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Determination of eight penicillin antibiotics in pharmaceuticals, milk and porcine tissues by nano-liquid chromatography

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ABSTRACT

This study describes the ability of nanoscale liquid chromatography (nano-LC) coupled with UV or mass spectrometry (MS) for the simultaneous determination of eight common penicillin antibiotics (amoxicillin, ampicillin, penicillin G, penicillin V, oxacillin, cloxacillin, nafcillin and dicloxacillin) in commercial samples (pharmaceuticals, milk, porcine tissues (liver and kidney)) for the first time. Material types of the on-column polymeric frits (polystyrene-based and polymethacrylate-based monoliths) and the packed stationary phase materials (C8 and C18 particles of 3 μm) used in the nano-LC for the influence of penicillin separation were evaluated. The nano-LC and MS parameters such as the composition and flow rate of mobile phase, capillary voltage and temperature of dry gas were examined in order to acquire high separation resolution and detection sensitivity for penicillin analyses. Furthermore, a home-made in-line filter (a nylon membrane of 0.2 μm pore size), was first used to connect with the flow cell of high sensitivity UV detector or the nanoelectrospray needle in MS detection. The result indicated it could effectively improve the reproducibility of penicillin mass signals or prolong the lifetime of the flow cell. The nano-LC methods provided good quantitative precisions in the range of 89.5–111.2% for UV detection at 0.5 μg/mL penicillins, and 83. 1–94.9% for MS detection at 5 μg/L penicillins), respectively, as well as offered stable retention repeatabilities (the relative standard deviation (RSD) of retention time was lower 0.30% in both the UV and MS detections). Compared to other LC–MS methods, the proposed nano-LC systems provided better detection sensitivity for these penicillins (the limits of detection (LOD) was of 2.27–4.06 μg/L for UV mode, and 0.01–0.51 μg/L for MS mode) when either UV or MS detector was employed.

1. Introduction

Penicillin antibiotics have been used extensively in both human and veterinary medicine practices to inhibit the infections of bacteria and fungi. Despite the positive effects of these antibiotics, the occurrence of penicillin residues in food or in discharge to aquatic environment after consumption is a serious health hazard [1–4]. So far, most of the analytical methods reported in the literature for penicillin group residue analysis are based on liquid chromatography (LC)[5–23], in which only a few papers on the usage of LC coupled with UV detection were reported because of poor detection sensitivities and qualitative abilities [22,23]. Although mass spectrometry (MS) or tandem mass spectrometry (MS–MS) has been employed as a major detection mode, however, inadequate detection sensitivity is still a concern if no sample preconcentration method was employed (limits of detection (LODs) were around 43 μg/L for ion trap MS [6]). In order to improve the detection ability for trace-level penicillins, a sample concentration step such as solid-phase or liquid–liquid extraction prior to LC separation is often necessary, and in these cases their LODs were reduced to sub-μg/L range [9–12,17,20].

Recently, a great amount of attention has been paid to the development of capillary and nanoscale LC (capillary-/nano-LC) systems because they can provide higher sensitivity than classic LC [24–26]. It has been reported that sensitivity can be improved hundreds of times by using a 100-μm I.D. capillary column instead of a 4.6-mm I.D. column [27,28]. In addition, the usage of capillary- or nano-LC allows further reduction in solvent consumption and to make possible the analysis of very small amount of solute. Because of the use of a wide LC microspheres with high separation efficiency for stationary phase, the packed column is major use of capillary-/nano-LC and capillary electrochromatography (CEC) so far. In order to retain successfully micrometer-sized particles inside narrow capillary tubes, different retaining frits such as sol–gel or polymeric frits have been proposed. Svec et al. first proposed macroporous polymer frits in CEC by the UV photopolymerization of a solution of glycidyl methacrylate and trimethylolpropane trimethacrylate [29]. Recently, Rocco and Fanali showed the possibility to work in CEC without external pressure but just used a packed capillary with a polymeric frit [30]. These frits taken in capillaries to retain

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particles of chromatographic packing have been demonstrated to be stable and reproducible. Compared to CEC, however, the usage of polymeric frits in capillary-/nano-LC was not reported except by Ding and co-workers [31]. In this case, an on-column frit was prepared by thermo-polymerization of a butylmethacrylate and an ethylenedimethacrylate solution.

To our knowledge, several high sensitivity nano-LC–MS methods have been reported for the analyses of pharmaceutical and biological compounds at trace levels [32–36]. As far as we know, however, this type of penicillin antibiotic separations by capillary- or nano-LC has not been reported to date [5–23].

In this study, analytical methods for the simultaneous separation and identification of penicillin residues in commercial products (pharmaceuticals, bovine milk, porcine tissues (liver and kidney)) were developed based on both high sensitivity nano-LC–UV and nano-LC–MS methods. Eight common penicillin antibiotics (nafcillin, dicloxacillin, ampicillin, oxacillin, penicillin V, cloxacillin, penicillin G, and amoxicillin) were chosen as analytes in this study. A 100 μm I.D. capillary column packed with of 3 μm C8 or C18 silica bonded stationary phase was used as separation column. Two different polystyrene-based and polymethacrylate ester-based porous monoliths were used as on-column frits, and their effects on penicillin separations were compared. This polystyrene-based polymeric monolith was first used as on-column frit of capillary column either in CEC or capillary/nano-LC. Furthermore, several parameters such as compositions of nano-LC mobile phase, MS parameters and arrangement of capillary column coupled to nano-electrospray ionization (nano-ESI) source or UV detector (i.e., the usage of a home-made in-line filter), were examined in order to achieve optimal penicillin analyses.

