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Complete List of Authors:	Yoshii, Tomoka; Keio University Faculty of Science and Technology Graduate School of Science and Technology, Department of Applied Chemistry Nakano, Kohei; Keio University Faculty of Science and Technology Graduate School of Science and Technology, Department of Applied Chemstry Okuda, Tomoaki; Keio University, Applied Chemistry Citterio, Daniel; Keio University, Department of Applied Chemistry Hiruta, Yuki; Keio University Faculty of Science and Technology Graduate School of Science and Technology, Department of Applied Chemistry

ARTICLE

Evaluation of separation performance for eggshell-based reversed-phase HPLC columns by controlling particle size and application in quantitative therapeutic drug monitoring

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Tomoka Yoshii,[a] Kohei Nakano,[a] Tomoaki Okuda, [a] Daniel Citterio [a] and Yuki Hiruta*[a]

Eggshell-based reversed-phase packing materials were applied to an analytical column for high-performance liquid chromatography. Commercially available eggshell powder was classified by a cyclone system to obtain three types of particles with different diameters (arithmetic mean ± standard deviation: 4.3 ± 3.8, 5.6 ± 3.3, and 9.5 ± 5.5 μm). Sedimentation separation removed tiny particles from each sample, resulting in particles with arithmetic means of 6.6 ± 5.5, 7.3 ± 4.5, and 10.2 ± 5.0 μm, respectively. The unclassified particles and three particle types treated with sedimentation separation were subsequently packed into analytical columns (4.6 × 150 mm I.D.), and their separation efficiencies were evaluated by comparing their height equivalent to a theoretical plate (HETP). The column without sedimentation separation exhibited the highest HETP, whereas the columns with sedimentation separation showed better separation efficiency and lower back pressure. The column with the best separation efficiency was applied for the separation of 10 alkylbenzenes and 5 steroids, and all peaks were observed with complete separation (peak resolution: R_S >1.5). Finally, the column was used for quantitative analysis of voriconazole, an azole antifungal agent, and imatinib, a first-generation molecularly targeted drug for cancer treatment, in spiked whole blood. Excellent accuracy (99.1–102.8%) and precision (0.6–1.9%) were observed for the spiked drugs and long-term stability (>3000 column volumes of mobile phase flow) indicated good applicability of the developed eggshell-based column as an analytical column for routine analyses of therapeutic drugs in blood.

Introduction

Octadecyl-modified silica (ODS), a mesoporous silica bead modified with hydrophobic octadecyl groups, is widely used as a stationary phase for reversed-phase high-performance liquid chromatography (HPLC). The silica gel used as a column packing material has a porous structure[1] and its large surface area makes it suitable as a stationary phase in reversed-phase chromatography, wherein analytes are separated by distribution equilibria. In addition, these materials can be easily modified with organic substituents via chemical bonding using silane coupling reagents.[2,3] Although silica gel-based column packing materials are common stationary phases in reversed-phase chromatography, column chromatography originated with liquid adsorption column using chalk (calcite crystals of calcium carbonate).[4] It has also been reported that seashells, which are mainly composed of calcium carbonate, can be applied as a stationary phase in normal-phase flash chromatography.[5] These reports of calcium carbonate-focused chromatography indicate that it has great potential as a stationary phase for column chromatography.

We previously developed a reversed-phase HPLC column based on synthesized calcium carbonate microspheres modified with the amphiphilic copolymer, poly(maleic acid-*alt*-1-octadecene) (PMAcO).[6,7] By adjusting the microsphere synthesis conditions, including the solution pH and stirring speed, we fabricated columns packed with different-sized microspheres. The column packed with the smallest particles showed a high separation efficiency, comparable to that of the commercially available ODS columns. We also developed preparative columns based on eggshells modified with PMAcO, referred to as Eggshell-PMAcO.[8,9] Eggshells are biominerals composed mainly of calcium carbonate.[10] Eggshell surface treatment with a chelating agent and weak acid is effective for improving the separation efficiency of eggshell-based columns. Eggshell-PMAcO columns were used for the separation of neutral and basic drugs, and the purification of pharmaceutical intermediates was also achieved. However, their separation efficiency is insufficient for application in analytical columns. If the particle size of eggshells can be classified, the separation efficiency of the Eggshell-PMAcO column can be improved. It is expected that an improved analytical column could be applied to the quantitative analysis of drugs.

