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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.

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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

pressure of 5000 psi (340 bar). The pore size and the morphology of the monolithic frits were studied with mercury intrusion porosimetry (AutoPore IV 9500, Micrometritics, Norcross, GA, USA) and scanning electron microscopy S-4100 (Hitachi, Tokyo, Japan).

2.3.3. Preparation of packed column

The pretreated column with outlet frit was then packed with an ethanol slurry (25 mg/mL) of 3 μm C18 silica particles using a LC pump (Model 260D, ISCO, Lincoln, NE, USA) at 5000 psi for 2 h then followed by 1000 psi for 3 h. The packed column, which was filled with silica particle bed, was cut at 10 cm in total length, and was washed with methanol (20 min) and then water (20 min) at a constant pressure of 2000 psi using an LC pump. The packed capillary with no inlet frit was directly connected to autosamplers, and it could provide good performance and repeatability of replicated separations over 109 times. For example, randomly picking up run 1, run 49 and run 109, the RSD of the plate numbers was in the range of 1.78–4.16% for the eight analytes, while the RSD of the retention time was in the range of 0.46–0.87%. Once the capillary needed to be removed from the autosampler, the flow rate of mobile phase was first set as 0 μL/min, and took off the packed column after the column pressure approached to 0 bar.

2.3.4. Preparation of in-line filter

Before the packed column was coupled to UV detector or a nanoelectrospray ionization source, a filter of nylon membrane (0.2 μm pore size, 47 mm diameter, GE Water & Process Technology, West Oakville, ON, USA) was inserted behind the outlet end of the packed column without any adhesion. The column was then connected directly to the flow cell (UV detector) or the nanoelectrospray needle (MS detector), this configuration can effectively improve the penicillin signal stability and prolong life time of the flow cell and nanoelectrospray needle. This nylon membrane filter was called as in-line filter in the study. This term was referred to previous reports on LC, in which it was used to improve peak shape or to prevent column from particulates in LC system. Our home-made in-line filter had different design and much simpler than commercial product [39,40].

2.4. Apparatus and operating conditions for nano-LC

All nano-LC experiments were performed with an UltiMate 3000 nano LC system (Dionex, Amsterdam, The Netherlands) equipped either with a UV detector model VWD-3000 (Dionex) or an ion trap mass spectrometer model Esquire 2000 (Bruker Daltonics, Leipzig, Germany). Both the nano-LC and UV detector was controlled by Chromeleon software suite (Dionex), whereas Data Analysis software was used for the mass instrumental control and MS data analysis. The Dionex UV detector that mounted with a z-shaped flow cell (3 nL in cell volume and 10-mm in path length) was fixed at 200 nm. The ion trap mass spectrometer was coupled to nano-LC system through an on-line nanoelectrospray (Bruker) when MS detection was used. A packed capillary column (10 cm length; 100 μm I.D.; 3 μm C18 silica particle) with an outlet frit of polystyrene-based monolith was taken in separation column. The optimal gradient elution for penicillin separation was performed with an initial of 0% acetonitrile (i.e., 0.1% FA aqueous solution), then was linearly increased to a final of 90% acetonitrile in 20 min. Samples and standards were injected in the range of 0.05–1 μL by autosamplers (Dionex) with 1 μL sample loop. The mobile phase was renewed every 7 days.

The MS and MS–MS detection were performed in the MRM (multiple reaction monitoring) mode. Since all penicillins had relatively strong molecular ion signals which exhibited as $[M+H]^+$ form, thus these molecular ion peaks (i.e., 366 *m*/*z* for amoxicillin, 350 *m*/*z* for ampicillin, 351 *m*/*z* for penicillin V, 335 *m*/*z* for penicillin G, 402 *m*/*z* for oxacillin, 436 *m*/*z* for cloxacillin, 470 *m*/*z* for dicloxacillin, and 415 *m*/*z* for nafcillin) were selected as monitored mass signals in the following nano-LC–MS experiments. Positive ions were generated through the application of 1.8 or 1.5 kV for the spray needle (New objective, Woburn, MA, USA), and end plate off-set was fixed at −0.5 kV. Nitrogen gas was used as dry gas at 280 °C with a flow rate 5 L/min, and no nebulization gas was needed for nano-LC–MS. Scanning mass range was from 100 to 550 *m*/*z* with a maximum accumulation time of 200 ms. Isolation time was determined by the average migration time of each penicillin. Isolation width was kept at 4 Da, and collision activation energy of 1 eV was used for MS–MS detection. The MS–MS spectra of these penicillins showed that each penicillin has a unique product ion spectrum, and the result was consistent to that of previous LC–MS–MS result [16,17].

3. Results and discussion

3.1. Effect of types of on-column polymeric frits

In this study, a porous polymeric monolith was used for on-column outlet frit to retain C18 silica particles of 3 μm I.D. within the capillary. Two monolithic polymers, polystyrene- and polymethacrylate-based monoliths, were used as porous frits, and their influence on penicillin separation was compared. The results indicated that no obvious difference in penicillin separation was observed when either the polystyrene- or polymethacrylate-based monolith was employed as the outlet frit (Fig. 1a). The results of the monolithic pore size measured by mercury intrusion porosimeter indicated that the polystyrene-based monolith had a smaller pore diameter and narrower pore size distribution when compared to that of polymethacrylate type (for example, 67.8 nm vs. 80.5 nm, Fig. 1b). As a result, the polystyrene-based monolithic frit, which may possibly provide a better retaining ability for C18 silica particles, was used in the study. When only a polystyrene-based monolithic frit was present inside the capillary (i.e., no C18 particle was exist) during penicillin separation, the result demonstrated that the porous frit had a relatively weak separating ability for these penicillins (Fig. 1a, the lowest profile). Consequently, the short polymeric monolith inside the capillary was simply used to retain the C18 particles, and the penicillin separation in the packed column was mainly relied upon the reversed-phase partition between the C18 particles and mobile phase.

