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A biomimetic hybrid material consisting of CaCO₃ mesoporous microspheres and an alternating copolymer for reversed-phase HPLC

Inspired by CaCO₃ based biominerals like eggshells, alternating amphiphilic copolymer-modified monodisperse mesoporous CaCO₃ microspheres were applied as a HPLC stationary phase for analyses of basic drugs requiring alkaline mobile phases.

As featured in:

A biomimetic hybrid material consisting of CaCO₃ mesoporous microspheres and an alternating copolymer for reversed-phase HPLC†

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We developed a biomimeral-inspired hybrid material composed of CaCO₃ and an organic polymer as a column packing material for HPLC. This material combines a hierarchical mesoporous structure and the functionality of the polymer. The surface of monodispersed mesoporous CaCO₃ microspheres was modified with poly(maleic acid-alt-1-octadecene) (PMACO) comprising hydrophobic alkyl chains and anionic carboxylate groups. PMACO adsorbed onto the surface of CaCO₃ through electrostatic interaction between Ca²⁺ sites and carboxylate groups, resulting in an octadecene coated microsphere interface. These microspheres were applied as a HPLC column and exhibited reversed-phase retention behavior in the separation of alkylbenzenes. This column showed high alkaline mobile phase resistance compared with the conventionally applied ODS column packing material. Quantitative analysis of the basic antidepressants clomipramine and imipramine spiked into whole blood was achieved with an alkaline mobile phase, demonstrating the potential of the biomimeral-inspired material as a HPLC stationary phase for practical applications in routine analyses of basic drugs requiring alkaline mobile phases.

Introduction

Biominerals are inorganic–organic hybrid materials with hierarchically organized structures from nanoscopic to macroscopic scales. For example, eggshells, seashells and corals have mesostructures of CaCO₃ nanocrystals, which are elaborately controlled by biopolymers. Focusing on eggshells as an example, their structure has pores that are necessary for gas exchange allowing respiration, and the presence of biopolymers prevents virus invasion. Furthermore, they provide relatively high mechanical strength to protect the inner shell. These characteristics of hierarchical structures combining CaCO₃ nanocrystals and biopolymers enable the control of substance distribution and provide mechanical strength. Therefore, material scientists have been attracted by the elaborate architecture and formation processes of these biominerals, attempting to develop new fabrication technologies and to obtain novel functionalities through imitation of these features.

Paying attention to the hierarchical structure of biominerals to control the distribution of substances, a normal phase chromatography stationary phase based on biopolymer-removed crushed seashells has been developed. Although it was shown that the hierarchical structure of biominerals can be applied to a chromatographic separation material, the functionality of the biopolymer of biominerals like egg shells in terms of controlling substance distribution was not fully used for separation purposes.

In order to control the morphology of CaCO₃, crystallization methods involving anionic small molecules or anionic polymers were studied by mimicking the formation process of biominerals. We have also reported that monodispersed mesoporous CaCO₃ microspheres with 20 nm mesopores and controllable particle size can be obtained by a biomimetic process. In this method, an anionic polymer, poly(sodium 4-sulfonate) (PSS), controls crystal growth (i.e. assemblage of 20 nm CaCO₃ nanocrystals) by electrostatic adsorption, resulting in the formation of a metastable phase vaterite crystal. In addition, we have modified these microspheres with thermo-responsive poly(N-isopropylacrylamide) (PNIPAAm) to transport chemical substances from an organic to an aqueous phase by temperature-switching. However, after modification with PNIPAAm, the particular space was fully filled with the thermo-responsive polymer, resulting in the loss of the hierarchical pore structure that is important in controlling substance distribution.

Cooperative interaction between the hierarchical structure and organic molecules is important to obtain the functions of biominerals and the high mechanical strength as in the case of
As with CaCO₃-based biomimetics wherein hierarchical structures are controlled by electrostatic adsorption between CaCO₃ crystals and biopolymers while stably maintaining the morphology, the imitation of their formation process allows the modification of synthetic CaCO₃ mesoporous microspheres with an anionic polymer by electrostatic adsorption. By this method, mesoporous CaCO₃ particles can be stably modified, due to polymer adsorption at multiple sites on the surface of CaCO₃, while effectively retaining the mesopores.

In addition, CaCO₃ is stable against dissolution in alkaline solutions, since it is a basic salt. On the other hand, the use of ODS is limited due to poor hydrolytic stability against alkaline mobile phases. The fact that alkaline mobile phases have to be avoided when using low alkaline resistant silica gel column materials is a disadvantage leading to low separation performance for basic analytes. By replacing ODS with the polymer-modified CaCO₃ particles, it is possible to realize an alkaline stable packing material overcoming the disadvantages of conventional stationary phases.