To the best of our knowledge, no studies regarding the application of biominerals, such as eggshells, as packing materials in analytical columns for HPLC have been reported. Therefore, this study focuses on the classification of eggshell powder for application in Eggshell-PMAcO columns for

[a]*Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan. Email: hiruta@applc.keio.ac.jp*

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analytical applications, such as therapeutic drug monitoring (TDM). TDM is critical for assessing drug concentrations in patient blood to determine appropriate dosages.[11] Drug concentrations in blood must be maintained at effective levels because low drug concentrations are ineffective, whereas high drug concentrations are toxic. Routine TDM allows for a rational dosage for individual patients, maximizing the therapeutic efficacy while minimizing drug toxicity.[12] Various TDM methods have been developed, including immunological and chromatographic methods.[13–15] HPLC is more versatile than other methods because it does not require a specific analyte ligand, such as an antibody. Herein, eggshells were collected and sorted by size using a cyclone system to investigate the effect of the particle size on the column separation efficiency. The cyclone process requires only simple equipment (cyclone) that can classify powders using airflow and centrifugal forces. To improve the separation efficiency and reduce column back pressure, tiny particles in the eggshell powder were removed via the simple and eco-friendly sedimentation separation method. Columns packed with Eggshell-PMAcO of different particle sizes were compared in terms of height equivalent to a theoretical plate (HETP) and back pressure at various mobile phase flow rates. The column with the best separation efficiency was applied to the separation of alkylbenzenes and steroids. Finally, the column was applied for quantitative analysis of voriconazole, an azole antifungal, and imatinib, a first-generation molecularly targeted drug for cancer treatment in spiked whole blood.

Experimental

Materials and reagents

Eggshell powder, Calhope (the eggshell membrane has been removed) was purchased from Kewpie egg (Tokyo, Japan). Sodium hypochlorite pentahydrate ($NaClO \cdot 5H_2O$), HPLC-grade chloroform, HPLC-grade methanol, HPLC-grade acetonitrile, and sodium hydroxide solution (5 M) were purchased from Nacalai Tesque (Kyoto, Japan). Poly (maleic anhydride-*alt*-1-octadecene) (PMAO, Mn: 30000–50000) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone was purchased from Kanto Chemical (Tokyo, Japan). 1,1,2,2-tetrabromoethane, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, hexylbenzene, heptylbenzene, octylbenzene, nonylbenzene, decylbenzene, prednisolone, hydrocortisone, hydrocortisone acetate, dexamethasone, testosterone, voriconazole, and imatinib were purchased from Tokyo Chemical Industry (Tokyo, Japan). Naphthalene, toluene, and sodium tetraborate were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Porcine whole blood with 0.3% of sodium citrate as an anticoagulant was purchased from Tokyo Shibaura Zouki (Tokyo, Japan) Ultrapure water (18.2 MΩcm) was obtained from a PURELAB flex water purification system (ELGA, Veolia Water, Marlow, U.K.). C18 column, Inertsil ODS-4 (150 mm × 4.6 mm I.D.) was purchased from GL Science (Tokyo, Japan).

Eggshell classification by cyclones

An overview of the classification system is presented in Fig. S1. Three different cyclone sizes (URG-2000-30ENB, URG-2000-30EH, and URG-2000-30ED) were connected, starting with the largest one, followed by the tissue quartz filter (PALLFLEX, 2500QAT-UP, 254 mm I.D.), valve, and pump in series with electrically conductive silicone tubes. Air was pumped into the cyclone system at a flow rate of 10 or 20 liters per minute (LPM), and commercial eggshell powder was fed directly into the first cyclone inlet. The eggshell particles captured by the second cyclone were collected.

Removal of organic impurities

Organic impurities in the eggshells were removed as described in our previous study.[8,9] Eggshells (7.0 g) were added to a 5 wt% NaClO aqueous solution (100 mL). After 48 h, all particles were collected by suction filtration and dried at 60 °C.

