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HIGH PERFORMANCED/PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT:

In assisted reproductive technology (ART), controlled ovarian hyperstimulation (COS) plays an important role in guiding treatment progress and determining success. Many ovulation-inducing agents are used in this process, including human menopausal gonadotropin (HMG), recombinant follicle-stimulating hormone (r-FSH), recombinant luteinizing hormone (r-hLH), urinary human chorionic gonadotropin (u-hCG), and recombinant human chorionic gonadotropin (r-hCG). HMG, which comes from the urine of postmenopausal women, mainly contains FSH, LH, and hCG. While bioactivity-based potency units and immunoassay-based ELISA methods remain the gold standard for quantifying protein therapeutics, LC-MS/MS is emerging as a game-changing technology. Its unique strengths are exceptional specificity and absolute quantification capabilities, independence from specialized reagents, powerful multiplexing potential, and ability to provide structural insights, establishing it as an essential

tool for both orthogonal verification and cutting-edge research in the field. This study used reverse-phase liquid chromatography with mass spectrometry (RP-LC-MS/MS) and a label-free quantitative proteomics method to analyze imported r-hFSH, r-hLH, r-hCG (Gonal-F, Luveris, and Ovidrel), three domestic HMG products (Le Baode, Man Fuxin, and Xin Yunle), and u-hCG (Li Zhu). The results showed that the purity of the main component in imported gonadotropin drugs was higher than 98.55%. On the other hand, the main element of domestic u-HMG is FSH, with small amounts of hCG and very little LH. These results reveal significant differences in the composition of imported and domestic gonadotropin products, underscoring the need to further evaluate their biological activity and clinical effectiveness. This study provides important insights into drug selection for ART, with practical value for improving the quality of HMG drugs and making better use of them in clinical settings.

KEYWORDS: Antimicrobial drugs; Box-Behnken design; CaFRI; Greenness assessment; RP-HPLC. Gallic acid determination; Halloysite nanotubes; Magnetic solid-phase extraction (MSPE); Molecularly imprinted polymers.

INTRODUCTION:

Scope of Chapter

The development of high-performance liquid chromatography (HPLC) packings and instrumentation over the past 25 yr has revolutionized the efficiency and speed of separation of molecules in general and peptides in particular. This development has also seen a tremendous output of published literature on the topic of HPLC of peptides, perhaps making the decision as to how best to approach a particular separation problem seem formidable to the novice, or even experienced HPLC user. Fortunately, regardless of whether high-performance approaches are utilized for routine peptide separations or for such state-of-the-art areas as proteomics, capillary methods, biospecific interactions, and so on, the fundamentals of chromatographic protocols remain the same. Thus, it is not the purpose of this chapter to present a comprehensive review of HPLC of peptides. Indeed, there is a wealth of relevant material accessible in the literature. For instance, several useful articles and reviews on HPLC of peptides can be found In addition, represent excellent resource books in this area. Finally, offers an extensive source of information on the early development of HPLC of peptides.

This chapter is aimed at laboratory-based researchers, both experienced chromatographers and those with limited exposure to high-performance separation approaches, who wish to

learn about peptide analysis by HPLC, based on representative examples from research carried out in our laboratory with general applicability. Standard analytical applications in HPLC of peptides will be stressed, together with novel approaches to separations and modest scale-up for preparative purification of peptides. In addition, the value of the complementary technique of capillary electrophoresis (CE) for peptide separations will be demonstrated. Finally, in order to maximize the "user friendliness" of this chapter, only nonspecialized columns, mobile phases, and instrumentation readily available and easily employed by the researcher are described.

Detection Methods for Profiling and Quantification:

HPLC-UV (ultraviolet detection):

