



Reversed-Phase Preparative and Process Chromatography

A Powerful but Often Overlooked Method for Protein Purification

Reversed-phase chromatography on 300 Å pore-size adsorbents, first introduced by Vydac in 1981, is a very useful high-resolution method for protein separations. The power of reversed-phase for protein chromatography is demonstrated, for example, by the separation of species-specific insulins, many of which differ by a single amino acid residue (Fig. 1), the separation of reduction products which differ mainly in conformation from native insulin (Fig. 2), the separation of ribosomal proteins (Fig. 3), and the separation of various apolipoproteins from human serum (Fig. 4).

Subtle differences in conformation can permit protein separation by reversed-phase chromatography because retention depends

on the "hydrophobic footprint" of a protein molecule – the minority of hydrophobic amino acid residues that are actually accessible at the surface of the folded protein. Since sample loading occurs at low organic concentration, this can reflect subtle differences in native structure.

A rule of thumb for developing preparative and process purifications is that a method that succeeds in revealing an impurity will often be the best method for removing that impurity. The frequent use of reversed-phase for protein analysis would therefore argue for its utility in purifications. To be fair, chromatography on Vydac reversed-phase adsorbents is already used in process purification of

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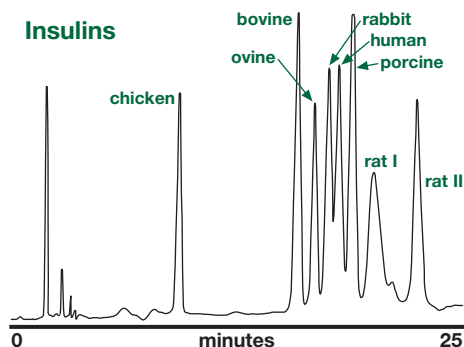


Figure 1. Separation of species-specific insulins, some of which differ by a single amino acid, demonstrates the ability of reversed-phase HPLC to separate very similar polypeptides. Column: Vydac 214TP54 (C₄, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Mobile phase: Gradient from 27 to 30% ACN with 0.1% TFA over 25 min. From J. Rivier and R. McClintock, *J. Chrom.* 268, 112-119 (1983).

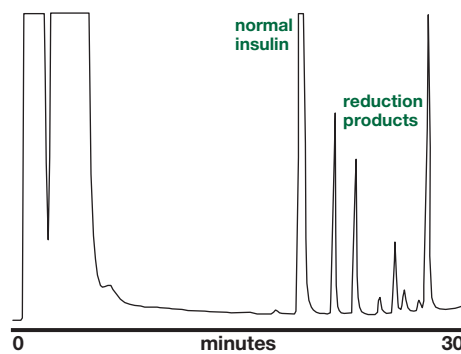


Figure 2. Separation of insulin and its partial reduction products. In a study of bridged peptides, disulfide bonds were reduced and the reduction products separated from normal insulin by C18 reversed-phase HPLC. Column: Vydac 218TP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Flow: 1.0 mL/min. Mobile phase: A=0.1% TFA, B=0.092% TFA in 60:40 ACN:H₂O. Gradient from 35 to 85% B in 25 min. Data from W. R. Gray, *Prot. Sci.* 2, 1732-1748 (1993).

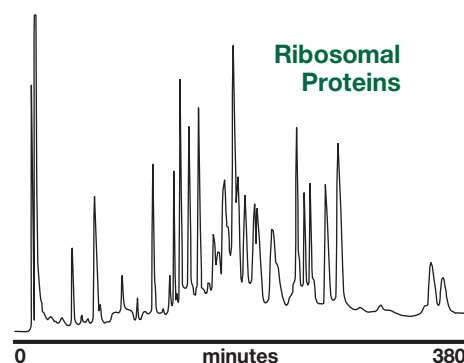


Figure 3. Separation of ribosomal proteins. Column: Vydac 214TP54 (C₄, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Mobile phase: Gradient from 10 to 38% isopropanol with 0.1% TFA over 355 min. From R.M. Kamp, A. Rosenthal, D. Kamp, and B. Wittman-Liebold, *J. Chrom.* 317, 181-192 (1984).