3.2. Effect of nano-LC chromatographic parameter on penicillin separation

In the proposed nano-LC method, several chromatographic parameters such as stationary phase material, gradient elution program and column temperature had to be examined in order to successfully separate the eight penicillins. Previous LC methods studied on penicillin analyses all used C18 silica particles as stationary phase, but no C8 stationary phase was employed. Thus, the influence of stationary phase type (C8 and C18 silica particles) on penicillin separation was evaluated. Under the same gradient condition, the C18 stationary phase provided a baseline separation for the eight analytes, and a higher separation efficiency was achieved especially for both Amp and Amo pencillins when compared to that of C8 packed column (for example the smallest plate height was 4.3×10^{-5} cm for Amp, and 9.3×10^{-5} cm for Amo). Thus, the packed column prepared by C18 stationary phase was employed in the study.

Next, gradient elution program used in the nano-LC was studied in order to optimize penicillin separation. A binary gradient composed of 100:0 0.1% FA aqueous solution/acetonitrile was used as the initial condition, and the mixture was varied linearly to a mobile phase composed of 10:90 0.1% FA aqueous solution/acetonitrile (i.e.,

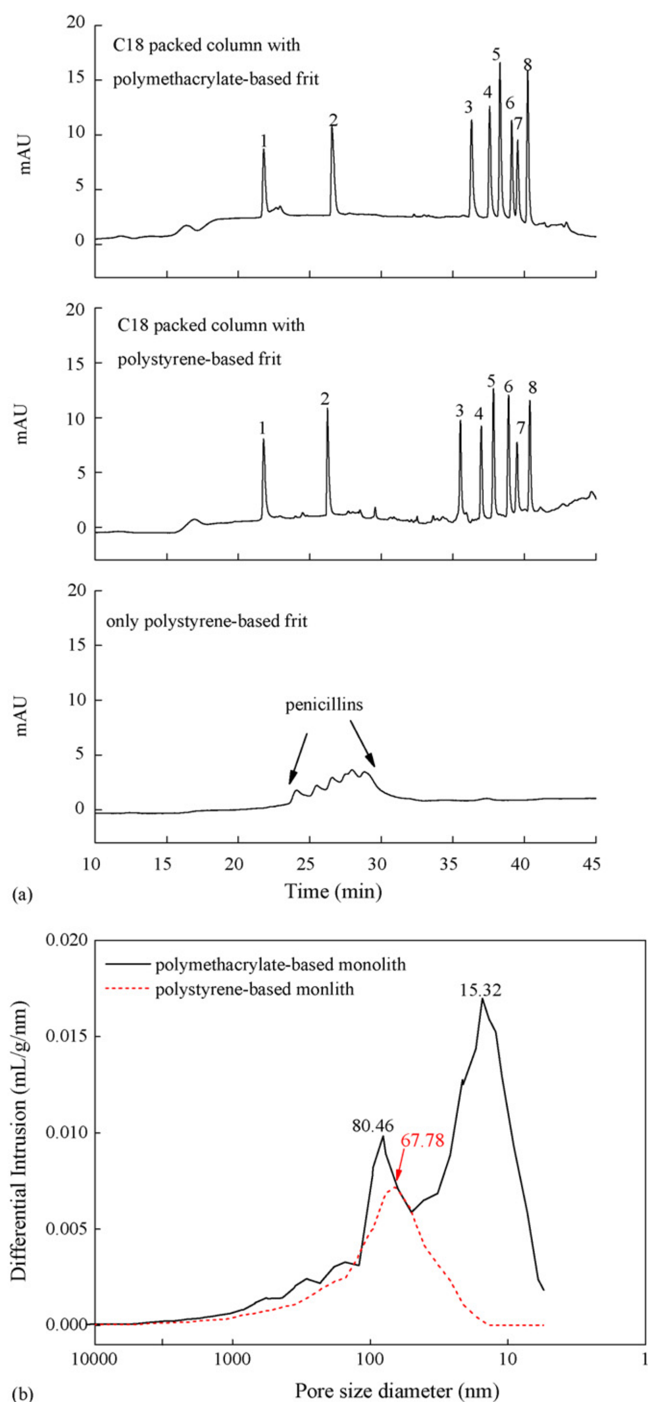

Fig. 1. Effect of different on-column frits on penicillin separation. (a) The chromatograms of nano-LC–UV were obtained by a 10-cm C18 packed column with polystyrene-, polymethacrylate-based monolithic frit, or by only polystyrene-based frit without C18 particles. The distribution of the polymeric frit pore size measured by mercury intrusion porosimeter is shown in (b). An initial 0% acetonitrile (i.e., 0.1% FA aqueous solution) was used, then it was linearly increased to a final of 90% acetonitrile in 20 min with a flow rate of 0.2 μL/min, and this was employed as the gradient elution program in the nano-LC system. The column temperature was kept at 25 °C; 200 nm wavelength was used for penicillin detection; and the amount of sample injection was maintained at 0.05 μL. Amo (1), Amp (2), PG (3), PV (4), Oxa (5), Clo (6), Naf (7) and Dic (8).

final condition) at various ramping time (5–20 min). The measurements were finally performed with ramping time of 20 min, because it enhanced peak intensity and provided better resolutions for the analytes, even though this ramping time of 20 min caused a longer retention time (∼40 min). The flow rate that was used for conducting mobile phase was also varied, and with an increase in the flow rate of mobile phase from 0.15 to 0.35 μL/min, the separation was speed up from 50 to 27 min. Since the nano-LC system employed a 100 μm I.D. capillary as separation column, a larger flow rate was likely to lead a higher column backpressure. As a result, the binary gradient elution was carried out at a flow rate of 0.2 μL/min in the study. Further trial was conducted to determine the effect of column temperature on analyte separation. The column temperature was controlled by oven in the study. Interestingly, the use of 20 °C led to an increase in the separation efficiency and reduced the retention time for Amo (the plot of the theoretical plate number (N) of Amp and Amo vs. column temperature is shown in Fig. 2), whereas the separation of the other seven analytes was not altered by column temperature. Both Amo and Amp, which carried positive charges in the mobile phase composed of 0.1% FA, have the same structure except for an extra hydroxyl group bonded to the benzene ring on Amo (Fig. 2). A higher column temperature likely caused the hydroxyl moiety to dissociate in a larger extent, and thus more hydrophilic and charged Amo zwitterions were formed inside the column. These charged Amo should not follow a reversed partition with the C18 particles, thus a broader peak shape observed in high temperature for Amo was reasonable. Consequently, the nano-LC was performed under 20 °C column temperature.