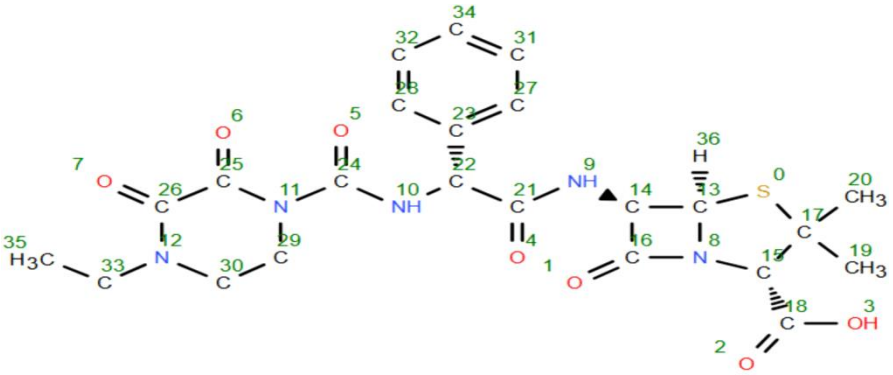
Among all HPLC detectors, the most simple and the most widely used is UV . Although it suffers from some limitations, particularly for NPs that do not possess UV chromophores, it has the best combination of sensitivity, linearity, versatility, and reliability of all the LC detectors developed so far. Most NPs absorb UV light in the range of 200–550 nm, including all substances having one or more double bonds and all substances that have unshared electrons. Thus, even compounds having weak chromophores, such as triterpene glycosides, can be successfully detected by UV at short wavelengths (203 nm) . In this case, however, several mobile-phase constituents that exhibit high UV cut-offs should be avoided because they might blind the detection of NPs with weak chromophores.

In UV detection, the relationship between the intensity of light transmitted through the detector cell and the solute concentration is given by Beer's Law . The two factors that control the detector sensitivity are the magnitude of the extinction coefficient of the analyte of interest at a given wavelength and the path length of the light passing through the UV cell. The sensitivity will increase with increasing path length, but a compromise must be found with the cell volume in order to avoid peak dispersion.

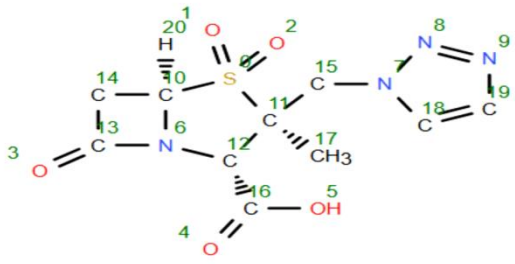
Three types of UV detectors are available: fixed wavelength, multiple wavelength, or photodiode array (DAD). The fixed-wavelength detector is the least expensive and has higher intrinsic sensitivity because the light is emitted at specific wavelengths with given lamps. However, the multiple-wavelength detector is more versatile and can often compensate for its lower sensitivity when a wavelength with the highest extinction coefficient for the solutes of interest is chosen. The photodiode array will be treated in the hyphenation section, as it can provide complete UV spectra of the constituent during separation. Limits of detection (LODs) can reach 10^{-8} g/mL, with a linear dynamic range of about three orders of magnitude.

Eco-optimized RP-HPLC method for chiral tazobactam and piperacillin drugs:

The PIP/TAZ antimicrobial combination has very strong activity against Gram-negative bacteria, making it a preferred prophylactic treatment for liver transplanted patients . A high incidence of post-transplant infections is present in pediatric liver transplant patients. This necessitates adequate drug exposure. Due to its kidney-primarily excretion path, PIP/TAZ can cause renal workload and acute kidney injury if used excessively. Aside from these factors, numerous other factors can compromise renal function after transplantation, including postoperative hemodynamic instability, nephrotoxic immunosuppressive medications, and drug interactions. The physiological changes associated with pediatric liver transplant recipients may alter the pharmacokinetics of piperacillin/tazobactam, requiring individualized dosing. A number of studies have shown that dose adjustments are often required when pediatric patients undergo complex abdominal surgery or transplants, as well as adult liver transplant recipients suffering from Gram-negative infections, biliary tract infections, enterobacteria, and ventilator-associated pneumonia



(a)



(b)

METHODS:

Size-Exclusion HPLC

Aqueous SEC is generally employed for peptide/protein separations and/or molecular weight determinations. Unique tertiary or quaternary structures can be demonstrated by molecular weight determinations in the presence and absence of denaturants in SEC. Such applications