Tiny particle removal

Optimal conditions for the removal of tiny particles (theoretically, ≤4 μm) were determined by Stokes' law shown in Eq. (1), where t, η, L, ρ_1, ρ_2, G, and d are the sedimentation time, solvent viscosity coefficient, sedimentation distance, powder specific gravity, solvent specific gravity, gravitational acceleration, and particle size, respectively.

$$t = 18\times\eta\times10^8\times L/(\rho_1-\rho_2)G\times d^2 \ldots (1)$$

Small particles in the NaClO-treated eggshells were removed by sedimentation separation, as shown schematically in Fig. S2. NaClO-treated particles (6.0 g) and water (700 mL, equivalent to 11.0 cm height) were added to an 800 mL beaker (175 mm × 90 mm I.D.). The mixture was stirred at 500 rpm for 2 min to finally obtain the eggshell suspension.

After standing for 100 min to remove ≤4 μm particles, 10 cm of water from the top (equivalent to 636 mL) was pumped out, and 10 cm of new water was added. The process of stirring for 2 min, standing for 100 min, removing 10 cm of water from the top, and adding new water was repeated three times, followed by suction filtration and drying of the eggshells at 60 °C.

Six eggshell powders with different particle size distributions were obtained using cyclone classification and sedimentation separation: Untreated, 20 LPM, 10 LPM, Sed, 20 LPM + Sed, and 10 LPM + Sed. The treatments performed on each particle are schematically summarized in Fig. S3.

Modification of PMAcO onto eggshells

PMAcO was prepared by hydrolysis of PMAO in the same way as our previous study.[8] PMAcO (0.5 g) was dissolved in acetone (100 mL). Eggshells (5.0 g of Untreated, Sed, 20 LPM + Sed, 10 LPM + Sed, respectively) were added to the acetone solution and stirred vigorously for 24 h. The obtained Eggshell-PMAcO particles (PMAcO-modified eggshells) were collected by suction filtration and dried at 60 °C.

Material characterization

Eggshells were observed by a scanning electron microscope (SEM, SU-70, Hitachi, Tokyo, Japan) after coated with osmium for 5 s at 10 mA by an osmium coater (HPC-1S, Vacuum Device, Ibaraki, Japan). Particle size distributions of eggshells were

measured by analyzing SEM images by the image processing software ImageJ (National Institutes of Health). The organic contents in the particles were evaluated by comparing the contents in the 200–585 °C regime of the thermogravimetric curves obtained by thermogravimetric analysis (TG, STA7200, Hitachi, Tokyo, Japan).

Column packing

Eggshell-PMAcO particles were dispersed in 15 mL of 1,1,2,2-tetrabromoethane/chloroform (83/17, v/v). The slurry was filled into a slurry reservoir (Chemco Scientific) connected to the stainless steel column blank (150 mm × 4.6 mm I.D., Chemco Scientific). Methanol was flowed through the slurry reservoir using a HPLC pump (LC-20AR, Shimadzu) for 1 h, followed by a 30-min flow of water/methanol (50/50, v/v). The pressure was maintained at 45 MPa during column packing.

Chromatographic analyses

All chromatographic analyses were conducted at 40 °C on a Prominence-i LC2030C (Shimadzu) system equipped with a UV-Vis detector. Chromatographic parameters were calculated the United States Pharmacopeia (USP) using the LabSolution software (Shimadzu) by the following equations.

Theoretical plate: $N = 16(t_R/W)^2$, where t_R and W are the retention time of the target analyte and the peak width, respectively.

HETP: $H = L/N$, where L is the column length.

Resolution: $R_S = 2(t_{R2} - t_{R1})/(W_1 + W_2)$, where $R_S \geqq 1.5$ indicates complete separation.

Pretreatment of voriconazole-spiked whole blood samples

Whole blood samples were pretreated according to the general protein precipitation method. First, 100 µL of 15, 30, and 100 µg/mL voriconazole in methanol or 100 µL of methanol as a blank were spiked into 2000 µL of porcine whole blood, resulting in 0.75, 1.5, 5.0, or 0 µg/mL voriconazole in the whole blood sample. Subsequently, 6900 µL of acetonitrile was added to each sample and mixed using a vortex mixer for 5 min. The resulting solutions were centrifuged at 4000 ×g for 15 min at room temperature. The supernatant, containing 0.15, 0.3, 1.0, or 0 µg/mL voriconazole, was filtered through a 0.2 µm membrane filter.