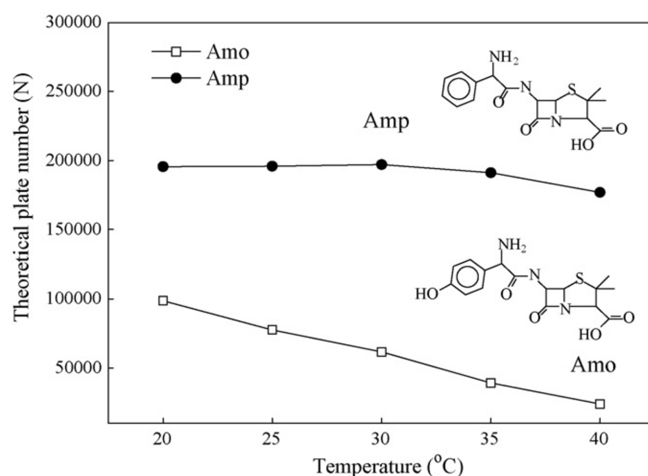

Fig. 2. Effect of column temperature on penicillin separation in the nano-LC–UV system. The column temperature was varied in the range of 20–40 °C. The plot of the plate number (*N*) of Amo and Amp vs. column temperature, and the chromatograms of penicillins are shown in (a) and (b), respectively. A 10-cm C18 packed column with polystyrene-based frit of 1 mm in length was used as the separation column. All other conditions were the same as in Fig. 1.

3.3. Effect of nano-LC mass spectrometer parameter

Next, an attempt was made to develop a nano-LC separation combined mass spectrometric detection for the eight penicillins. The composition of mobile phase used in nano-LC such as acid type (FA or acetic acid) and acid concentration (0.01, 0.1 or 1.0% FA) were varied, and their influence for penicillin's mass signal was compared. Since the optimal mobile phase solution for penicillin separation proposed in the above nano-LC–UV system (i.e., Section 3.2) with an initial of 0% acetonitrile (i.e., 0.1% FA aqueous solution), then was linearly increased to a final of 90% acetonitrile in 20 min, provided the highest mass sensitivities, and thus was also employed as the running solution in the following nano-LC–MS system. Several instrumental parameters of mass spectrometer including capillary voltage, flow rate and temperature of dry gas, were evaluated in order to acquire the highest mass signals for the eight tested compounds. The results indicated that positive ions of the eight penicillins were readily generated through the applica-

tion of 1.8 or 1.5 kV (i.e., capillary voltage) for the spray needle in the nano-ESI source, however, the use of 1.8 kV (vs. 1.5 kV) led to an increase in signal intensity for Amo and Amp by 34.1% and 59.7%, respectively, but the signal intensity decreased by 14.0% to 54.0% for the other six penicillins. These observations were likely due to that both Amo and Amp were eluted out of the LC capillary with a mobile phase composed of higher aqueous solution when compared to that of other analytes, thus a higher capillary voltage was necessary for Amo and Amp. As a result, a capillary voltage of 1.8 or 1.5 kV was used to apply to the spray needle in order to obtain the best mass signals (1.8 kV for Amo and Amp, and 1.5 kV for the other six tested analytes). In addition, nitrogen gas was used as desolvation gas (i.e., dry gas) at a flow rate of 1–7 L/min (1, 3, 5 and 7 L/min). Basically, all of the analytes' signals were enhanced with dry gas flow rate when it was increased from 1 to 5 L/min. Once the flow rate of dry gas was higher than 5 L/min, the analyte signals of most penicillins became small except for Amp, however, the signal to noise ratio (S/N ratio) of Amp was not further enhanced when the dry gas flow rate was more than 5 L/min. Consequently, the flow rate of dry gas was kept at 5 L/min in the study, where all analytes had the highest mass signal to noise ratio. Subsequently, the temperature of dry gas in the nano-ESI also needed optimization in order to achieve stable mass signals. The results showed that the mass signals of penicillins varied with the temperature of dry gas, and higher mass signal was also obtained when the temperature of dry gas was kept either at 180 or 280 °C. Since 280 °C provided a higher signal stability, thus it was chosen as the optimal dry gas temperature.