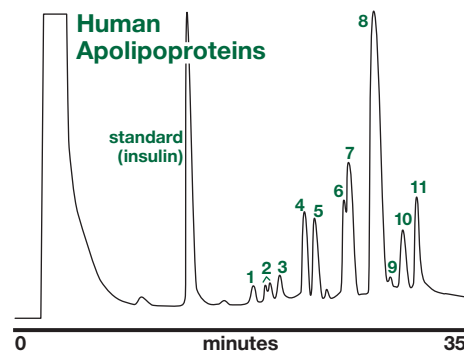


Figure 4. Separation of apolipoproteins isolated by gradient ultracentrifugation from human serum. Column: Vydac 218TP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Flow: 1.2 mL/min. Temperature: 50°C. Mobile phase: A = 25% ACN/0.1% TFA in water. B = 58% ACN/0.1% TFA in water. Gradient from 0 to 100% B in 33 min. Peaks: 1. apoC-IIIa; 2. apoC-IIIb; 3. apoC-IIa; 4. apoC-IIIc; 5. apoC-I; 6. apoC-IIb; 7. apoA-Ia; 8. apoA-Ib; 9. apoA-IIc; 10. apoA-IIa; and 11. apoA-IIb. Reproduced with author's permission from Hughes, et. al., *J. Lipid Res.* 29, 363-376 (1988).

Reversed-Phase Preparative and Process Chromatography

(continued from page 1)

several FDA-approved bio-pharmaceuticals, and new reversed-phase purifications of protein products are reported regularly. However it also appears that reversed-phase methodology, while highly regarded as an analytical method and in spite of its prodigious resolving power, is often dismissed or overlooked as a candidate method for purification.

Why is this?

We believe it reflects some apprehensions about reversed phase that actually have simple answers.

Don't reversed-phase conditions denature proteins and destroy activity?

Although the organic solvents used in reversed-phase elution may disrupt tertiary structure, most proteins will refold and regain activity when returned to aqueous buffers. Some proteins, especially those with disulfide bridges that help maintain structure, will either not lose activity or refold very rapidly. For others, proper refolding is often favored by gradual removal of solvent at moderate temperatures. A little experimentation will usually discover the best conditions.

Use of organic solvents does provide some advantages. For example microbial growth and endotoxin contamination are less likely in organic-aqueous mixtures than in purely aqueous mobile phases. Reversed-phase chromatography can also provide an effective method for endotoxin removal.

Isn't reversed-phase chromatography too expensive for process-scale use?

Bulk adsorbents for preparative and process reversed phase are available in quantity at lower cost than adsorbents typically used to pack analytical HPLC columns. It is often possible to choose solvents for reversed phase that are not more expensive than buffer components used in other forms of chromatography.

The separation power of reversed phase as well as its utility in desalting, endotoxin removal, and ability to produce highly concentrated fractions often allows reversed-phase chromatography to replace other steps that taken together are more expensive in a purification process.

Aren't organic solvents toxic? Don't they cause waste disposal problems?

Proper choice of solvents is the key. While the acetonitrile and methanol often used for analytical reversed-phase can be problematic, process protein separations can normally be performed with less expensive and nontoxic ethanol or isopropanol. Ethanol is often a good choice because it is available in USP grade at reasonable cost, is familiar to the FDA, and is environmentally innocuous. For ion pairing, acetic acid is a good choice to replace the TFA commonly used in analytical separations.

In developing reversed-phase protein separations, whether analytical or preparative, it helps to have a partner that is experienced in this technology. As the leading provider of 300 Å reversed-phase adsorbents for protein and peptide chromatography, Vydac has more experience than any other supplier. In addition, we provide a full range of 300 Å reversed-phase packed columns plus bulk adsorbents for preparative and process applications. Our bulk materials include larger silica particle sizes – 10 µm, 10-15 µm, 15-20 µm, and 20-30 µm – with the same 300 Å pore size and bonded reversed-phase chemistries available in Vydac 5 µm adsorbents for analytical protein and peptide HPLC. This availability of identical chemistries simplifies method development by allowing separations to be initially scouted on analytical-size columns, then scaled up, with appropriate adjustments in conditions of elution, to virtually any scale necessary for purification of larger quantities.

