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Use of a short analytical column for the isolation and identification of degradation products of ICI 200 880, a peptidic elastase inhibitor

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ABSTRACT

This paper describes a new approach to maximize sample throughput in an analytical column for the isolation of dilute peptidic compounds. This approach exploits the chromatographic behavior of peptidic compounds which is a large change in retention relative to the change in mobile phase strength. A a result, a small decrease in solvent strength gives added retention without the loss of solubility of the sample. This same behavior also allows the use of an injection solvent which is only slightly weaker than the mobile phase to concentrate the peaks at the head of the column in a solvent overload mode. A short column allows the use of large capacity factors while maintaining reasonable elution times. In addition, cleanup of the column can be accomplished by a single injection of strong solvent in the large loop. This approach was applied to the isolation and identification of degradation products of a peptidic elastase inhibitor, ICI 200 880.

INTRODUCTION

The identification of synthetic impurities and degradation products is part of the characterization of drug substances. These compounds may be present in the drug substance or product at levels under 1%. Some techniques may enhance the concentration of the impurities and degradation products. However, usually these compounds remain as relatively dilute components of the sample. In order to ensure identification and structural verification, sufficient quantities of each unknown must be isolated. This could be as much as 1 mg [1]. In order to obtain these quantities, a preparative strategy to maximize sample throughput is needed.

One approach which has been reported is to use preparative or semi-preparative columns [2]. While a linear relationship exists between sample size and the size of the column with good column technology, scale-up from analytical methods directly has limitations [3]. In addition, preparative chromatography is operated in an over-

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load condition further limiting scale-up. As a result, this approach requires additional development work.

An alternative preparative approach which utilizes an analytical column is displacement chromatography [4,5]. Displacement chromatography involves concentration of the sample on the column using a weak solvent. The components of the sample are displaced from the column using a displacer added to the mobile phase. This approach also requires additional development and column regeneration.

A separation based on the analytical method would be ideal. In order for this to be practical, the loading onto an analytical column would have to be increased and the regeneration between runs minimized. The loading on the column could be optimized by either sample (concentration) overload or volume (solvent) overload conditions [6]. However, both conditions are limited by the resolution between peaks. For reversed-phase separations, the solubility of the analytes usually limits concentration overloading. Since the analytes to be isolated are dilute, optimizing solvent overload conditions offers the best opportunity to maximize throughout. Utilizing a weak injection solvent, the analytes would concentrate at the head of the column similar to displacement chromatography. The limit on the weakness of the solvent is the solubility of the sample.

Peptides have a unique retention characteristic under reversed phase conditions. Namely, there is a large change in retention time with only small changes in organic modifier concentration under isocratic conditions [7]. One approach to control this phenomenon in analytical methods is to use short columns in the gradient mode [8]. However, this characteristic provides the opportunity to increase volume overload without a significant loss of solubility. By operating at high capacity factors (>20), it should be possible to load large volumes of sample onto the column with the sample in the mobile phase. With these high capacity factors, the effect is preconcentration of the band at the head of the column. By reducing the length of the column to a minimum, total run times would be reduced to practical limits.

Another increase in throughput can be realized as a result of the short column. With a reduced void volume and the use of a large loop, a step change gradient of the mobile phase can be utilized to facilitate cleanup using a strong solvent injection. The column should rapidly equilibrate after the injection.

This paper reports the successful application of the use of a short column, operated at large capacity factors, to the isolation and identification of degradation products of ICI 200 880, a peptidic neutrophil elastase inhibitor.

EXPERIMENTAL

Reagents

Acetonitrile was HPLC grade, and acetic acid, acetone, ethyl acetate, glycerol, hydrochloric acid, methanol, phosphoric acid and sodium phosphate were reagent grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). Deuterated dimethylsulphoxide (DMSO-d_6) was 99.9% atom% ^2H from MSD Isotopes (Montreal, Canada). Deionized water was prepared using a Millipore (Bedford, MA, U.S.A.) Milli-Q cartridge deionizer system. ICI 200 880 and synthetic samples of products I and III (see Fig. 1) were provided by Medicinal Chemistry Department, ICI Americas (Wilmington, DE, U.S.A.). Product II was not synthesized.

Chromatographic instrumentation

All chromatographic analyses and isolation procedures were performed on a modular chromatographic system consisting of a Spectra-Physics (San Jose, CA, U.S.A.) Model 8800 pump, a Rheodyne (Cotati, CA, U.S.A.) Model 7010 Injector and a Kratos (Ramsey, NJ, U.S.A.) Model 773 variable-wavelength detector. The loop size on the injector was either 50 μl (analytical mode) or 5 ml (preparative mode). Injections were performed using a Hamilton (Reno, NV, U.S.A.) Model 1005TEFLL 5-ml gas-tight syringe. Analog data from the variable-wavelength detector were converted to digital data and processed using a VG Laboratory Systems (Altrincham, UK) Multichrom chromatographic data acquisition system on a Digital Equipment Corporation (Maynard, MA, U.S.A.) MicroVAX II processor.

The column was a 3.3 cm × 0.46 cm I.D. column packed with 3-μm Supelcosil LC-8 DB from Supelco (Bellefonte, PA, U.S.A.). The mobile phase for the isolation of the preparative separations was acetonitrile–0.1 *M* acetic acid (20:80). The flow-rate was 2.0 ml/min. The back pressure was about 90 bar.

Chromatographic retention studies

The mobile phase was a solution of 0.1 *M* acetic acid and acetonitrile. The concentration of acetonitrile was varied from 18 to 30% (v/v). Analytical samples (1 mg/ml) of ICI 200 880 and products I and III were prepared and injected.