3.4. Effect of nylon membrane filter on the stability of penicillin signals

So far, the operation conditions for nano-ESI coupled to MS have been optimized by the previous examination in the study. The repeatability of the proposed nano-LC–MS method had to be evaluated prior to applying it in routine analysis. The results, however, indicated that the mass signal intensities significantly decayed with the operation time of this nano-LC–MS (Fig. 3a). In order to make sure the decrease of mass sensitivity was not owing to the use of a home-made packed capillary as the separation column in the nano-LC system, thus a commercial C18 capillary column (LC Packings, Dionex) was used instead of the home-made capillary. Unfortunately, similar signal decay was still observed in the commercial C18 column. This phenomenon was possibly due to the spray needle used in the nano-ESI becoming blunt after repeated applied voltage or was clogged by some particulates produced in the nano-LC system. Similar to an in-line filter used in traditional LC system, in which some particulates can be avoided from clogging the separation column, in the case a nylon membrane filter (0.2 μm pore size), it was considered to play the function of in-line filter in the nano-LC–MS. This nylon membrane filter was placed between the C18 separation column and the spray needle of nano-ESI without adhesive (its configuration was shown in Fig. 4), and the reproducibility of mass sensitivity of all penicillins was markedly improved by the usage of the in-line filter (Fig. 3b). Note that the standard concentration for Fig. 3a was 1 μg/mL, while it was 20 or 200 times lower for Fig. 3b (i.e., 50 μg/L (5 μg/L for Amp and Naf)). Furthermore, the retention time of the tested analytes was not altered by the insertion of this nylon filter. On the other hand, the in-line filter behind the chromatographic column was also used to couple with the flow cell of high sensitivity UV detector, in which there were very low inner volume (3 nL) and narrow connection tube (<20 μm I.D.) in the flow cell. The images of scanning electron microscopy (SEM) or optical microscopy (Fig. 4b) also indicated that the nylon filter had the smallest interval between the linked nodules when compared to that of the packed material and on-column polymeric frit. Thus, this in-line filter should effectively avoid some particulates from

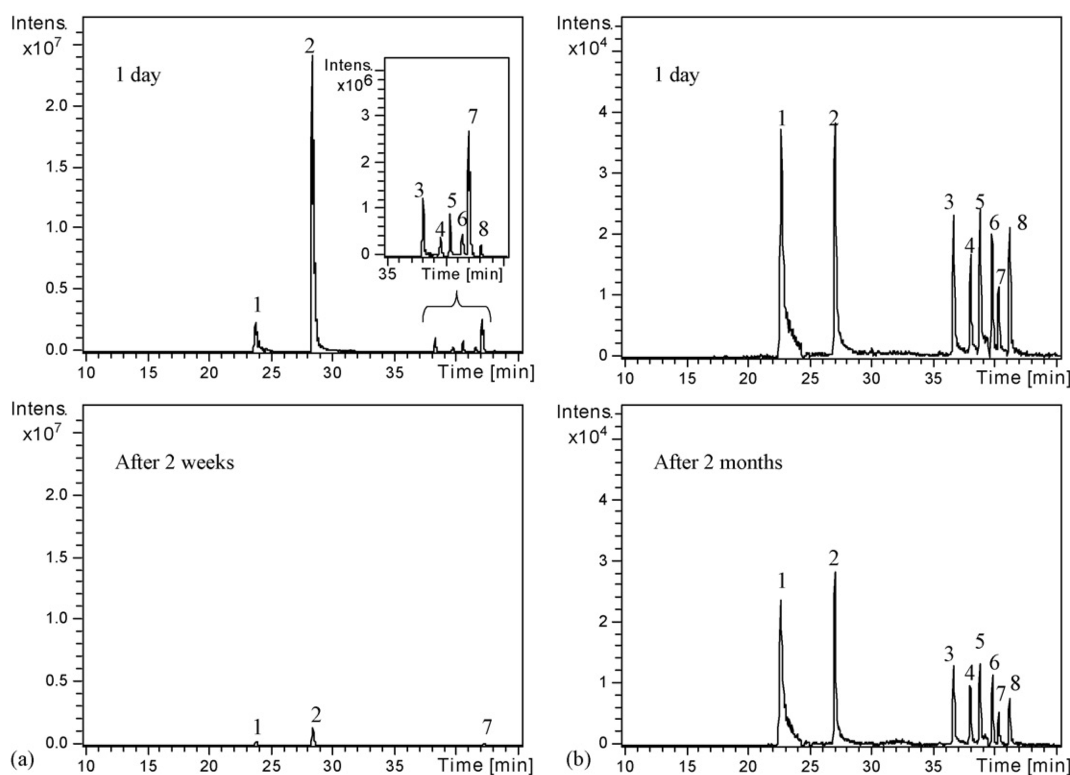

Fig. 3. Effect of in-line filter on mass signal stability of penicillins under nano-LC–MS. Chromatograms were obtained (a) without, and (b) with nylon membrane filter used as in-line filter. Penicillins were determined by ion trap mass spectrometer under the scanning mode of precursor ion. The concentration of penicillin standard was maintained at (a) 1 μg/mL, and (b) 50 μg/L (5 μg/L for Amp and Naf) for each analyte. The amount of sample injection was 0.5 μL; the flow rate and temperature of dry gas were kept at 3 L/min and 180 °C, respectively. All other conditions were the same as in Fig. 2.