Vydac has a history of supporting process reversed-phase customers. We have the capacity to supply bulk reversed-phase adsorbents in 100 kg quantities with extensive regulatory support information for GMP applications. Our technical department can assist you in developing separations, scaleup strategies, and obtaining adsorbents in sufficient quantities to meet process needs.

Seminars Available

Vydac's technical staff is able to provide technical seminars at customer locations on a variety of HPLC separations-related topics. To explore potential technical seminar opportunities, contact Vydac via email to experts@vydac.com or call Vydac's Technical Department at (800) 247-0924.

Sources for Current Vydac Information

The 2000/2001 Vydac Catalog



The new 2000/2001 Vydac Catalog was printed and distributed in February. If you have not received a copy, please contact Vydac to request one.

The Vydac Website

All Vydac publications are available in electronic form for viewing and downloading on the Vydac website. For immediate information, go to URL

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New Reversed-Phase Polymer Packings for Preparative Protein and Peptide LC

- acid and alkali stable, pH 0 to 14
- heat stable to 80°C
- 300 Å pore size for proteins and peptides

Vydac's 259VHP polymer-based reversed-phase adsorbent is a unique and versatile tool for protein and peptide chromatography. Its exceptional chemical

resistance makes it ideal for separations of difficult proteins or peptides that require harsh conditions to maintain solubility, and also for more routine applications where it is desired to clean and sanitize columns between runs, for example by washing with strong alkali.

The 259VHP polymer has a 300 Å pore size to provide access to all adsorbent surfaces for large peptides and proteins. It is mechanically stable under normal HPLC operating pressures and produces resolution comparable to 300 Å silica-based reversed-phase adsorbents.

The 259VHP material has been available for several years in the form of

5 µm diameter particles for analytical and semipreparative HPLC. Now Vydac has introduced two larger particle sizes for scaleup applications – 8 µm and 15 µm.

The chemistry of the larger particle sizes is identical to that of the 5 µm particles, allowing easy scaleup with minimal modifications to conditions developed using analytical columns. Comparisons of peptide and protein separations run under identical conditions on different 259VHP particle sizes are shown in Figures 5 and 6 on this page.

Vydac can supply 259VHP media prepacked in columns or as bulk adsorbents. Please contact Vydac for pricing on bulk materials.

Peptides

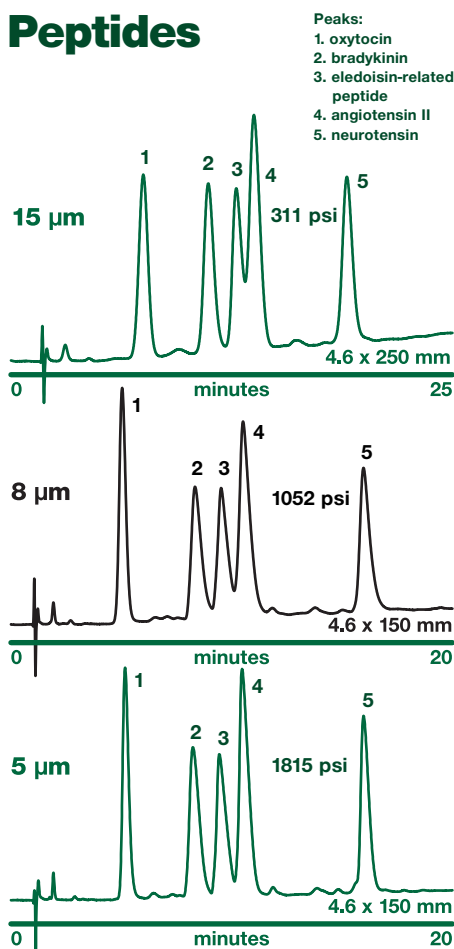


Figure 5. Peptides on 259VHP. A reversed-phase separation of five peptides was compared on 259VHP adsorbents of three different particle sizes. Conditions identical for all three columns. Column sizes and backpressures as shown. Detection: 220 nm. Flow: 1.5 mL/min. Mobile phase: A=0.1% TFA (w/v) in water. B = 0.1% TFA (w/v) in 25:75 water:ACN. Gradient: Linear, 20% to 40% B over 30 minutes.