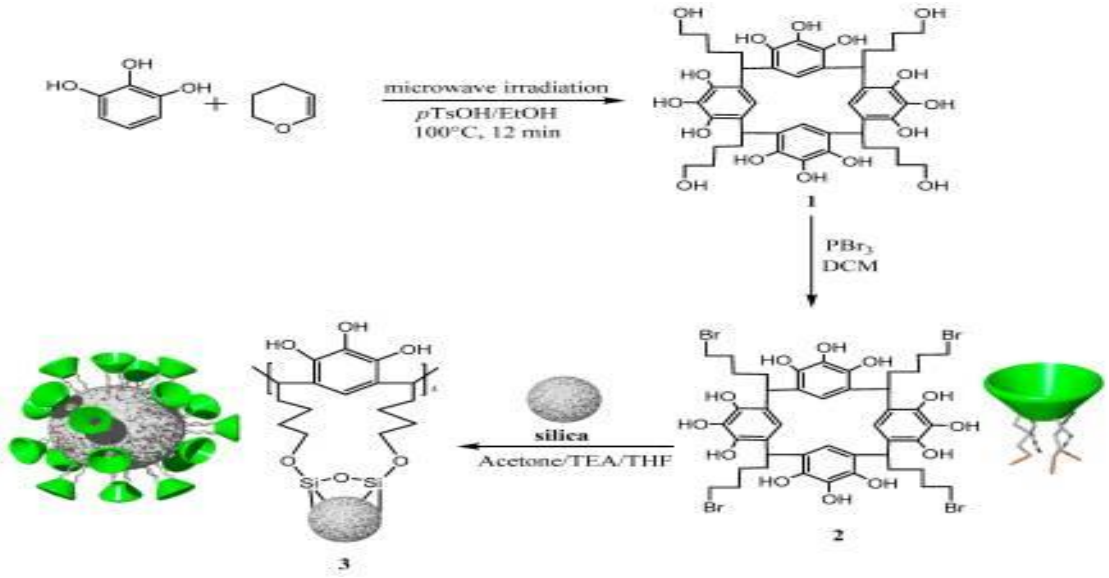
require ideal SEC behavior, i.e., separations should be based solely on solute size. However, most modern high-performance SEC columns are anionic (i.e., carry a negative charge) to a greater or lesser extent . Such a property may lead to interaction with positively charged side-chains in peptides and proteins unless such undesirable electrostatic interactions are suppressed. Because electrostatic effects are minimized above an eluent ionic strength of about 0.05 , aqueous phosphate buffers (pH 5.0–7.5) containing 0.1–0.4 salts are commonly employed as the mobile phase for SEC of peptides and proteins . Of course, peptide–protein or protein–protein interactions may be eliminated if the salt concentration is too high when electrostatic interactions are a dominating factor to the interaction.

RESULTS & DISCUSSION

C-Alkylpyrogallol[4]arenes (PgCn) are a subgroup of calixarene macrocycles, which share two key features. The first is they comprise twelve hydroxy groups at the upper rim of their cup-like structure, which results in many hydrogen-bonding interactions. Second is the alkyl groups that radiate from the spheroidal assembly, which range in length from ethyl to undecyl . This abundance of potential hydrogen bonding sites led to the synthesis of a number of pyrogallol[n]arene derivatives to study not only chromatographic applications of pyrogallol[n]arenes but also application in the field of separation science.

Synthesis of Silica-Bound C-Butylpyrogallol[4]arene Stationary Phase *3a* and *3b*

C-Hydroxybutylpyrogallo[4]arene **1** was prepared from pyrogallol and 2,3-dihydropyran under microwave irradiation and converted to the tetrabromo derivative **2** with phosphorus tribromide in 64% yield (Scheme 1). The ^{1}H NMR for *C*-bromobutylpyrogallol[4]arene **2** revealed the presence of two singlet signals for hydroxy protons in the downfield region, as well as multiplet signals for the bridge proton and the methylene protons adjacent to bromine. The multiplet signals for the rest of the aliphatic side chain protons are in good agreement with the proposed structure The mass spectrometric analysis (ESI-MS) of **2** confirmed the presence of the molecular ion as five m/z peaks, $[M]^{+}$ to $[M+8]^{+}$, in an appropriate ratio for a tetrabromo compound . The silica-bound *C*-butylpyrogallol[4]arene HPLC stationary phase is reported as phase **3a**, while the silica-bound C-butylpyrogallol[4]arene flash stationary phase is reported as phase **3b.**



High-Pressure Liquid Chromatography chromatogram of Ivy leaf extract:

High-Pressure Liquid Chromatography results were expressed in these selective chromatograms. shows the chromatogram for the Ivy extract. The identification was done through the retention time and wavelengths of the UV spectrum for both standards and samples. Eight peaks were detected, where 3 of them were identified: 4-Hydroxyphenyl acetic acid at 13.52 minutes, rutin at 31.51 minutes, and Hesperidin at 42.97 minutes. 4-Hydroxyphenyl acetic acid is a potent antioxidant, while Rutin has antioxidant and anti-inflammatory effects and it might also offer some protection against cancer and other diseases. Rutin (rutoside, quercetin-3-O-rutinoside) is the glycoside combining the flavonol quercetin and the disaccharide rutinose (α-L-rhamnopyranosylcentrifugation at-(1→6)-β-D-glucopyranose). It is a flavonoid glycoside found in a wide variety of plants· Hesperidin is a flavanone glycoside found in citrus fruits and many other plants, and its aglycone is hesperetin. Hesperidin plays a role in plant defense.

MATERIAL & METHODS:

This study was approved by the institutional review boards of the New York University Langone Medical Center (NYU) and Columbia University Medical Center (CUMC). GCB and finger stick blood (FSB) were collected from patients in the general practice clinics at the NYU College of Dentistry. FSB was collected by registered nurses or trained nursing students and GCB was collected by dental providers or trained dental and dental hygiene students. HbA1c was measured at CUMC.

Specimen Collection

GCB Specimens

All dentists and dental hygienists were trained to collect the GCB sample from patients while they were seated in the dental chair. After probing and selecting a site that exhibited erythema and/or edema, the dental provider isolated the area using cotton rolls to prevent saliva contamination, scaled the site, dried the area with gauze to eliminate contaminants from the tooth, and then re-probed the site, thereby leading to a steady flow of debris-free blood. The dental provider collected blood using a micropipette and transferred to 2–3 discs on Whatman 903 filter paper. The blood was allowed to dry at room temperature for an hour before refrigeration in anticipation of transfer to the laboratory for HbA1c analysis.

FSB Specimens

FSB was also collected from patients while they were seated in the dental chair. After the side of the fingertip was cleaned with an alcohol prep pad, the alcohol was allowed to evaporate, and after the skin was dry, the side of the fingertip was punctured with a sterile lancet. The first drop of blood was wiped away with a sterile gauze pad. With the patient's palm face down, the patient's blood was collected using a micropipette and transferred to 2–3 discs on Whatman 903 filter paper. As with the GCB specimen, the FSB was allowed to dry at room temperature for an hour before refrigeration in anticipation of transfer to the laboratory for HbA1c analysis.

Whole Blood Specimens (WBS)

Blood for HbA1c analysis was collected at CUMC by venipuncture in 5ml EDTA vacutainer tubes from Becton-Dickinson. Dried blood spots from these specimens were obtained by spotting enough blood on Whatman 903 filter paper to completely cover the disc.

Instrumentation and Reagents:

Procedure

For GCB, FSB and WB Specimens Spotted on Filter Paper

The accuracy of the HbA1c result is dependent on the collection method of blood specimens which, when conducted properly, avoids introduction of contaminants during specimen collection and enables the collection of a sufficient volume of blood that is spotted on the filter paper. If there was sufficient blood to cover the entire disc, one 3/16 inch punch was used. If a small amount of blood was on the disc, 2–4 punches may have been necessary. The disc(s) from each filter paper spot were placed into a vial containing 1 ml of diluent. After

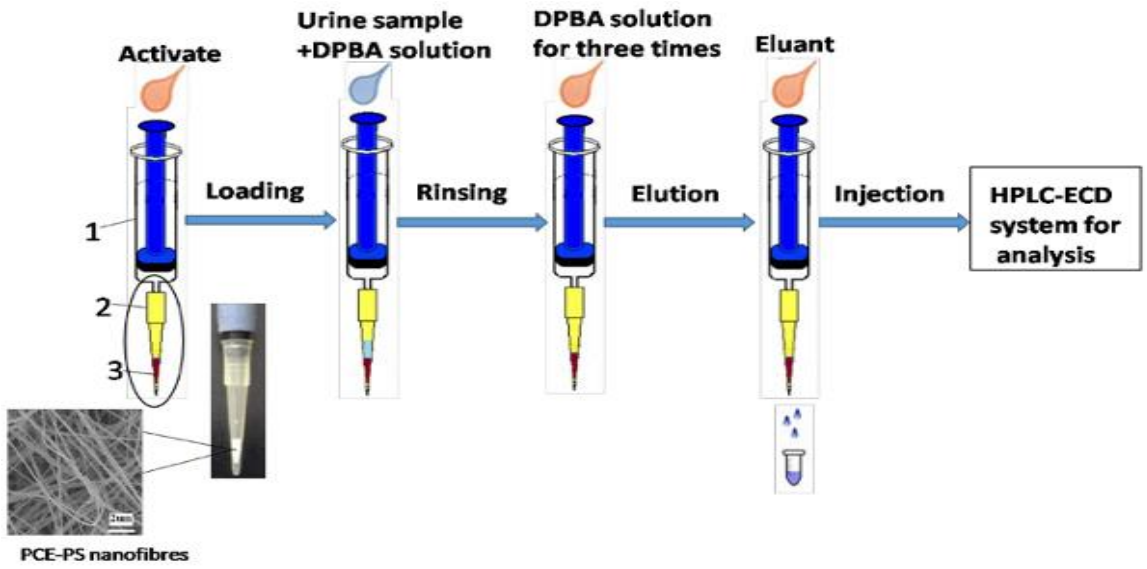
standing at room temperature for 1 hour, the disc(s) was removed from the vial, the vial inverted and placed on the analyzer.