Pretreatment of imatinib-spiked whole blood samples

Whole blood samples were pretreated in the same manner as voriconazole-spiked whole blood samples.[16] Porcine whole blood (2000 µL) was spiked with 100 µL of 15, 30, and 60 µg/mL imatinib in methanol, or 100 µL of methanol as a blank, resulting in 0.75, 1.5, 3.0, or 0 µg/mL imatinib in the whole blood. The supernatant, containing 0.15, 0.3, 0.6, or 0 µg/mL imatinib, was filtered through a 0.2 µm membrane filter.

Results and discussion

Fabrication and characterization of Eggshell-PMAcO

Commercial eggshell particles (Untreated)were classified using three cyclones connected in series to investigate the effect of particle size on the separation efficiency of the resulting column. In this system, large particles were removed by the largest cyclone, followed by the removal of small particles by the smallest cyclone or filter. The size of the particles captured by the middle cyclone was controlled by adjusting the flow rate to 20 or 10 LPM. SEM images of the Untreated eggshell powders and those captured by the second cyclone are shown in Fig. 1(a–c) and Fig. S4(a–c). Untreated eggshell powders contain particles of various sizes, whereas those classified with cyclones showed a more uniform size distribution. The size distributions of the particles were analyzed by ImageJ using SEM images (Fig. S5 and Table 1). The classified particles exhibited larger arithmetic mean particle sizes than the Untreated particles. The cyclone collection efficiency ($D_{p50\%}$) can be defined by the Lapple Model shown in Eq. (2), where μ, b, N_e, V_i, and ρ_p are the air viscosity, inlet width, number of effective turns, inlet velocity, and particle density, respectively, which is one of the theoretical formulas for cyclones.[17] Slower flow rates during classification yielded a larger arithmetic mean particle size, in accordance with Eq. (2). Consequently, three different particle sizes were successfully obtained using the cyclone system.

$$D_{p50\%} = (9\mu b/2\,\pi N_e V_i \rho_p)^{1/2} \quad \ldots (2)$$

Sedimentation separation was performed on the three different eggshell particle sizes to reduce the number of tiny particles, as shown in Fig. 1(d–f) and Fig. S4(d–f). Tiny particles in the packing material can cause poor separation efficiency owing to particle inhomogeneity and the undesired increase in the back pressure of the column. The particle size distributions after sedimentation separation are shown in Fig. 2 and Table 1. The percentage of particles ≤4 µm classified by sedimentation separation was low compared to that without sedimentation (Table S1). Four types of particles with different size distributions were modified with PMAcO to obtain Eggshell-PMAcO. The TG analysis results for the four types of particles before and after PMAcO modification confirmed the successful modification of the eggshell surface (Table S2). The weight of PMAcO relative to the weight of the particles ranged between 0.3-0.4%.

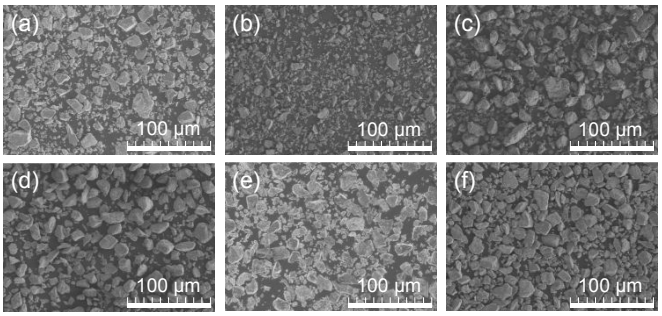


Fig. 1 The effect of cyclone classification and sedimentation separation on the particle size of eggshells. SEM images of (a) Untreated, (b) 20 LPM, (c) 10 LPM, (d) Sed, (e) 20 LPM + Sed, and (f) 10 LPM + Sed.

Table 1. Particle size of eggshells analyzed by SEM images shown in Fig. 1.