Proteins

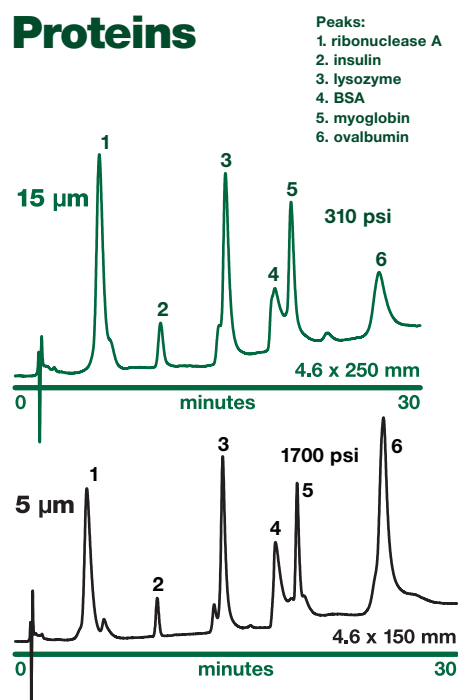


Figure 6. Proteins on 259VHP. A reversed-phase separation of six proteins was compared on 259VHP adsorbents of two different particle sizes. Conditions identical for both columns. Column sizes and backpressures as shown. Detection: 220 nm. Flow: 1.5 mL/min. Mobile phase: A = 0.1% TFA (w/v) in water. B = 0.1% TFA (w/v) in 25:75 water:ACN. Gradient: Linear, 33% to 80% B over 30 minutes.

Ordering Information

Cat. No.	Description
259VHP5415	Analytical Column, Polymer Reversed Phase, 300 Å, 5 µm, 4.6 mm i.d. x 150 mm
259VHP810	Semiprep Column, Polymer Reversed Phase, 300 Å, 8 µm, 10 mm i.d. x 250 mm
259VHP822	Prep Column, Polymer Reversed Phase, 300 Å, 8 µm, 22 mm i.d. x 150 mm
259VHP1522	Prep Column, Polymer Reversed Phase, 300 Å, 15 µm, 22 mm i.d. x 250 mm

Other column sizes and bulk adsorbent are available for both analytical and preparative applications.

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Also available...

RamPak™

Axial-compression system for preparative scale-up



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- versatile
- reliable and cost-effective

Through a partnership with Varian Instruments, Inc., Vydac is proud to offer RamPak column packing systems.

These unique, pneumatically controlled axial-compression systems facilitate easy, reliable on-site packing of high-performance preparative and process columns. They are ideal for use with Vydac bulk adsorbents.

A selection of column dimensions, from 41 mm to 150 mm ID, provides versatility to meet current and future requirements. Varying the amount of packing slurry determines the column length, up to 350 mm. RamPak columns can be tailored to maximize throughput while reducing costs.

For more information and quotations, contact Vydac Technical Support.

"RamPak" is a trademark of Varian Instruments.

History of Vydac – A Commitment to Support and Technical Excellence

Beginnings

Vydac, also known as The Separations Group, Inc., was founded in Hesperia, California, in 1971 as a company dedicated to the research, development and manufacture of separation materials for high performance liquid chromatography.

The first products developed by Vydac were based on the deposition of silica on glass spheres. The silica layer was then chemically bonded using organosilane reagents to create an organic film on the surface. The interaction of molecules with the organic film on the silica surface produced reversed-phase chromatographic separations. These "pellicular" HPLC adsorbents provided high mechanical stability, but relatively low capacity, making them useful primarily for analytical separations.

Separations for Biotechnology

A totally silica-based product was developed and introduced in 1980. Designated "TP" for "totally porous" (as opposed to pellicular), this 300 Å pore-size silica, similarly bonded with organosilanes, had wide applicability for separation of large molecules compared to the 60 Å to 100 Å pore-size HPLC silicas otherwise available. Its introduction coincided with a very rapid phase of growth in biotechnology. Vydac TP silica adsorbents became accepted as the standard and most widely utilized media for reversed-phase separations of proteins, peptides, and oligonucleotides, finding application in analysis and purification of molecules from both natural and synthetic sources.