Diluted samples (10 μg/ml) of products I and III were prepared over the range of 10 to 22% acetonitrile in 0.1 *M* acetic acid. Large volumes (5 ml) were injected in the acetonitrile–0.1 *M* acetic acid (20:80) mobile phase to determine the extent of concentration of these analytes at the head of the column.

Degradation products and isolation procedures

Solid-state degradation was produced by heating ICI 200 880 drug substance (80 and 90°C) for three months. Solution degradation products were produced in a 10 mg/ml solution of ICI 200 880 in a 50 m*M* sodium phosphate buffer at pH 7 heated at 80 or 90°C for two to four days.

The degraded solid-state sample was dissolved in acetonitrile and diluted with 0.1 *M* acetic acid to a final concentration of 15% acetonitrile. The solution degradation products were isolated by acidification with phosphoric acid and extraction with ethyl acetate. The ethyl acetate layer was evaporated to dryness at 40°C with a nitrogen stream using an Organomation Associates (New Berlin, MA, U.S.A.) Model 111 N-Evap. The residue was dissolved in acetonitrile and diluted with 0.1 *M* acetic acid to a final concentration of 15% acetonitrile for injection.

After injection, the analytes were collected (rejecting the beginning and end tails of each peak) as each analyte eluted from the column. After the last peak to be collected had eluted, 5 ml of acetonitrile–water (45:55) was injected to elute the remaining peaks (including ICI 200 880) in the sample. The collected portions of each degradation product were pooled and the mobile phase was reduced by evaporation. The residue solution was acidified with phosphoric acid and extracted with ethyl acetate. After evaporation of the ethyl acetate, the residue for each degradation product was dissolved in acetonitrile, diluted with 0.1 *M* acetic acid and reinjected for final purification. The collected portions were concentrated, acidified and extracted with ethyl acetate as above. The ethyl acetate layers were evaporated and transferred to sample vials with acetone.

Mass spectral analysis

Positive ion liquid secondary ion mass spectra (+L-SIMS spectra) were measured using a VG Analytical (Manchester, UK) Model 70-250/SEQ double focusing tandem hybrid mass spectrometer equipped with a standard VG cesium ion gun. The cesium ion gun was operated at 35 kV with an anode emission current of approximately 2 μA. Samples were first dissolved in methanol and then mixed with approximately 1 μl of glycerol (doped with 0.1 *M* HCl) on the surface of the L-SIMS target. The mass spectrum was scanned at a rate of 3 s/decade from *m/z* 950 to 100. Data were collected by multichannel averaging and subsequently mass assigned using a VG OPUS data system.

NMR analysis

The proton NMR spectra were obtained at 400 MHz on a Bruker (Rheinstetten, West Germany) Model AM-400 spectrometer. The samples were dissolved in 0.5

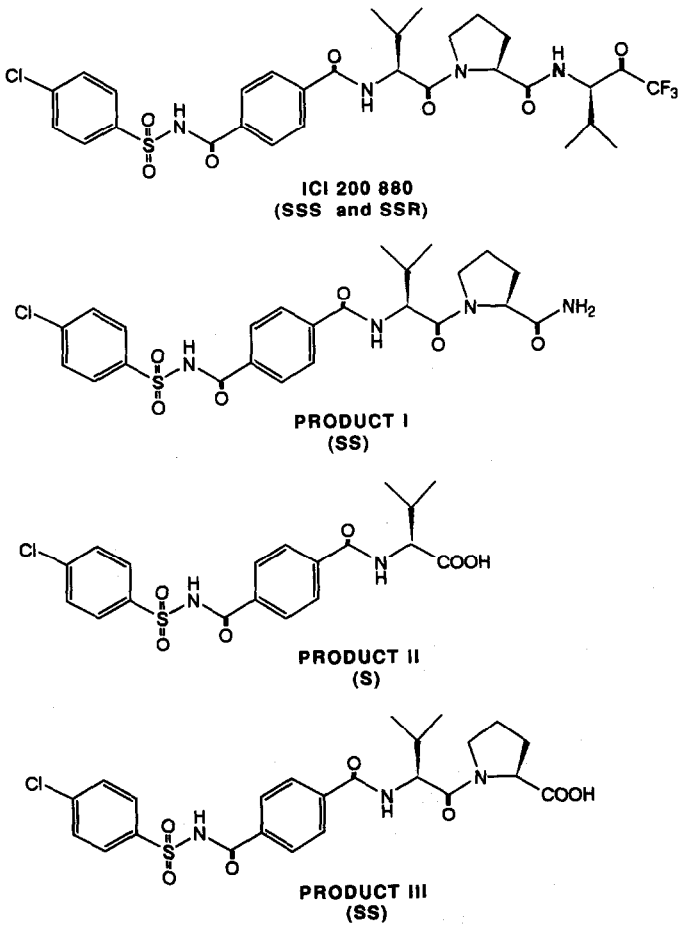

Fig. 1. Structure of ICI 200 880 and its degradation products (isomeric designations in parentheses).

ml DMSO-d_6. The spectra were run in a 5-mm diameter NMR tube at 25°C with a spectral width of 7046 Hz and memory size of 32K words, resulting in a digital resolution of 0.43 Hz/pt.

RESULTS AND DISCUSSION

Chromatographic behavior of components

The peptidic drug substance, ICI 200 880 (Fig. 1) exists as a pair of interconverting diastereoisomers. The SSS isomer is designated as isomer 1 and the SSR isomer is designated as isomer 2. The two isomers are well separated chromatographically, but elute as broad peaks with apparent separation efficiencies on the order of one tenth of that of test solutes on the column. At least two secondary equilibria appear to be the cause of this loss of efficiency. The first is slow positional isomerization around the proline ring [9,10]. The second is the hydration and dehydration of the carbonyl group during the separation process between the hydrophobic stationary phase and the hydrophilic mobile phase.