We have developed a biomimetic-inspired inorganic–organic hybrid material by modifying the surface of monodisperse mesoporous CaCO₃ microspheres through electrostatic adsorption of a regularly alternating copolymer having both anionic groups and functional groups, while maintaining the mesoporous structure. Whereas the anionic groups serve the purpose of adsorbing the polymer to the crystal surface, the alternating functional groups impart their functionality. Thus, efficient modification and functionalization of the surface of the monodisperse mesoporous CaCO₃ microspheres were simultaneously achieved. Especially, maleic anhydride-based alternating copolymers can obtain various functionality by radical copolymerization of maleic anhydride with styrene, α-olefins, or vinyl ethers.

In this work, poly(maleic acid-alt-1-octadecene) (PMAcO), consisting of regularly alternating hydrophobic alkyl chains and two carboxylate units, was used (Fig. 1). The presence of the octadecyl groups on the surface of the monodisperse mesoporous CaCO₃ microspheres imparts a strong hydrophobic character, similar to the octadecyl groups of ODS, and enables the application as a versatile packing material in reversed-phase HPLC.

**Results and discussion**

**Preparation and characterization of amphiphilic polymer modified monodisperse CaCO₃ microspheres**

Bare monodisperse mesoporous CaCO₃ microspheres were prepared by a simple mixing method of two solutions at room temperature according to the literature. CaCO₃ crystal growth during microsphere synthesis was controlled with PSS, followed by the decomposition of PSS using aqueous NaClO solution and calcination. PMAcO was obtained by the hydrolysis of repeating maleic anhydride units in poly(maleic anhydride-alt-1-octadecene) (PMAO), as shown in Scheme 1. Hydrolysis was confirmed by FT-IR spectra (Fig. S1, ESI†), in which the peak at 1780 cm⁻¹ derived from maleic anhydride decreased, while the peak at 1729 cm⁻¹ derived from maleic acid was enhanced. The surface of the bare CaCO₃ microspheres was modified with PMAcO by simply dispersing the microspheres in an organic solution of PMAcO. PMAcO adsorbed onto the surface of CaCO₃ through electrostatic interaction between cationic calcium sites and anionic carboxylate groups, resulting in an octadecyl-coated microsphere interface referred to as “CaCO₃–PMAcO”.

Particle morphologies of bare CaCO₃ and CaCO₃–PMAcO particles were observed by scanning electron microscopy (SEM),
as shown in Fig. 2. Both before and after modification with PMAcO, monodisperse spherical particles with identical average particle diameters of 3.5 ± 0.4 μm and 3.5 ± 0.6 μm, respectively, were observed (n = 400, measured from Fig. 2D and H).

The particle size distributions are shown in Fig. S2 (ESI†). CaCO₃ microspheres obtained by the applied synthesis method have been reported to consist of aggregates of 20 nm-sized CaCO₃ crystals,21 which was also observed for the modified CaCO₃–PMAcO particles (Fig. S3, ESI†), confirming that the morphology of the mesoporous microspheres was retained after modification with PMAcO. According to the XRD patterns (Fig. 3A), the bare CaCO₃ particles were assigned to be pure vaterite, with the crystal structure unchanged by modification with PMAcO. FT-IR spectra (Fig. 3B) showed the appearance of peaks at 2924 and 2858 cm⁻¹ derived from –CH₂– moieties after modification with PMAcO. In addition, the mass reduction in the thermogravimetric (TG) curves in the 240–540 °C range (Fig. 3C) indicated the presence of 5 wt% PMAcO on the surface of CaCO₃ microspheres, which was comparable to oleic acid for CaCO₃ surface modification prepared in analogy to the CaCO₃–PMAcO particles (Fig. 3D). These results demonstrate that PMAcO was successfully modified onto the surface of CaCO₃ microspheres while maintaining their properties. The pore size distributions of CaCO₃ microspheres before and after PMAcO modification were obtained by nitrogen adsorption–desorption isotherms (Fig. 3E). While the pore size distributions hardly changed upon modification with the polymer, the pore diameter decreased from 22 to 16 nm (Fig. 3F). The thickness of an octadecanoic acid monolayer on CaCO₃ has been reported to be ≈2.6 nm.22 Therefore, these results infer that a uniform monolayer of repeating octadecene units was formed on the surface of the pores by PMAcO modification. Since ODS

![Fig. 2](image)

Fig. 2 SEM images of (A–D): bare CaCO₃ particles and (E–H): polymer-modified CaCO₃–PMAcO particles.