In 1985 Vydac introduced a 90 Å pore size silica for separation of small molecules, and in 1992 new polymer based materials (VHP) were introduced for high-performance ion-exchange separations, and eventually polymer-based reversed-phase applications. The designation

"VHP" was for "very highly porous" relating to the very open 900 Å pore structure of the polymeric ion exchange materials.

Technical Competence

Throughout its history Vydac has been an innovator in the field of silica and polymer chemistries applied to HPLC separations. We have expanded from four employees in 1980 to approximately 25 today with a primary objective of maintaining a core group of highly competent chemists and technical support personnel not only to develop unique materials but also assist Vydac customers in their application to real-world separation problems. By virtue of our continuing involvement and concern with customer applications, we have accumulated a base of experience that we are happy to apply in helping chemists and biotechnologists meet new separation challenges.

Customer Support

Much of our experience is reflected in Vydac technical publications which include the *Vydac Catalog*, an extensive list of *Application Notes*, this newsletter *Vydac Advances*, and our comprehensive *Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC* – an excellent introduction and reference source for anyone involved in the field.

Vydac technical publications in print are available free for the asking. All publications are also available on the Vydac website (<http://www.vydac.com>) for viewing and can be conveniently downloaded when the need for information won't wait for the mail.

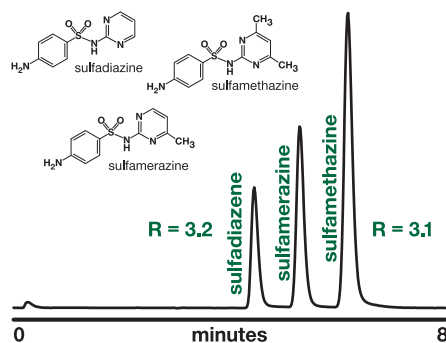
Vydac technical staff is available for consultation by telephone, via email at experts@vydac.com, and also has the capability to present technical seminars at customer locations.

SelectaPore™ 300P is C₁₈ Reversed-Phase Column of Choice for USP Analyses

Vydac's SelectaPore column line consists of three C₁₈ reversed phases for small molecule analysis. Variations in selectivity based on bonding chemistry and pore size improve the chances of detecting contaminants in new pharmaceutical products. For routine analyses of pharmaceuticals, on the other hand, analytical conditions and suitability requirements are specified by USP. Vydac's SelectaPore 300P column is recommended for USP analyses. The polymeric C₁₈ phase of this column contributes stability and long column life. The specially treated base silica minimizes tailing for alkaline analytes, and the 300 Å pore size provides uniform surface access for large multi-ring molecules. Performance is guaranteed to meet USP suitability criteria, as indicated by the examples shown here.

Trisulfapyrimidines

USP requires R not less than 3.0.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 254 nm. Flow: 1.0 mL/min. Mobile phase: 86:13:1 water:ACN:glacial acetic acid. Isocratic.

Ordering Information

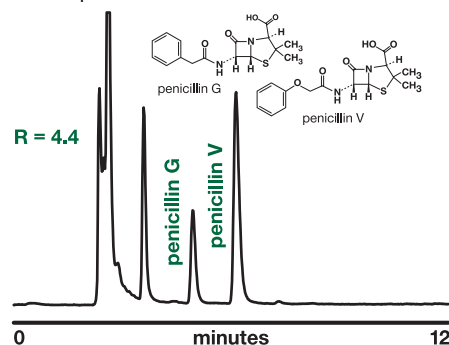
Cat. No.	Description
218WP54	Column. SelectaPore 300P. Polymeric C ₁₈ . 300 Å. 5 µm. 4.6 mm i.d. x 250 mm.

For information about other Vydac SelectaPore columns, request a copy of the SelectaPore Brochure.