The drug substance has a large change in capacity factor with relatively small

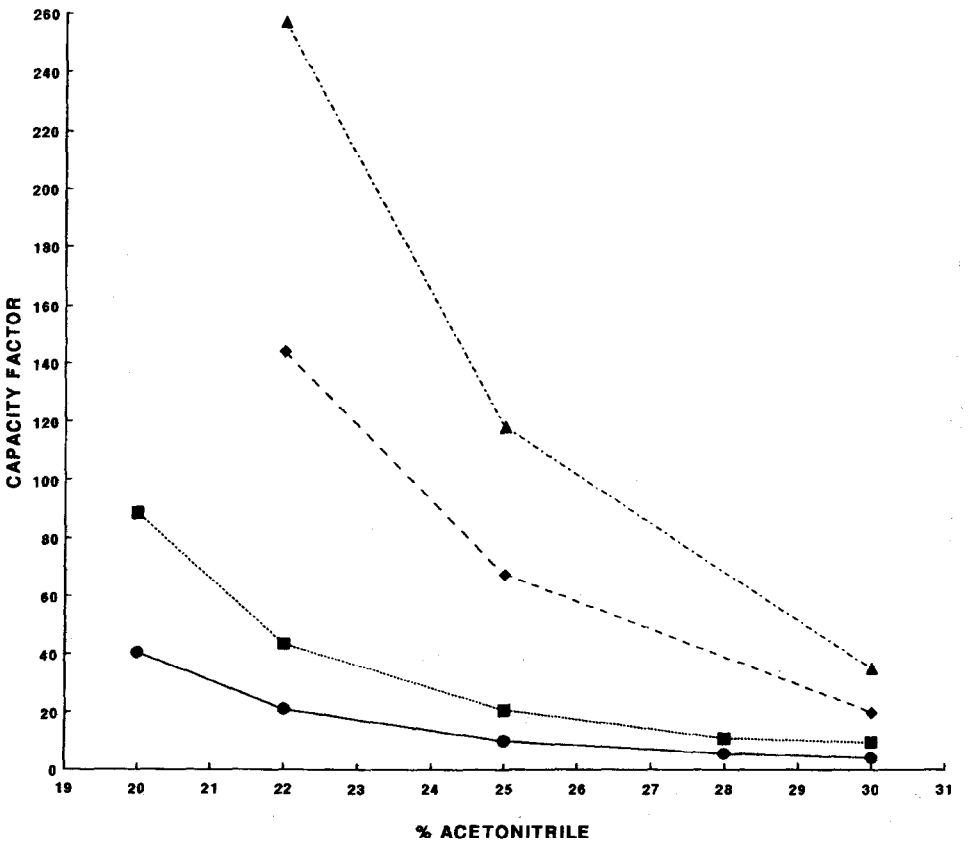

Fig. 2. Effect of solvent strength on the retention of ICI 200 880 isomers and its degradation products on the column. ● = Product I; ■ = product III; ◆ = isomer 1; ▲ = isomer 2.

changes in acetonitrile concentration in the mobile phase. This is shown in Fig. 2. Even at 30% acetonitrile, the capacity factors for the two isomers are large. With a standard 25-cm column, with a void volume of about 2.5 ml, it would take over 40 min for the second isomer to elute with a flow-rate of 2 ml/min. Using a 3.3-cm column, isomer 2 eluted in less than 10 minutes.

The elution behavior of the degradation products was observed to parallel that of ICI 200 880 as seen in Fig. 2, although the large changes were at lower concentrations of acetonitrile. As a result, at concentrations of 20% acetonitrile, all components had capacity factors greater than 40 and the drug substance is essentially retained on the column while the degradation products elute.

In a solvent overload condition, the injection solvent can have a dramatic effect on peak width. The peak width would be equal to the peak width of the analytical peak (normal band spreading) plus the width caused by the large injection volume [6]. For a 5-ml injection, this would be an additional 5 ml of width or 2.5 min at 2 ml/min flow-rate. For the 3.3-cm column, this is over 10 column volumes. By reducing the