2. Experimental

2.1. Chemicals and reagents

Dicloxacillin (Dic), ampicillin (Amp, pK_{a2} 7.4), amoxicillin (Amo, pK_{a2} 7.20), cloxacillin (Clo), and penicillin G (PG) were purchased from Sigma (St. Louis, MO, USA). Penicillin V (PV) was bought from ICN (Bryan,OH,USA). Oxacillin (Oxa) was obtained from Fluka (Buchs, Switzerland). Nafcillin (Naf) was purchased from MP (Eschwege, Germany). Since these penicillins have a carboxylic acid moiety bonded to a thiazolidine ring in their structures, thus their pK_a (or pK_{a1}) are around 2.6. The eight penicillin standards used as tested analytes in the study were individually dissolved in deionized water at a stock concentration of 2 mg/mL. C18 stationary phase (Nucleosil, 3 μm I.D., 100 Å pore) was bought from CROM (Herrenberg, Germany). All other chemicals were reagent-grade.

2.2. Sample pretreatment

Several commercial pharmaceutical, full-cream bovine milk and porcine tissue (liver and kidney) samples used as testing samples were obtained from supermarkets in Taiwan. The pharmaceutical samples were mixed with a suitable amount of deionized water and sonicated for 15 min. The resulting clear liquid was filtered through a 0.2 μm membrane filter and it was then directly analyzed by nano-LC without any other treatment. The extraction procedure for porcine tissues and milk were followed the previous methods [8,17], but some part was modified in the study. Milk samples (20 mL) were centrifuged by a high speed centrifuge (RC-5B, Sorvall Instrument, Haverhill, MA, USA) for 30 min at 16,000 rpm (4 °C). The clear centrifuged liquid was mixed with 0.1 M phosphate buffer (pH 8) in the volume ratio of 1:1, and then diluted with the same volume of *n*-hexane followed with mixing with vortex for 10 min. The resulted solution was centrifuged for 20 min at 16,000 rpm (4 °C). The clear aqueous layer was then collected and was ready for solid-phase extraction (SPE) procedure described below.

C18 column (LC-18; Supelco, Bellefonte, PA, USA) used in SPE was conditioned prior to use by washing with methanol (10 mL), deionized water (10 mL) then followed with 0.1 M phosphate buffer (pH 8, 10 mL). After the addition of the centrifuged aqueous phase, the extraction column was washed with deionized water (2 mL) followed with air flush to dry. The absorbed penicillins were then eluted from the column with 5 mL of acetonitrile at a rate of approximately 0.5 mL min^{-1}. The eluted solution, which contained the penicillins, was dried with nitrogen at 40–45 °C.

The porcine liver and kidney samples (2 g) were vortex-mixed with 10 mL of methanol for 10 min. The mixture was centrifuged for 20 min at 6000 rpm. The clear liquid phase was collected and then was dried with nitrogen at 40–45 °C.

The final dry extract (milk or porcine tissue) was dissolved with 20 mL of deionized water followed with a 0.2 μm membrane filtration, and then was ready to be analyzed by nano-LC.

2.3. Preparation of outlet frit and packed column

Two major steps are involved in the preparation of columns. The first entails the in situ formation of porous monolith (the outlet frit) inside a fused-silica capillary by using previously reported methods for the preparation of polymeric monolithic columns [37,38]. In the second step, the fabrication of packed column is proceeded by slurry packing procedure.

2.3.1. Pretreatment of capillary column

Fused-silica capillary was conditioned by washing first with 0.1 M sodium hydroxide (2 min), followed by deionized water (10 min), and finally with methanol (2 min). After the capillary was dried by N_2 gas, it was filled by syringe injection with 3-trimethoxysilyl propyl methacrylate (MSMA) mixed with methanol in a volume ratio of 1:1. Both ends of the capillary were then sealed and submerged overnight in a 35 °C water-bath. Finally, the capillary was washed with methanol (5 min), then with water (5 min), and dried by N_2 gas.

2.3.2. On-column outlet frit

Two different porous monoliths, polystyrene- and polymethacrylate ester-based porous monoliths were used as on-column frits of the packed column, and their preparation was carried out according to the following procedure. Solution composed of monomers, porogenic solvents and initiator (benzoperoxide (BPO), 1.0% (w/w), of monomer) was used to prepare the polymeric frits. The solution for polystyrene-based monolith was made by mixing BPO, monomer solution (styrene (1200 μL) and divinylbenzene (1800 μL)), and porogenic solvent (cyclohexanol (1750 μL) and *N*,*N*-dimethylacetamide (1750 μL)). Alternatively, the solution for polymethacrylate-based monolith was made by mixing BPO, monomer solution (ethylene dimethacrylate (914 μL) and butyl methacrylate (1611 μL)), and porogenic solvent (*n*-propanol (3913 μL) and 1,4-butanediol (1262 μL)). The mixture solutions were sonicated for 15 min until they became homogeneous, then they were used to fill the preconditioned capillary (15 cm) to a total length of 15 mm by syringe injection. When both ends of the capillary were sealed with adhesive resin, the capillary were submerged in a 70 °C water-bath for 17 h (polystyrene-based monolith) or 20 h (polymethacrylate-based monolith). An on-column monolithic frit of 1 mm was then fabricated by using ceramic knife to cut the extra polymeric monolith at the 14 mm position of the column. An LC pump was used to wash the monolithic frit with methanol prior to introduction of C18 stationary phase. The prepared on-column frits, polystyrene- and polymethacrylate-based monoliths, were able to resist a high