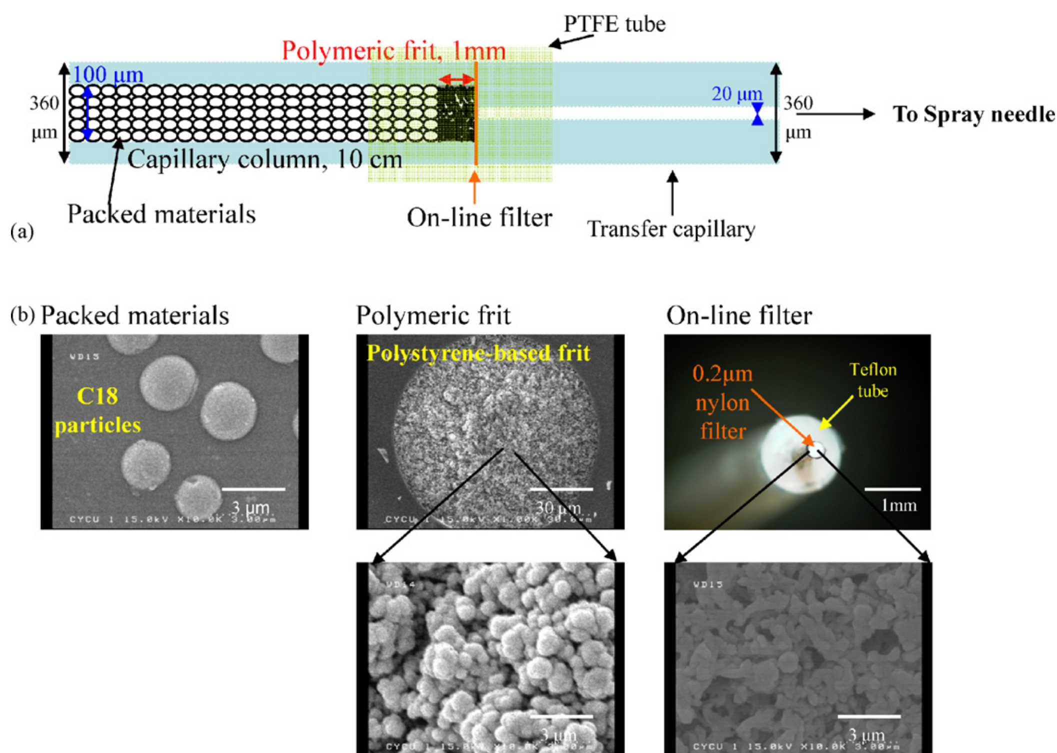

Fig. 4. Schematic diagram of an in-line filter connected with the C18 capillary column and spray needle. (a) A 10-cm C18 packed capillary was coupled with a nano-ESI spray needle through a nylon filter. SEM or optical microscope images of C18 particles, on-column frit and nylon filter are shown in (b).

entering the spray needle or the flow cell, and prevent them from being clogged. This study demonstrated that the usage of in-line filter could further prolong the lifetime of the expensive spray needle and flow cell.

3.5. Comparison of nano-LC–UV and nano-LC–MS for penicillin analyses

The qualitative and quantitative performance of the proposed nano-LC–UV and nano-LC–MS methods under optimal conditions are shown in Tables 1 and 2, respectively. The LODs of penicillin antibiotics were in the range of 2.27–4.06 µg/L for UV mode, and 0.01–0.51 µg/L for MS mode (S/N = 3). Compared to the detection sensitivity of penicillins obtained with the UV detection, the nano-LC–MS method provided about 4- to 340-fold reduction in detection limit and without loss of separation resolution. Previous LC–MS studies on the analyses of these penicillin compounds reported their detection limits to be around 43 µg/L when no sample preconcentration method was employed [6]. In the best case, the detection limits of penicillins were reduced to 0.1–2.1 µg/L range if SPE sample concentration step or high sensitive mass spectrometry such as triple quadrupole (QqQ) and quadrupole time of flight (Q-TOF) was used in LC–MS system [9–12,16,17,20]. Consequently, the nano-LC methods from this study did provide better detection ability for the analysis of penicillin compounds when either UV or MS spectrometer was employed as the detection mode. In addition, the RSDs of peak area on LC–MS method were in the range of 1.87–6.88%, and 3.54–7.92% at the penicillin standards of 5 and 25 µg/L, respectively ($n = 5$). On LC–UV method, they were of 0.17–1.03%, and 0.11–1.10% at 1.0 and 2.5 µg/mL penicillins, respectively ($n = 5$).

The quantitative precisions of the nano-LC methods are also shown in Table 3, that were comparable with previous LC–MS works (in the range of 74–115%) [6,12,16–18,21]. The results indicated that both nano-LC–UV and nano-LC–MS methods could be used to determine penicillin antibiotics in samples with satisfactory recoveries. Consequently, the above results indicated that the proposed nano-LC–UV and nano-LC–MS methods should be acceptable for the detection of trace-level penicillins. Compared to conventional LC–UV or LC–MS method, the proposed nano-LC methods had a higher separation efficiency ($N > 260{,}000$ plates for penicillin compounds except for Amo (8000 plates) and Amp (33,000 plates), and better selectivity (baseline separation for all of the eight penicillins), even a longer separation time (42 min in the study vs. 35 min in the previous report [18]). In this study, the nano-LC separation conditions were optimized to obtain the best resolution and baseline separation for the eight penicillins. With a mass selective second dimension (mass extraction), however, the high resolution separation would not be necessary if components have different masses.

3.6. Real sample separation by optimal nano-LC methods

The chromatograms of penicillin standards and commercially available samples (pharmaceuticals, milk and porcine tissues (liver and kidney) derived by the optimal nano-LC methods are shown in Fig. 5 (chromatograms shown on the top were measured by UV detection, and others was obtained by MS detection). The penicillin compounds in all real samples were identified carefully by migration times and/or comparisons of parent ion spectra (MS spectra) and product ion scan spectra (MS–MS spectra). The result indicated that Amp or Dic was determined by this nano-LC method without any interference as the penicillin compounds present in the pharmaceutical and porcine tissue samples (Fig. 5a–c medium and right profiles), but no penicillin residues was founded in the two tested bovine milk samples (Fig. 5d, left). In addition, in order to examine the separation and detection ability for all penicillin compounds, milk sample (free-penicillin antibiotics) spiked with eight penicillins of 5 µg/L was also separated by the nano-LC method. Consequently, these trace-level penicillin compounds in the milk sample were successfully determined by the nano-LC method either in MS (Fig. 5d) after a simple SPE. In this case, the

Table 1
Qualitative and quantitative performance of nano-LC–UV for penicillin compounds.