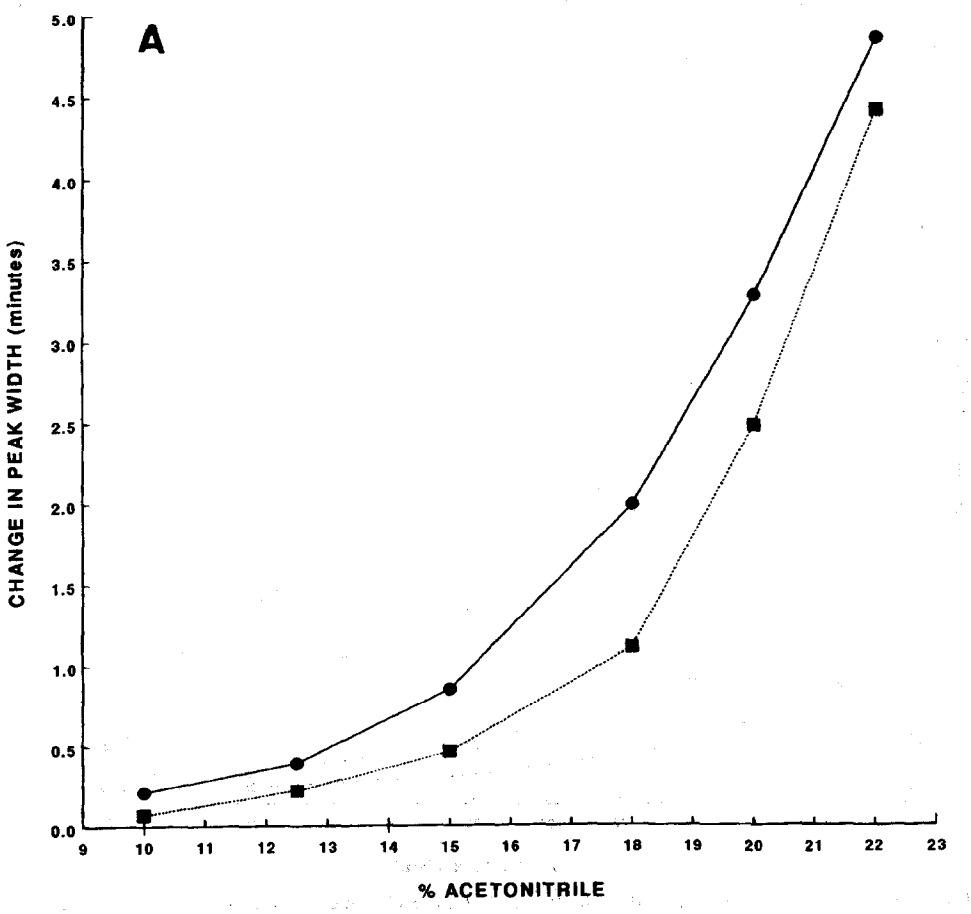

Fig. 3.

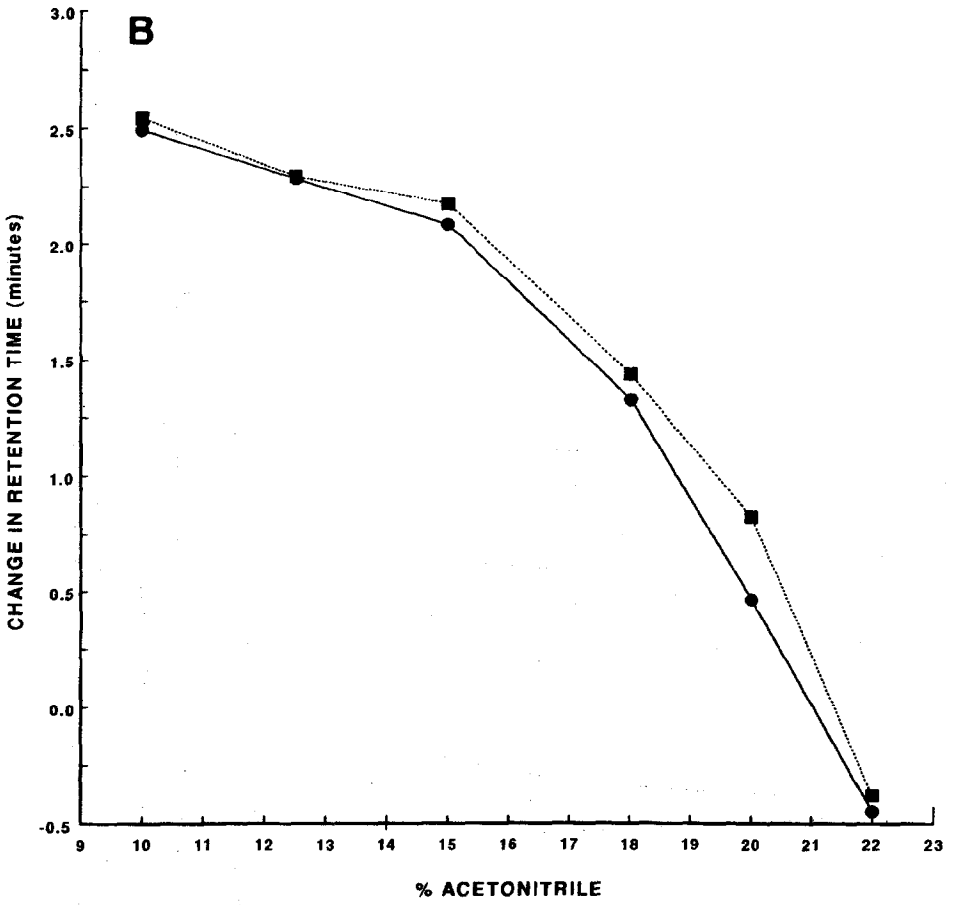

Fig. 3. Effect of injection solvent on sample concentration on column. (A) Effect on peak width. (B) Effect on retention time. ● = Product I; ■ = product III.

solvent strength of the injection solvent, a concentrating effect can be observed.

Fig. 3A shows this concentrating effect on peak width for products I and III (Fig. 1). As the solvent strength of the injection solvent increases from 10 to 22%, the peak widths increase. At 15% acetonitrile, the peak width is only 0.85 min (1.7 ml) more than the analytical peak for product I and only 0.41 min (0.92 ml) more for product III. This is only 34 and 18% of the 5 ml volume injected, for products I and III, respectively. It can be seen that at the longer retention of product III, there is a larger concentrating effect. Even when the injection solvent approaches the mobile phase at 20% acetonitrile, some concentrating effect is realized at high capacity factors. However, at slightly higher acetonitrile concentrations, the peaks broaden even more than the 5-ml volume, with the resultant loss of resolution.