	Particle size / μm arithmetic mean ± standard deviation
Untreated	4.3 ± 3.8 (*n* = 1558)
20 LPM	5.6 ± 3.3 (*n* = 1172)
10 LPM	9.5 ± 5.5 (*n* = 510)
Sed	6.6 ± 5.5 (*n* = 823)
20 LPM + Sed	7.3 ± 4.5 (*n* = 743)
10 LPM + Sed	10.2 ± 5.0 (*n* = 527)

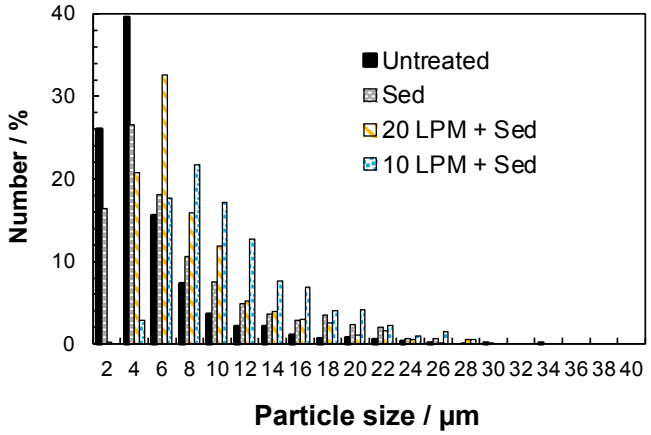


Fig. 2 Particle size distributions of eggshells analyzed by SEM images shown in Fig. 1.

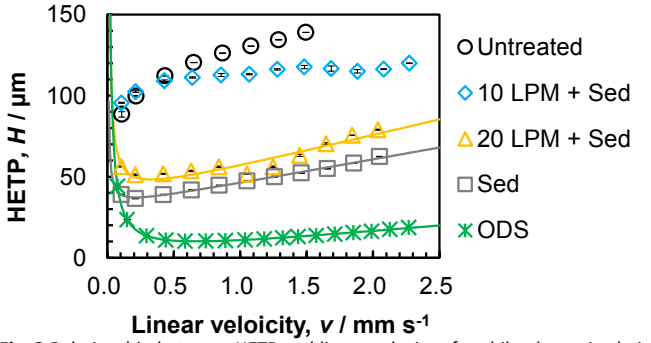


Fig. 3 Relationship between HETP and linear velocity of mobile phase. Analytical conditions: water/methanol (60/40, v/v) for eggshell-PMAcO columns and water/methanol (20/80, v/v) for ODS column, 0.1 mg/mL naphthalene, 10 μL injection, detected at 254 nm, *n* = 3.

Column performance comparison

The Eggshell-PMAcO particles were packed into a stainless steel column blank (4.6 x 150 mm I.D.). The back pressure of the four columns was proportional to the flow rate of the mobile phase (Fig. S6). Columns prepared with sedimentation separated Eggshell-PMAcO showed lower back pressure compared to the "Untreated" column, indicating that tiny particle removal effectively reduced the back pressure. Compared to the columns packed with sedimentation separated materials, the back pressure increased with decreasing arithmetic mean particle size, identical to that of general packing materials. The separation efficiencies of the Eggshell-PMAcO columns and a commercial ODS column were compared with the HETP calculated from chromatograms of naphthalene. The HETPs were plotted as a function of the mobile phase linear velocity (*v*), as shown in Fig. 3. The "Sed", "20 LPM + Sed" and "ODS" traces were fitted to the van Deemter equation shown in Eq. (3).

$$H = A + B/v + Cv \quad \ldots (3)$$

The *A*, *B*, and *C* terms (flow path heterogeneity, analyte longitudinal diffusion, and analyte mass transfer into the packing material pores, respectively) were calculated using the least square curve fitting method (Table S3). The "10 LPM + Sed" and "Untreated" plots were not fitted to van Deemter equation because no local minimum values were obtained, resulting in negative *B* terms by the least square curve fitting. "Untreated" showed the lowest separation efficiency despite being packed with the smallest arithmetic mean particle size. This suggests that the presence of tiny particles induced poor column performance. Although inferior to commercially available ODS columns, tiny particle removal by sedimentation separation was effective for improving column performance. Comparing the three columns packed with sedimentation separated materials, the column with the smaller arithmetic mean particle size showed a higher separation efficiency, identical to that of general packing material.

The results presented herein demonstrate that sedimentation separation is more environmentally-friendly and simpler than the reagent-based process used previously[8,9] to improve the separation efficiency of packing materials.