Penicillin compounds	Nano-LC–UV								
	LOD[a] (μg/L or pg) (S/N = 3)	LOQ[a] (μg/L or pg) (S/N = 10)	Retention time[a] (min) (RSD%)	Repeatability of peak area[b] (RSD%)		Calibration curves[c]	Coefficient of determination for calibration curves (r^2)	Standard deviation of slope (S_m)	Standard deviation of intercept (S_b)
				Concentration level 1	Concentration level 2				
(1) Amoxicillin	4.06	13.54	22.82 (0.27)	0.44	0.35	$y = 8.8398x + 1.3519$	0.998	0.26	0.69
(2) Ampicillin	3.45	11.50	26.78 (0.05)	0.37	0.56	$y = 6.1608x + 0.5821$	0.999	0.13	0.33
(3) Penicillin G	2.54	8.47	36.46 (0.07)	0.64	1.10	$y = 5.4067x + 0.942$	0.997	0.18	0.46
(4) Penicillin V	2.45	8.17	37.83 (0.07)	0.63	0.59	$y = 5.9082x + 1.9451$	0.998	0.17	0.44
(5) Oxacillin	2.40	8.01	38.55 (0.06)	0.17	0.14	$y = 7.0923x + 1.6906$	0.995	0.28	0.71
(6) Cloxacillin	2.35	7.83	39.47 (0.06)	0.37	0.12	$y = 6.3412x + 1.3143$	0.996	0.22	0.56
(7) Nafcillin	2.32	7.73	39.99 (0.06)	1.03	0.17	$y = 4.2043x + 1.1629$	0.999	0.07	0.17
(8) Dicloxacillin	2.27	7.57	40.81 (0.08)	0.46	0.11	$y = 7.0916x + 1.5069$	0.997	0.22	0.55

[a] Values were determined by 2.5 μg/mL of each penicillin standard in a 1 μL loop with full loop injection for nano-LC–UV. The values in parentheses are the relative standard deviations of retention times. $N = 5$.
[b] The values were the relative standard deviations of peak area obtained at 1 μg/mL (concentration level 1) and 2.5 μg/mL (concentration level 2) of each penicillin standard ($n = 5$), respectively.
[c] The calibration curves were constructed from three replicate measurements at each concentration in the range of 0.05–5 μg/mL(0.05, 0.5, 1, 2.5 and 5 μg/mL) for nano-LC–UV.

Table 2
Qualitative and quantitative performance of nano-LC–MS for penicillin compounds.

Penicillin compounds	Nano-LC–MS								
	LOD[a] (μg/L or pg) (S/N = 3)	LOQ[a] (μg/L or pg) (S/N = 10)	Retention time[a] (min) (RSD%)	Peak area repeatability[b] (RSD%)		Calibration curves[c]	Coefficient of determination for calibration curves (r^2)	Standard deviation of slope (S_m)	Standard deviation of intercept (S_b)
				Concentration level 1	Concentration level 2				
(1) Amoxicillin	0.29	0.97	22.9 (0.24)	3.68	4.75	$y = 62.586x + 0.0883$	0.994	2.75	0.13
(2) Ampicillin	0.01	0.04	27.1 (0.17)	6.32	3.54	$y = 386.79x + 0.0258$	0.997	11.46	0.05
(3) Penicillin G	0.16	0.54	36.6 (0.12)	1.87	4.30	$y = 26.94x + 0.0271$	0.998	0.63	0.03
(4) Penicillin V	0.51	1.71	38.0 (0.12)	5.32	3.63	$y = 9.5619x + 0.0132$	0.997	0.33	0.02
(5) Oxacillin	0.15	0.48	38.8 (0.14)	6.88	7.92	$y = 39.551x - 0.0047$	0.999	0.79	0.04
(6) Cloxacillin	0.26	0.87	39.7 (0.11)	2.93	7.72	$y = 21.885x + 0.0172$	0.997	0.69	0.03
(7) Nafcillin	0.07	0.23	40.2 (0.00)	4.25	6.34	$y = 82.701x + 0.0044$	0.996	2.99	0.01
(8) Dicloxacillin	0.44	1.46	41.1 (0.11)	5.41	6.92	$y = 9.9067x + 0.0208$	0.991	0.54	0.02

[a] Values were determined by 2.5 μg/L of ampicillin and nafcillin standards, and 25 μg/L of other penicillin standards in a 1 μL loop with full loop injection for Nano-LC–MS. The values in parentheses are the relative standard deviations of retention times. $n = 5$.
[b] The values were the relative standard deviations of peak area obtained at 5 μg/L of each penicillin standard (0.5 μg/L for ampicillin and nafcillin) (concentration level 1), and 25 μg/L of each penicillin standard (2.5 μg/L for ampicillin and nafcillin) (concentration level 2) ($n = 5$), respectively.
[c] The calibration curves were constructed from five replicate measurements at each concentration in the range of 0.1–10 μg/L (0.1, 0.25, 0.5, 2.5 and 10 μg/ml) for ampicillin and nafcillin standards, and 1 to 100 μg/L (1, 2.5, 5, 25, and 100 μg/L) for other standards in the nano-LC–MS.

Table 3
Qualitative precisions of nano-LC methods[a].

Penicillin compounds	Nano-LC–UV[b]		Nano-LC–MS[c]	
	Recovery 1 (spiked 1 μg/mL) (%)	Recovery 2 (spiked 0.5 μg/mL) (%)	Recovery 1 (spiked 25 μg/L) (%)	Recovery 2 (spiked 5 μg/L) (%)
(1) Amoxicillin	110.8 (0.75)	89.5 (1.60)	114.5 (8.26)	87.7 (2.52)
(2) Ampicillin	103.58 (0.81)	100.4 (1.27)	109. 5 (9.00)	85.97 (6.79)
(3) Penicillin G	115.3 (0.55)	96.6 (0.38)	99.9 (9.70)	86.0 (8.03)
(4) Penicillin V	111.9 (0.08)	111.2 (0.59)	108.2 (9.71)	90.4 (9.34)
(5) Oxacillin	115.4 (1.07)	105.0 (0.90)	103.3 (1.72)	89.7 (8.55)
(6) Cloxacillin	115.7 (0.34)	108.87 (1.01)	117.2 (7.88)	94.9 (7.22)
(7) Nafcillin	109.5 (0.60)	96.7 (1.25)	115.0 (3.77)	83.1 (6.78)
(8) Dicloxacillin	113.6 (0.62)	108.4 (1.10)	113.8 (5.77)	89.4 (7.11)

[a] $n = 3$. The values in parentheses are the relative standard deviations of recovery. Recovery = (penicillin concentration obtained by nano-LC/spiked concentration of penicillin standard) × 100%.