The concentrating effect was also demonstrated by the retention time shifts of the components. In solvent overload conditions, the retention time (center of the band) will be equal to the retention time of the analytical separation plus one half the injection volume divided by the flow-rate. This is 1.25 min for a 5-ml injection. If the analyte is completely retained at the head of the column prior to elution, the retention

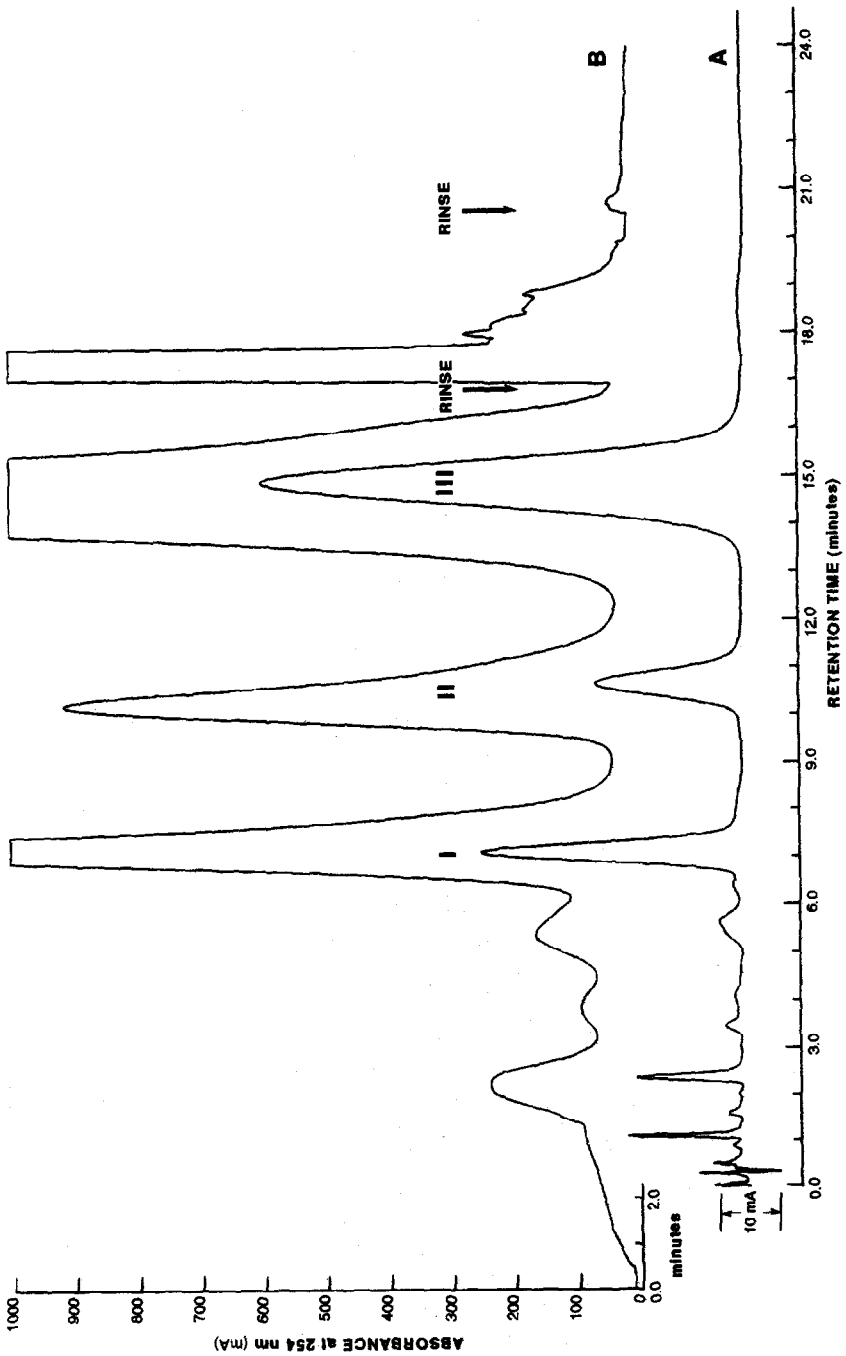

Fig. 4. Chromatograms of ICI 200 880 solid-state degradation products. (a) Analytical separation. (B) Preparative separation. Note the 2-min delay for the preparative separation. Rinse injections shown by arrows. See text for conditions. mA = 10^{-3} absorbance units.

time would increase to the limit of the time for the total injection volume to elute. This would be 2.5 min for a 5-ml injection (at 2 ml/min flow-rate). Fig. 3B shows the shift in retention times of product I and III with acetonitrile concentration in the injection solvent. The trend is according to the prediction. The retention times were shifted a maximum of 2.5 min at 10% acetonitrile. At 15% acetonitrile, the retention times were 2.1 and 2.2 min longer for products I and III, respectively. This is still over 80% of the maximum shift. The use of 15% acetonitrile in the injection solvent was a compromise between the concentrating effect and sample solubility.

Degradation products and isolation procedure

Three degradation products were observed in both solid-state and solution degraded samples (products I, II, and III). The retention times of the three products were similar from both the solid-state and solution studies, suggesting that the degradation products were the same.

In solution, a significant amount of degradation was observed. After four days at 80°C, only 25% of the compound remained. A single peak (product III) accounted for 70% of the 75% loss. Two other minor peaks (products I and II) were about 2 and 1% of the sample, respectively. While all three products were observed in the solid-state samples, much less degradation was observed. Products I, II and III were about 2, 1 and 8% of the sample, respectively.

The chromatograms in Fig. 4 illustrate an analytical and a 5-ml injection of the solid-state degraded samples. In the analytical separation, the peak widths were 1.3, 1.9 and 3.1 min for products I, II and III, respectively. The peak widths for the same components in the 5-ml injection were 2.05, 2.8 and 4.3 min, respectively. These values indicate that a 50 to 75% concentrating effect was realized for a 5-ml injection.