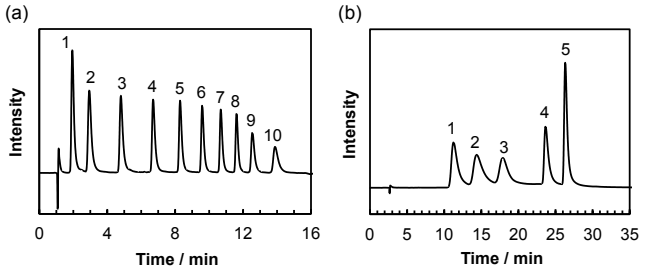


Fig. 4 (a) Chromatograms of alkylbenzenes (1: toluene, 2: ethylbenzene, 3: propylbenzene, 4: butylbenzene, 5: pentylbenzene, 6: hexylbenzene, 7: heptylbenzene, 8: octylbenzene, 9: nonylbenzene, 10: decylbenzene). Analytical conditions: water/methanol (70/30, v/v) for 1 min, followed by the gradient elution to 30/70 (v/v) for 9 min and finally 30/70 (v/v) for 6 min, 1.0 mL/min, 0.1 mg/mL alkylbenzenes, respectively, 10 μL injection, detected at 254 nm. (b) Chromatograms of steroids (1: prednisolone, 2: hydrocortisone, 3: hydrocortisone acetate, 4: dexamethasone, 5: testosterone). Analytical conditions: water/methanol (85/15, v/v) for 15 min, followed by the gradient elution to 70/30 (v/v) for 5 min and finally 70/30 (v/v) for 15 min, 0.4 mL/min, 0.1 mg/mL steroids, respectively, 10 μL injection, detected at 254 nm.

Separation of neutral hydrophobic compounds

The "Sed" column, which exhibited the best separation efficiency among the prepared columns, was applied to separate 10 alkylbenzenes and 5 steroids to further demonstrate the improved separation efficiency over our previous Eggshell-PMAcO column without classification[8] (Fig. 4). Despite slight tailing, all peaks were observed with a baseline resolution (R_S >1.5, Tables S4 and S5). The slight tailing of peaks is likely due to slight irregularity in packing of Eggshell-PMAcO caused by coexistence of tiny particles that have not been completely removed by sedimentation. In fact, our previous study[8] have shown that the interparticle gaps between eggshell particles measured by mercury porosimetry were not uniform. These analytes were separated previously using Eggshell-PMAcO column, but the obtained chromatogram peaks were excessively broad and tailing for use as an analytical column,

and a complete steroid separation was not achieved.[8] These results suggested that the separation performance of Eggshell-PMAcO was sufficiently improved for use as an analytical column by removing the tiny particles.

Quantitative analysis of voriconazole under neutral conditions

The "Sed" column was applied for quantitative analysis of voriconazole using a neutral mobile phase. Voriconazole is azole antifungal agent used for the treatment of invasive aspergillosis and other serious fungal infections.[18,19] TDM of voriconazole is recommended, as UK and Canadian guidelines specify concentrations of 1.0–5.5 and 1.5–5.0 μg/mL for the treatment of invasive fungal infections, respectively.[20,21] Fig. S7a shows the chromatograms of 0.1–2.0 μg/mL voriconazole using a neutral mobile phase, with sharp peaks obtained independent of the sample concentration. A calibration curve was obtained from the peak areas of the chromatograms (Fig. S7b). A good linear relationship was observed between the voriconazole concentrations and peak areas. Three concentrations of voriconazole-spiked whole blood samples at 0.75, 1.5, and 5.0 μg/mL were prepared to represent ineffective, effective, and addictive concentrations, respectively. After pretreatment with a protein precipitation method, the voriconazole-spiked whole blood samples were analyzed with the “Sed” column, resulting in the separation of the blood-derived components and voriconazole for all three different sample concentrations (Fig. 5). In addition, excellent accuracy and precision were obtained (Table 2), indicating that the prepared “Sed” column could be successfully applied for voriconazole quantification in whole blood with potential as an analytical column for TDM of voriconazole.