[b] The penicillin standard level of 1 or 0.5 μg/mL was repeatedly quantified on 3 different days by the nano-LC–UV method through the same calibration curves.

[c] The penicillin standard level of 25 μg/L (2.5 μg/L for ampicillin and nafcillin)(recovery 1) or 5 μg/L (0.5 μg/L for ampicillin and nafcillin) (recovery 2) was repeatedly quantified by the nano-LC–MS method through the same calibration curves.

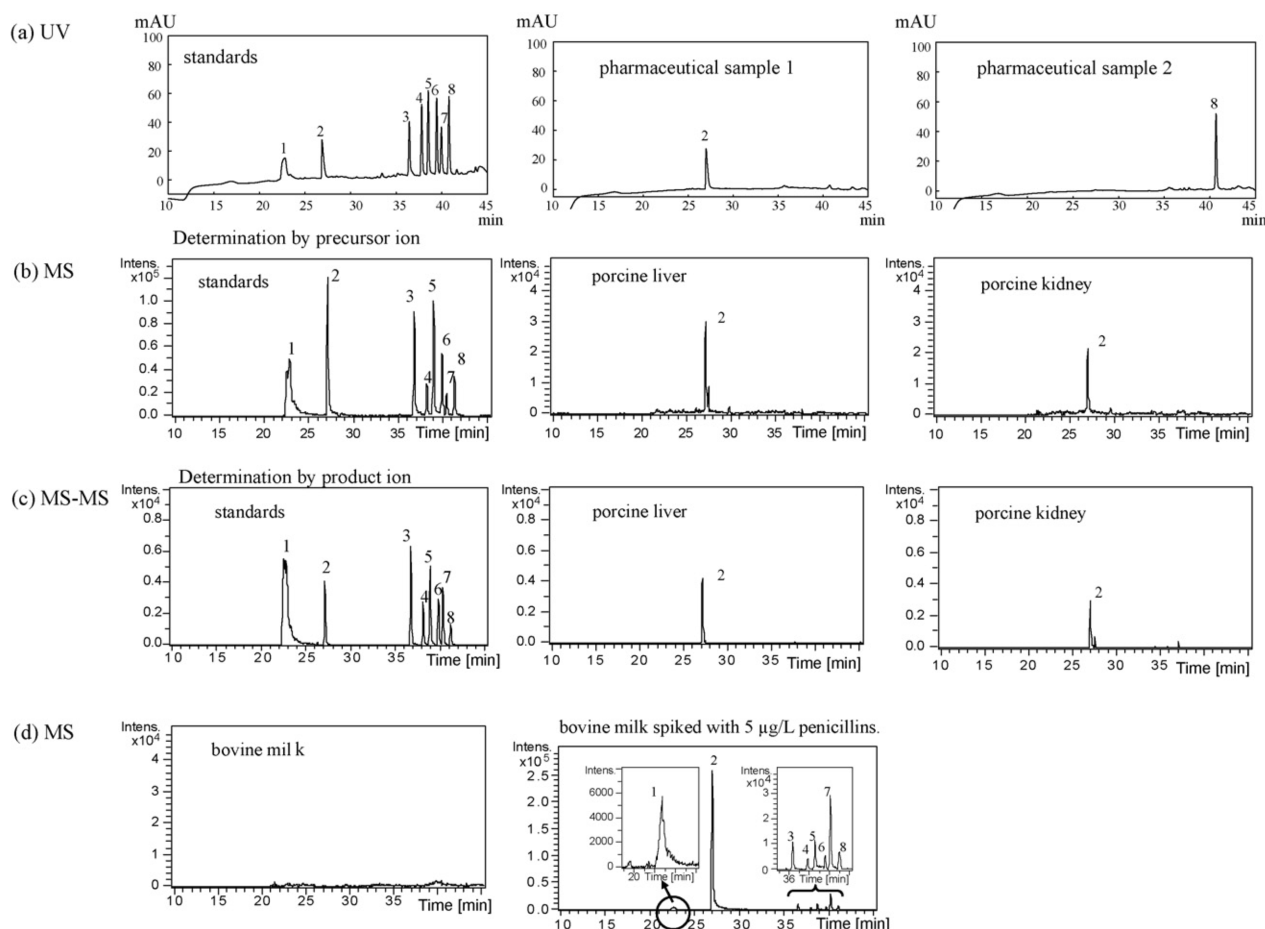

Fig. 5. The chromatograms of penicillin standards or commercial products determined by nano-LC methods. Penicillin signals were acquired by (a) UV (200 nm), and (b–d) MS detection. The concentration of penicillin standard was maintained at (a, left) 1 μg/mL, and (b–c, left) 25 μg/L (2.5 μg/L for Amp and Naf) for each analyte. Mass signal was determined by the precursor ions (b and d), and product ions (c). Samples were prepared by the procedure described in the Section 2. The amount of sample injection was 1 μL; the flow rate and temperature of dry gas were kept at 5 L/min and 280 °C, respectively. All other conditions were the same as in Fig. 1.

SPE step was taken in sample cleanup and matrix reduction, but the final dry extract was still dissolved with 20 mL of deionized water that had the same volume with the original sample. Thus the penicillin concentration in sample injection solution was not enhanced by the SPE step. By this way, that could clarify the high detecting sensitivity of the nano-LC method for the analysis of trace-amount penicillin antibiotics in the real samples.