The increases in retention times observed between the analytical and large-volume injection in Fig. 4 were 2.2, 1.7 and 1.9 min for products I, II and III, respectively. These values are close to the predicted value of 2.5 min for complete concentration at the head of the column. Note the 2-min offset of the time axis between the analytical and preparative chromatograms in the figure to account for the delay.

Fig. 5 illustrates similar behavior for the solution sample. The peak widths in the analytical separation were 1.3, 1.8 and 3.5 min for products I, II and III, respectively. The peak volumes for the 5-ml injection were 2.9, 2.75 and 6.2 min, respectively. These results indicate a 40 and 60% concentrating effect for products I and II. It appears that some concentration overload was observed for product III. Retention time increases for the products in the solution sample were also similar to those in the solid-state sample.

Since the 5-ml loop is over 10 column volumes (void volume was 0.4 ml), an injection of a strong solvent is an efficient way of cleanup of late eluting components. After the last peak of interest eluted, the column was rinsed by injection of 5 ml of acetonitrile–water (45:55). Figs. 4 and 5 demonstrate the effectiveness of this technique. The rinse was injected at 19 min (17 min on the analytical chromatogram time axis). An additional 5 ml were also injected at 23 min (21 min on the analytical time axis). Note a minimum of response after the second injection verifying the rinse was complete.

With a target of 1 mg for each component, it was necessary to inject and collect

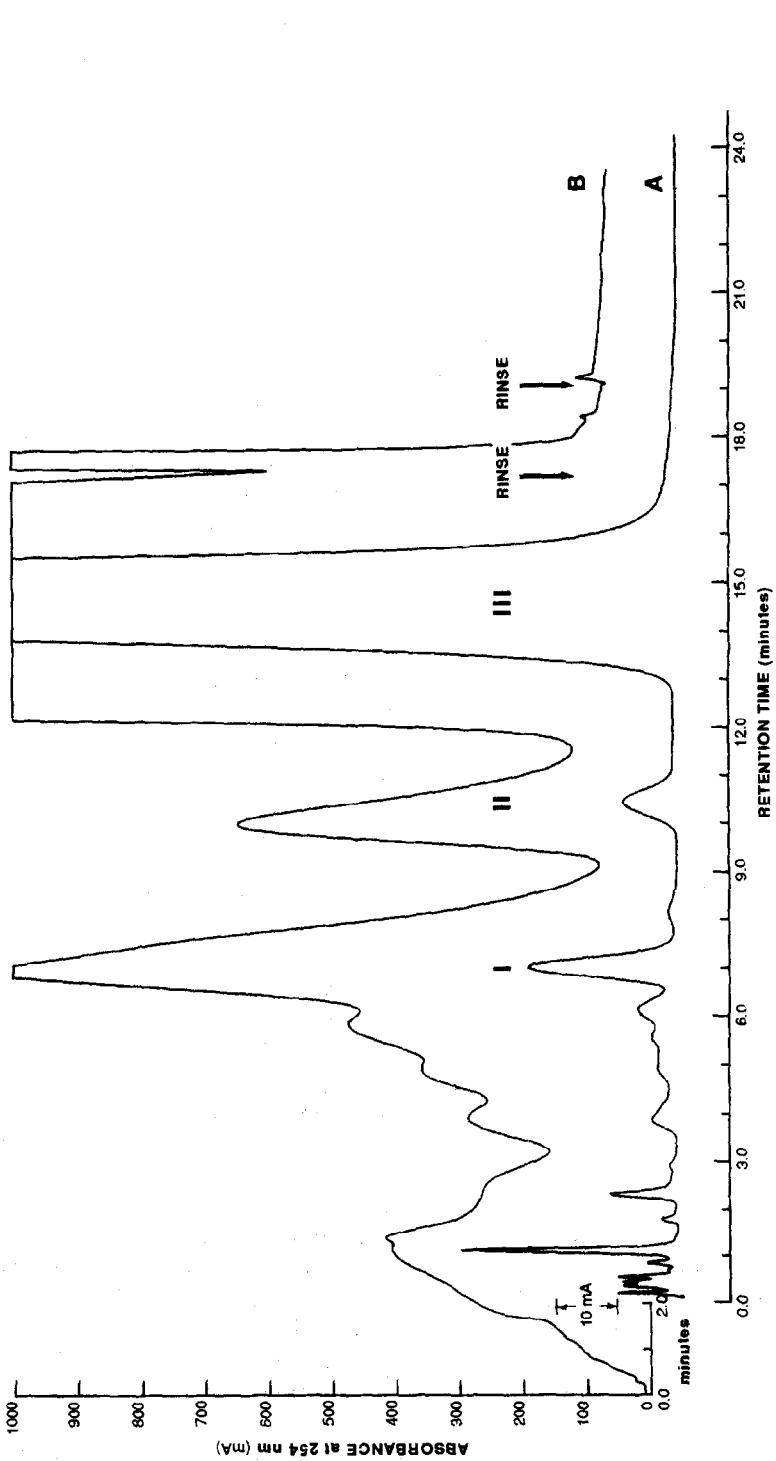

Fig. 5. Chromatograms of ICI 200 880 solution degradation products. (A) Analytical separation. (B) Preparative separation. Note the 2-min delay for the preparative separation. Rinse injections shown by arrows. See text for conditions.