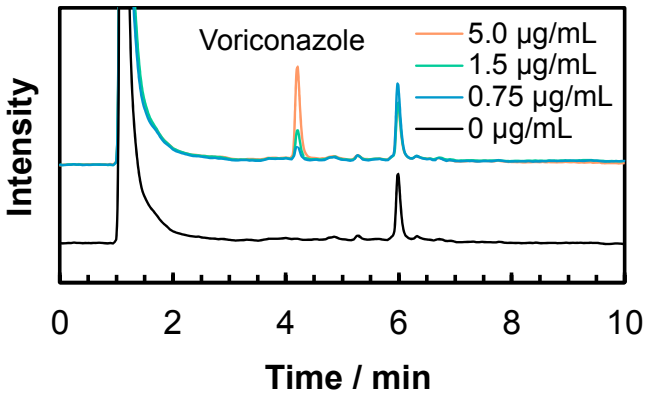


Fig. 5 Chromatograms of voriconazole spiked into porcine whole blood. Analytical conditions: water/methanol (90/10, v/v) for 1 min, followed by the gradient elution to 30/70 (v/v) for 4 min and finally 30/70 (v/v) for 5 min, 1.0 mL/min, 0–5.0 μg/mL voriconazole in whole blood (After pretreatment: 0–1.0 μg/mL), 10 μL injection, detected at 254 nm.

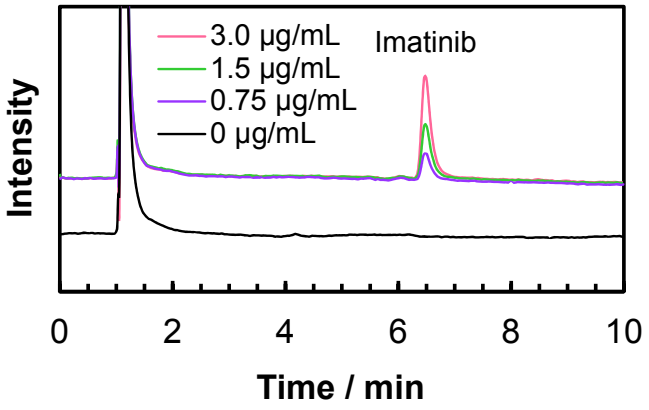
Table 2. Accuracy and precision of voriconazole from whole blood samples. (n = 3)

Concentration / μg/mL	Accuracy / %	Precision / %
5.0	99.7	0.8
1.5	99.1	0.7
0.75	101.3	1.7

Quantitative analysis of imatinib under alkaline conditions

The "Sed" column was also applied for quantitative analysis of imatinib using an alkaline mobile phase. Imatinib is a tyrosine kinase inhibitor designed to selectively interfere with key signal transduction pathways and is used to treat cancers such as chronic myeloid leukemia.[22–24] Detection and quantification of imatinib in biological fluids are often performed using HPLC.[25–31] A minimum dose of 1.0 μg/mL imatinib is required to achieve a molecular genetic response, while concentrations of >3.0 μg/mL increase neutropenia risks.[32] Imatinib is a basic compound with amine and pyridine structures that exhibit pK_{a1}, pK_{a2}, pK_{a3}, and pK_{a4} values of 8.07, 3.73, 2.56, and 1.52, respectively.[33] During the analysis of basic compounds, tailing of peaks can be caused by strong adsorption between the analytes and residual silanol groups on the common silica gel-based stationary phase.[34] Thus, the eggshell-based column was considered to be more suitable for long-term quantitative analysis of basic drugs because of its higher resistance to tailing peak generation when using alkaline mobile phases.[8] To elute neutral imatinib by suppressing adsorption, a pH 10.8 $Na_2B_4O_7$ buffer was used as an aqueous mobile phase. Fig. S8a shows the obtained chromatograms of 0.1–2.0 μg/mL imatinib using the alkaline mobile phase, yielding sharp peaks regardless of the sample concentration. Calibration was performed using the chromatogram peak areas, as shown in Fig. S8b. A good linear relationship was observed between the imatinib concentrations and peak areas. Three concentrations of imatinib-spiked whole blood samples at 0.75, 1.5, and 3.0 μg/mL were prepared, representing ineffective, effective, and addictive concentrations, respectively. After pretreatment using a protein precipitation method, the imatinib-spiked whole blood samples were analyzed using the “Sed” column with an alkaline mobile phase. Good separation of the blood-derived components and imatinib was achieved at all three concentrations (Fig. 6).

