) and the detection level was found to be 0.2%.

The fact that the separation of the two diastereomeric derivatives can be performed only at the unusually high pH of 9 merits special attention. In most of the hundreds of papers on the HPLC separation of peptides, including diastereomeric derivatives, slightly acidic or neutral buffers, preferably with ion-pairing anions such as phosphate, with or without organic modifiers, were used as the eluents[5,6]. With our highly polar peptide no modifier was found to be necessary to obtain suitable k' values. The presence of phosphate ion is essential; using ammonium carbonate buffer poor peak shapes and no resolution of the diastereomers were obtained. Equally necessary was the above-mentioned high pH. Table II shows the changes in the retention and separation parameters as a function of pH, and large changes take place

TABLE II

RETENTION AND SEPARATION DATA AT VARIOUS pH VALUES

pH	$k'_{Arg-Lys-Asp}$	$k'_{Arg-Lys-D-Asp}$	α
7.0	0.75	0.75	1.00
7.5	0.75	0.75	1.00
8.0	0.76	0.80	1.05
8.5	0.78	0.90	1.15
9.0	0.80	1.20	1.50
9.5	0.82	1.25	1.52

between pH 8 and 9. This is attributable to the changes in the ionization state of RGH-0205 in this pH range. The pK_a values of RGH-0205 are as follows[7]: 2.9 and 4.3 (carboxyl groups of the aspartic acid), 7.6 (α-NH_3^+ group of arginine), 10.6 (ε-NH_3^+ group of lysine) and about 14 [–NHC(=NH_2^+)NH_2 group of arginine]. From these data it is evident that the main change that occurs when the pH is raised from 8 to 9 is deprotonation of the α-amino group to form a neutral, zwitterionic species. The discussion of the exact mechanism of the separation will be the subject of another publication, where the retention and separation data of all diastereomers of RGH-0205 will also be presented. It is probable, however, even from these data that the separation of the two diastereomers at pH 9 is due to the above-mentioned deprotonation of the terminal α-NH_2 group, preventing its participation in the process of ion pairing so that ion-pair formation is limited to the side-chain positive centres and the two carboxylates bringing the molecules into the isoelectric state. Ion-pair formation in this state may be subject to steric hindrance, causing differences in the polarities, *i.e.*, the retention characteristics of the diastereomers.

In the course of a study aiming at finding the reasons for the formation of the LLD impurity in some of the batches of RGH-0205 it was necessary to investigate the optical purity of its protected form. It was found that, in accordance with the above-outlined separation mechanism, no separation took place at any pH with the LLL and LLD form of Boc-Arg(HCl)–Lys(Boc)–Asp(OBu^t)-OBu^t. The determination of the ratio of the diastereomers in this form was traced to the underivatized form of RGH-0205. The protecting group can be quantitatively removed by treatment with trifluoroacetic acid at room temperature and the neutralized reaction mixture is suitable for direct injection into the chromatograph. It was found that no racemization takes place during the removal of the protecting groups, hence this indirect method is suitable for the determination of the diastereomeric composition of the protected peptide. From the analytical point of view, the chromatographic separation after the splitting with trifluoroacetic acid at room temperature is a new example of a series of "retro-derivatization" reactions successfully used in our laboratory for the spectrophotometric and chromatographic determination of various steroid, benzhydrol and other derivatives[8–11].

CONCLUSIONS

The problem of the HPLC separation of a diastereomeric impurity in some batches of Arg–Lys–Asp can be solved by raising the pH of the eluent to 9. The

question of the extent to which this finding can be generalized, *i.e.*, the extent to which investigation at high pH can be recommended as a part of the impurity profiling of peptides, can only be answered when more data than we have at present are available. Work is in progress on this aspect.

On the other hand, it should be taken into account that working with eluents at pH 9, which is much above the usual pH range recommended for silica or modified silica columns, can drastically reduce the lifetime of the column. The solution to this problem may be the use of an alkali-resistant porous polystyrene–divinylbenzene column with which the optimal separation of diastereomeric di- and tripeptides was achieved in the pH range of the isoelectric point[12].

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