20 × 5 ml of a 1 mg/ml solution of the degraded samples, assuming a 1% level for each degradation product. For products I and III, this was easily achieved. Product II was the smallest product and about 500 μg was isolated from both solid-state and solution samples. Since product II has only 65% of the mass of ICI 200 880, the actual recovery is close to 80%. Since product III was a major component of the solution sample and the largest product in the solid-state sample, it was possible to collect enough of that product in fewer injections. For the rest of the injections of that sample, the injection of the strong solvent rinse was made after product II eluted, saving additional time.

Chromatographic analysis of the products indicated that product I isolated from the solid state did not contain any measurable impurities while it contained about 2% impurities when isolated from the solution sample. Product II contained about 0.5% impurities when isolated from either the solid state or solution sample. Product III did not contain any measurable impurities when isolated from either sample.

Spectral identification and confirmation

Mass spectral results. Representative + L-SIMS spectra obtained from the three major degradation products are shown in Fig. 6. The spectra obtained from either the solid-state or solution samples were similar. The major fragmentation pathways are summarized as inserts in each of the spectra. The fragmentations shown are fully consistent with the proposed structures. In addition to the identifications noted, the signal at *m/z* 133 is believed to originate from Cs^+, and the signal at *m/z* 185 corresponds to the protonated dimer of glycerol $(2G+H)^+$. Products I and III yield spectra which match the spectra obtained from corresponding synthetic standards.

NMR results. Fig. 7 shows the proton NMR spectra of products I, II and III. All of the spectra shown were of samples isolated from degraded solutions of ICI 200 880. The spectra obtained from the solid-state samples were similar. It can be seen that all significant signals are related to the samples with the exception of a peak for acetonitrile (2.1 ppm) used in the isolation and the large broad peak for residual water (3.4 ppm) which was in the sample and in the DMSO-d_6 solvent. There are also several small peaks unrelated to the compounds. However, they do not interfere with the identification of the sample and assignment of the spectra.

The identity of products I and III was verified by comparison of the spectra to spectra of authentic samples. The spectrum of the product II is consistent with a structure that results from loss of the proline function from product III. This interpretation is based on integration of the peaks, splitting patterns, and homonuclear decoupling experiments that determined proton–proton connectivities.

Based on the identification of the products and the chemistry that produced them, it is clear that each reaction would produce two products, one containing an ultraviolet chromophore and one which does not. Therefore, it can be inferred that each of the three identified products has another product associated with it. No attempt was made to find these products in this study.

CONCLUSIONS

The results of these studies demonstrate the effectiveness of the use of a short analytical column in the isolation of peptidic compounds for spectral identification.

The procedure is enhanced by the chromatographic behavior of the degradation products. That behavior of relatively large changes in retention with small changes in solvent strength allows solvent overload conditions and concentration at the head of the column. The short column allows for the use of large capacity factors and for a rapid column cleanup between injections.