Finally, the "Sed" column was applied for the routine analysis of imatinib in whole blood. An ineffective sample was first



analyzed, followed 20 min later by an effective sample, another 20 min later by an addictive sample, and again by an ineffective sample. A total of 180 analyses (60 times for each sample) were performed. Despite the continuous alkaline mobile phase flow, >3000 column volumes (CV), and 180 injections of samples containing blood-derived contaminants, negligible peak area and retention time changes were observed for imatinib (Figs. 7 and S9), resulting in consistent accuracy and excellent precision (Table 3 and Fig. S10). Imatinib in whole blood was also analyzed by a commercial ODS column in the same time-course to the "Sed column" (Fig. S11 and S12). After 30 measurements for each sample concentration, the peak of imatinib was no longer detectable (Fig. S11). Furthermore, after 36 measurements of each sample, the back pressure of the column increased so rapidly that the analysis could no longer be performed. These results indicate the potential of the eggshell-based analytical column for long-term TDM of imatinib under alkaline conditions.

Fig. 6 Chromatograms of imatinib spiked into porcine whole blood. Analytical conditions: 10 mM $Na_2B_4O_7$ buffer (pH = 10.8)/methanol (90/10, v/v) for 1 min, followed by the gradient elution to 70/30 (v/v) for 3 min and finally 70/30 (v/v) for 6 min, 1.0 mL/min, 0–3.0 µg/mL imatinib in whole blood (After pretreatment: 0–0.6 µg/mL), 10 µL injection, detected at 285 nm.

Table 3. Accuracy and precision of imatinib from whole blood samples. (n = 3)

Concentration / µg/mL	Accuracy / %	Precision / %
3.0	101.5	1.4
1.5	98.3	1.3
0.75	98.7	2.2

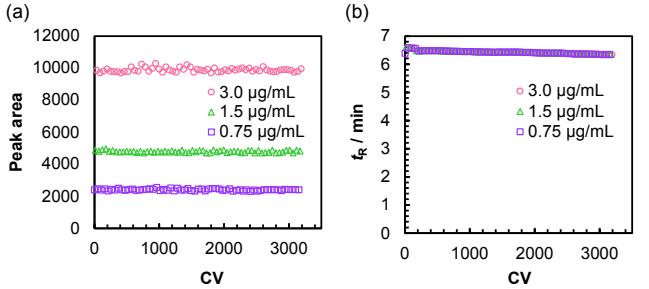


Fig. 7 Changes in (a) peak areas and (b) retention times during routine analysis of three different concentrations of imatinib-spiked whole blood samples. Analytical conditions: 10 mM $Na_2B_4O_7$ buffer (pH = 10.8)/methanol (90/10, v/v) for 1 min, followed by the gradient elution to 70/30 (v/v) for 3 min, 70/30 (v/v) for 6 min, and finally (90/10, v/v) for 10 min, 1.0 mL/min, 0.75–3.0 µg/mL imatinib in whole blood (After pretreatment: 0.15–0.6 µg/mL), 10 µL injection, detected at 285 nm.

Conclusions

Eggshell-PMAcO, an eggshell modified with an amphiphilic copolymer, PMAcO, was used as an analytical column packing material. The cyclone system successfully yielded three types of particles with different arithmetic mean diameters. Tiny particles were removed via sedimentation separation, resulting in decreased contents of ≤4 µm particles for all tested samples. Columns prepared with sedimentation separated materials exhibited better separation efficiency than the Untreated columns. The column that showed the best separation efficiency, the "Sed" column, completely separated 10 alkylbenzene and 5 steroids. Finally, the "Sed" column was used to monitor voriconazole and imatinib in whole blood. These drugs were separated from blood-derived components and their respective quantification was achieved with excellent accuracy and precision. Furthermore, their long-term stability in an alkaline mobile phase was confirmed via routine analysis of imatinib in an alkaline mobile phase. To the best of our knowledge, this study is the first that has applied a composite of eggshell and a copolymer as a packing material in an analytical column. Quantitative analysis of drugs in whole blood was achieved using neutral and alkaline mobile phases with this newly-prepared eggshell-based column.

Author Contributions

Tomoka Yoshii: Methodology, Investigation, Writing – Original Draft. Kohei Nakano: Methodology, Investigation. Tomoaki Okuda: Resources, Writing – Review & Editing, Supervision. Daniel Citterio: Writing – Review & Editing, Supervision. Yuki Hiruta: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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