For WB Specimens

The sample was prepared by mixing 5ul of blood with 1ml of diluent. All vials were mixed before placing them on the analyzer.

Extraction and Analysis with High-Pressure Liquid Chromatography of Catecholamine Neurotransmitters:

This protocol is a simple and convenient PFSPE method to pretreat urine samples and enrich five catecholamines for detection via an HPLC-ECD system; a diagram of the process is shown in. The protocol mainly includes four steps-activating, loading, rinsing, and eluting-coupled with a small quantity of PCE-PS nanofibers and a simple solid-phase extraction device. The morphology of PCE-PS nanofibers was assessed using a surface and porosity analyzer. The textural properties-the BET (Brunauer, Emmett, and Teller) surface area, pore volume and pore size-were 2.8297 $m^2 g^{-1}$, 0.009 $cm^3 g^{-1}$, and 12.76 nm, respectively. These data indicate that the material used in the protocol has nanoscale pores on the surface, which may contribute to the high adsorption efficiency and the lowered binding pH in the protocol.



Recent Advancement in Hplc:

New Stationary Phase Materials

The stationary phase is one of the most critical components of an HPLC column, as it directly influences the separation of analytes based on their chemical interactions. In recent

years, there has been significant innovation in the development of new stationary phase materials that offer enhanced selectivity, stability, and durability.

Major breakthrough has been the development of silica-based materials with improved surface properties. These materials have higher surface areas and better uniformity, leading to enhanced separation efficiency and faster analysis times. Moreover, new polymeric stationary phases have been introduced to increase the column's stability in extreme pH conditions, expanding the range of analytes that can be effectively separated.

A key advancement is the introduction of chiral stationary phases, which are crucial for separating enantiomers—molecules that are mirror images of each other but may have different biological activities. This is particularly significant in the pharmaceutical industry, where enantiomeric purity is critical for drug efficacy and safety.

Small Particle Size and High-Efficiency Columns

One of the most significant advancements in HPLC technology has been the reduction in the size of the stationary phase particles. Smaller particles result in a higher surface area for interactions between the analyte and the stationary phase, which leads to faster and more efficient separations. Sub-2-micron particles, which are used in ultra-high-performance liquid chromatography (UHPLC) columns, have become increasingly popular due to their ability to produce superior resolution in a shorter amount of time compared to traditional HPLC columns.

The development of new packing materials for these small particles has further reduced backpressure, making high-efficiency columns more practical and accessible for routine analyses. The application of sub-2-micron particles has enabled rapid separations without sacrificing the quality or resolution of the analysis, particularly in high-throughput environments

Core-Shell Technology

Core-shell technology represents a hybrid approach that combines the advantages of both large and small particles. In core-shell columns, the stationary phase consists of a solid, non-porous core surrounded by a porous shell. This design provides the high efficiency of small particles with lower backpressure, making it a highly desirable option for high-speed, high-resolution separations.

Core-shell columns have been particularly useful in applications requiring fast separations without sacrificing resolution, such as in the pharmaceutical industry and food safety testing.

The combination of high surface area for interactions and low resistance to flow leads to better sensitivity and reproducibility. This technology is also advantageous in applications involving complex matrices, where high-resolution separations are necessary to identify trace components in a mixture.

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