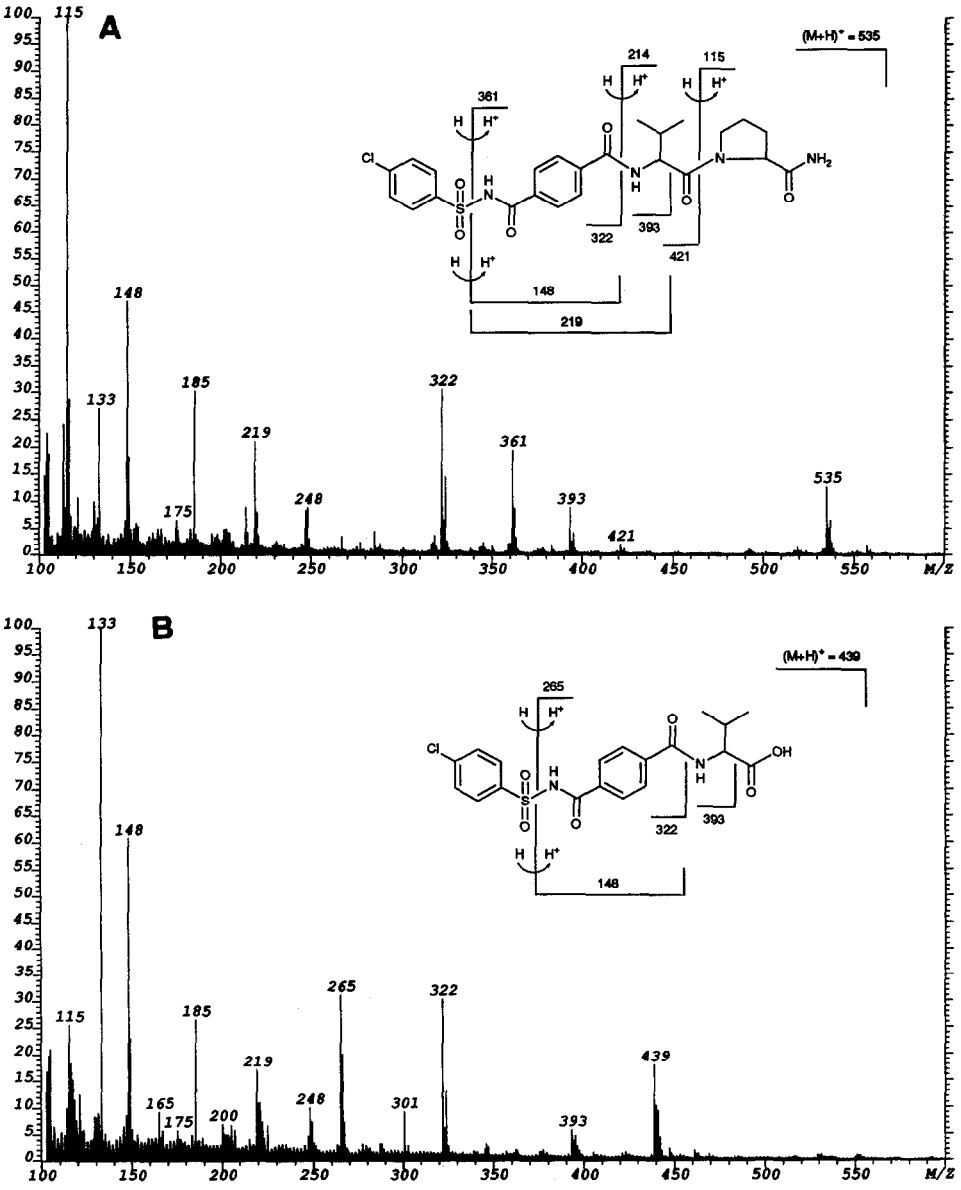

Fig. 6.

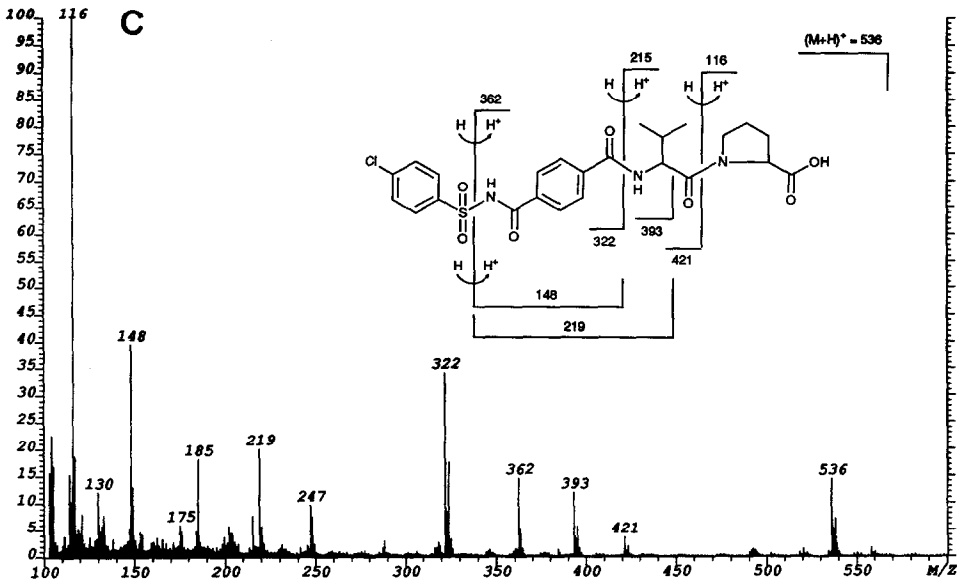

Fig. 6. Positive ion liquid secondary ion mass spectra of the isolated degradation products. (A) Product I; (B) product II; (C) product III.

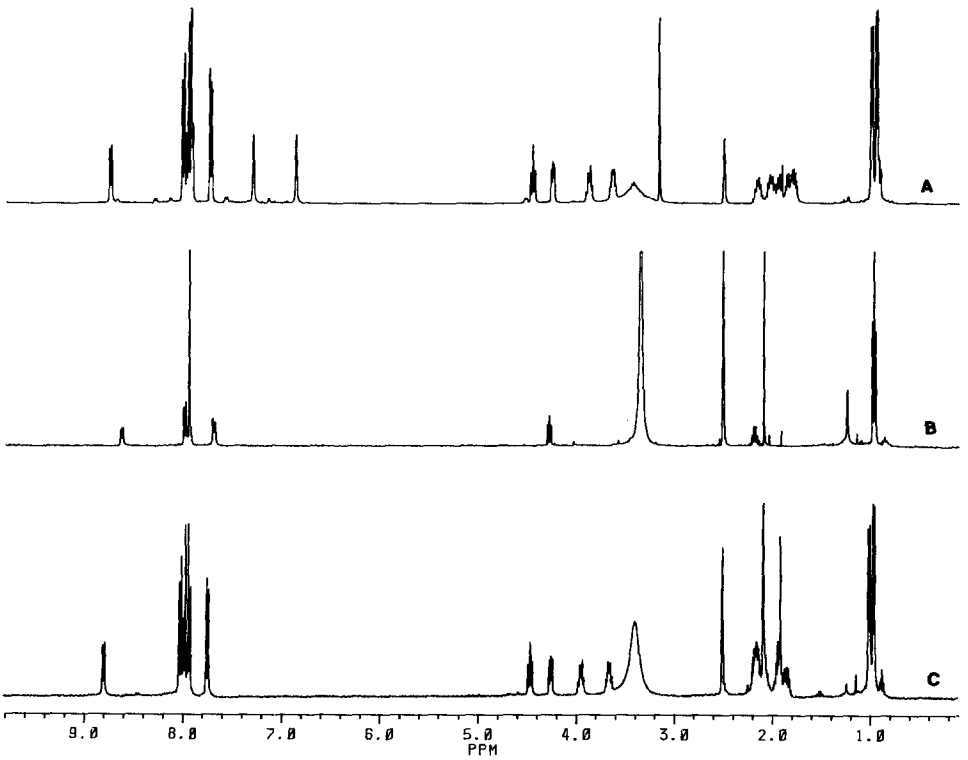

Fig. 7. Proton NMR spectra of degradation products. (A) Product I; (B) product II; (C) product III.

This procedure may also be applicable to the isolation of compounds with more normal chromatographic retention behavior. The extent of the success of this approach would be limited by solubility and any ability to concentrate the sample at the head of the column.

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