4. Conclusion

In this paper, a highly sensitive nano-LC method was developed for analyzing trace-level penicillin-related antibiotics present in several commercial products. The composition and flow rate of mobile phase, and column temperature were found to have apparent influences on penicillin separation in this nano-LC system. Mass

signal intensities of all penicillins were determined by the capillary voltage applied on the spray needle and the temperature of dry gas in the nano-ESI. Furthermore, an in-line filter composed of a nylon membrane with 0.2 μm pore size, which connected with the separation capillary column and the spray needle, was found to enable effective improvement of the mass signal stability in the nano-LC system. Finally, the proposed nano-LC method provided better detecting sensitivity for the eight penicillins either in UV or mass spectrometer detecting mode when compared to that of other LC–MS methods. In the present nano-LC–MS method, an ion trap mass spectrometry was used as detector. If a triple quadruple mass spectrometry is used, it should possible provide better signal reproducibility and lower LOD for penicillin analyses in the future.

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References

[1] C.K. Fagerquist, A.R. Lightfield, Rapid Commun. Mass Spectrom. 17 (2003) 660.
[2] M. Becker, E. Zittlau, M. Petz, Anal. Chim. Acta 520 (2004) 192.
[3] S. Riediker, A. Rytz, R.H. Stadler, J. Chromatogr. A 1054 (2004) 359.
[4] C.L. Flurer, Electrophoresis 24 (2003) 4116.
[5] R.M. Seifar, Z. Zhao, J. van Dam, W. van Winden, W. van Gulik, J.J. Heijnen, J. Chromatogr. A 1187 (2008) 103.
[6] Y.-A. Hammel, R. Mohamed, E. Gremaud, M.-H. LeBreton, P.A. Guy, J. Chromatogr. A 1177 (2008) 58.
[7] D. Lia, M. Yang, J. Hu, Y. Zhang, H. Chang, F. Jin, Water Res. 42 (2008) 307.
[8] K. Granelli, C. Branzell, Anal. Chim. Acta 586 (2007) 289.
[9] M.S. Díaz-Cruz, M.J. López de Alda, D. Barceló, J. Chromatogr. A 1130 (2006) 72.
[10] D.N. Heller, C.B. Nochetto, N.G. Rummel, M.H. Thomas, J. Agric. Food Chem. 54 (2006) 5267.
[11] T.A.M. Msagati, M.M. Nindi, Food Chem. 100 (2007) 836.
[12] O.J. Pozo, C. Guerrero, J.V. Sancho, M. Ibáñez, E. Pitarch, E. Hogendoorn, F. Hernández, J. Chromatogr. A 1103 (2006) 83.
[13] D.N. Heller, M.L. Smith, O.A. Chiesa, J. Chromatogr. B 830 (2006) 91.
[14] Y. Ito, T. Goto, H. Oka, H. Matsumoto, K. Takeba, J. Chromatogr. A 1042 (2004) 107.
[15] S. Bogialli, V. Capitolino, R. Curini, A.D. Corcia, M. Nazzari, M. Sergi, J. Agric. Food Chem. 52 (2004) 3286.
[16] D.M. Holstege, B. Puschner, G. Whitehead, F.D. Galey, J. Agric. Food Chem. 50 (2002) 406.
[17] S. Riediker, R.H. Stadler, Anal. Chem. 73 (2001) 1614.
[18] F. Bruno, R. Curini, A.D. Corcia, M. Nazzari, R. Samperi, J. Agric. Food Chem. 49 (2001) 3463.
[19] Y. Ito, Y. Ikai, H. Oka, H. Matsumoto, Y. Miyazaki, K. Takeba, H. Nagase, J. Chromatogr. A 911 (2001) 217.
[20] W. Ahrer, E. Scherwenk, W. Buchberger, J. Chromatogr. A 910 (2001) 69.
[21] E. Daeseleire, H.D. Ruyck, R.V. Renterghem, Rapid Commun. Mass Spectrom. 14 (2000) 1404.
[22] K. Takeba, K. Fujinuma, T. Miyazaki, H. Nakazawa, J. Chromatogr. A 812 (1998) 205.
[23] J.A. Tarbin, W.H.H. Farrington, G. Shearer, Anal. Chim. Acta 318 (1995) 95.
[24] M. Wilm, M. Mann, Anal. Chem. 68 (1996) 1.
[25] S.E. Martin, J. Shabanowitz, D.F. Hunt, J.A. Marto, Anal. Chem. 72 (2000) 4266.
[26] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, J. Sep. Sci. 30 (2007) 1589.
[27] J.P. Chervet, M. Ursen, J.P. Salzmann, Anal. Chem. 68 (1996) 1507.
[28] S. Hsieh, J.W. Jorgenson, Anal. Chem. 68 (1996) 1212.
[29] J.-R. Chen, R.N. Zare, E.C. Peters, F. Svec, J.J. Frechét, Anal. Chem. 73 (1987).
[30] A. Rocco, S. Fanali, J. Chromatogr. A 1191 (2008) 263.
[31] J. Ma, M. Ding, Y. Xu, L. Chen, Anal. Sci. 23 (2007) 371.
[32] Y. Song, Y.-M. Liu, J. Mass Spectrom. 43 (2008) 1285.
[33] G. D'Orazio, A. Cifuentes, S. Fanali, Food Chem. 108 (2008) 1114.
[34] T. Natsume, Y. Yamauchi, H. Nakayama, T. Shinkawa, M. Yanagida, N. Takahashi, T. Isobe, Anal. Chem. 74 (2002) 4725.
[35] I.R. Cañás, B. Hamilton, M.F. Amandi, A. Furey, K.J. James, J. Chromatogr. A 1056 (2004) 253.
[36] N. Rosales-Conrado, M.E. León-González, G. D'Orazio, S. Fanali, J. Sep. Sci. 27 (2004) 1303.
[37] H.-Y. Huang, C.-W. Chiu, I.-Y. Huang, J.-M. Yeh, Electrophoresis 25 (2004) 3237.
[38] H.-Y. Huang, Y.-C. Liu, Y.-J. Cheng, J. Chromatogr. A 1190 (2008) 263.
[39] R.D. Morrison, J.W. Dolan, LC GC N. Am. 26 (2008) 610.
[40] SGE Analytical Science-ProteCol™ In Line Filters, http://www.sge.com/products/protecol-in-line-filters.