Penicillin V

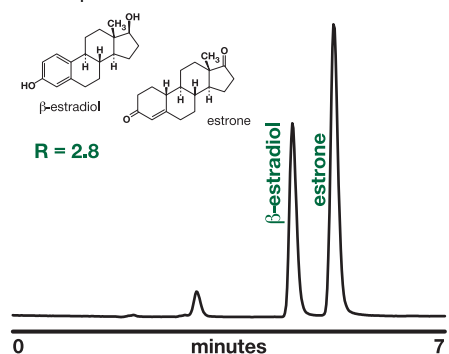
USP requires R not less than 3.0.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 254 nm. Flow: 1.0 mL/min. Mobile phase: 60:40 water:ACN with 1% HOAc. Isocratic.

β-Estradiol

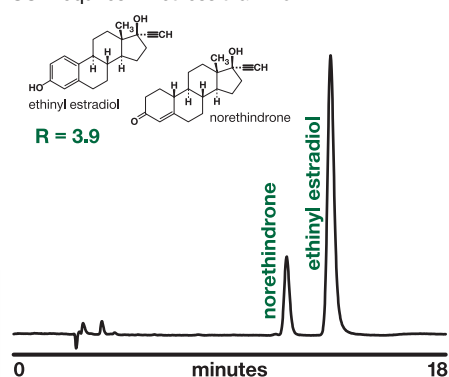
USP requires R not less than 2.0.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 205 nm. Flow: 1.0 mL/min. Mobile phase: 51:49 ACN:water. Isocratic.

Norethindrone and ethinyl estradiol

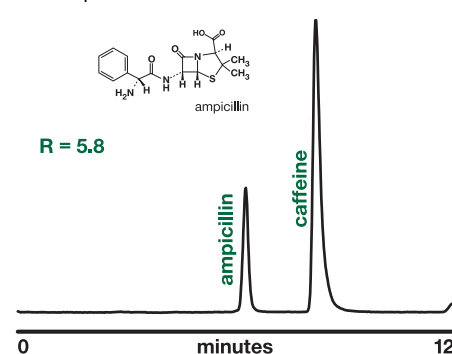
USP requires R not less than 2.0.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 200 nm. Flow: 1.0 mL/min. Mobile phase: 20 mM KH₂PO₄, pH 6.0, 35% ACN. Isocratic.

Ampicillin

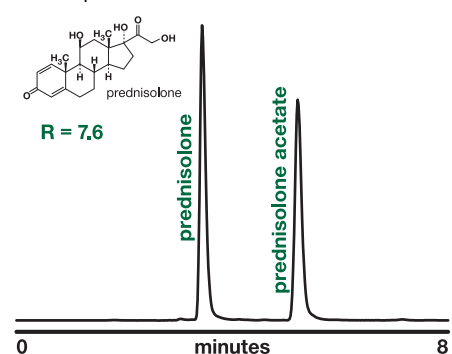
USP requires R not less than 2.0.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 254 nm. Flow: 1.0 mL/min. Mobile phase: 909:80:10:1 water:ACN:1MKH₂PO₄:1N acetic acid. Isocratic.

Prednisolone acetate

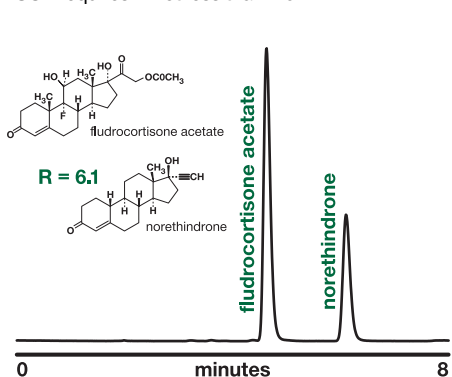
USP requires R not less than 2.0.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 254 nm. Flow: 1.0 mL/min. Mobile phase: 40% ACN in water (v/v). Isocratic.

Fludrocortisone

USP requires R not less than 2.5.



Column: Vydac 218WP54 (C₁₈, 300 Å, 5 µm, 4.6 mm i.d. x 250 mm). Detection: 254 nm. Flow: 1.0 mL/min. Mobile phase: 45:55 ACN:water. Isocratic.