![Fig. 3](image)

Fig. 3 Physical properties of bare CaCO₃ and CaCO₃–PMAcO microspheres: (A) XRD spectra, (B) FT-IR spectra, (C) TG curves, (D) TG curves of CaCO₃–PMAcO and CaCO₃–oleic acid before and after washing; ‘‘-W’’ indicates samples after washing, (E) N₂ adsorption–desorption isotherms, and (F) pore-size distributions.
particles used in HPLC column packings have a uniform particle size of 2–5 μm with 6–30 nm pores, the current CaCO₃–PMAcO microspheres with comparable uniform particle size and pore size fulfill the basic requirements to be applied as a HPLC column packing material. For successful application as a packing material for long-term reproducible HPLC analysis, durability against the mobile phase solvent is required. Since the PMAcO polymer has a plurality of adsorption sites, it is expected to be stably adsorbed onto the surface of CaCO₃. The durability of the PMAcO modification was experimentally evaluated by comparing the amount of organic components by means of the TG curves in the 240–540 °C regime recorded before and after washing with a water/methanol mixture (50/50, v/v) representing a common mobile phase. The results shown in Fig. 3D indicate that the amount of PMAcO did not change during the washing process. On the other hand, in a comparative experiment using oleic acid for CaCO₃ surface modification (prepared in analogy to the CaCO₃–PMAcO particles), a drastic decrease of the organic compound from 5 to 1 wt% upon washing was observed, corresponding to an 80% reduction. This significant difference is attributed to the fact that oleic acid only has a single carboxylate residue to interact with the CaCO₃ surface, in contrast to PMAcO with multiple binding sites, leading to the higher durability of the latter. Therefore, the biomimic inspired surface modification with the polymer by interaction between polymeric functional groups and calcium sites seems to contribute to the long-term durability of CaCO₃–PMAcO and, hence, its applicability as a packing material for HPLC.

**Evaluation of CaCO₃–PMAcO microspheres as a column packing material for HPLC**

CaCO₃–PMAcO microspheres were packed into a column (100 × 2.1 mm I.D.) and their performance as a stationary phase for HPLC was evaluated. Fig. S4A (ESI†) shows the relationship between the mobile phase mixing ratio and the back pressure for methanol/water mixtures. At a flow rate of 0.4 mL min⁻¹, the back pressure of the column was below 15 MPa (Fig. S4B, ESI†). Furthermore, the back pressure was found to be stable and, therefore, suitable for use with a conventional HPLC system. The separation mode of this column was evaluated through the elution behavior of naphthalene and alkylbenzenes (C₀–C₁₀) depending on the mixing ratio of methanol/water as the mobile phase. The retention factors of naphthalene and alkylbenzenes increased with decreasing proportions of methanol (Fig. S5, ESI† and Fig. 4A) and longer alkyl chains resulted in higher hydrophobicity (Fig. 4A). These results indicate that CaCO₃–PMAcO behaved as a reversed-phase mode stationary phase for the separation of naphthalene and alkylbenzenes, while pure CaCO₃ generally works as normal-phase mode stationary phase.⁷ The octadecene groups of the CaCO₃–PMAcO microspheres at the stationary phase to mobile phase interface performed a role identical to the octadecyl groups of ODS, resulting in reversed-phase characteristics. During the separation of a mixture of 11 alkylbenzenes, all peaks could be clearly observed with a baseline resolution (peak resolution Rₛ = 1.8–4.2 > 1.5) in the chromatogram (Fig. 4B).

The durability of the CaCO₃–PMAcO column against an alkaline mobile phase (mixture of pH 10.8 aqueous Na₂B₄O₇ buffer and methanol) was evaluated by monitoring the elution behavior of naphthalene. The deterioration of the column was estimated by the relative change of the retention time during the continuous flow of the mobile phase. A comparative study was carried out using an ODS column prepared from a commercially available 3 μm ODS silica packing material through the same procedure as that for the CaCO₃–PMAcO column. As shown in Fig. 4C and Fig. S6 (ESI†), the retention time and the number of theoretical plates of naphthalene were retained over 3500 column volumes (CVs) of alkaline mobile phase purging in the case of the CaCO₃–PMAcO column (RSD of retention time: 0.7%), while it continuously decreased when using the ODS packed column (over 35% decrease after 3500 CV purges). It is assumed that the amount of octadecyl groups on the silica surface gradually decreased due to the inherent solubility of silica in alkaline media, resulting in a reduction of hydrophobic interaction with naphthalene. In contrast, the performance of the CaCO₃–PMAcO packing in the alkaline mobile phase was preserved, since
the solubility of CaCO$_3$ decreases with increasing pH. These results indicated that CaCO$_3$-PMAcO packed columns show excellent durability at high pH and could be used for routine analyses requiring alkaline mobile phases.

**Quantitative analysis of basic antidepressants spiked into whole blood using a CaCO$_3$–PMAcO packed column**

As a proof-of-concept demonstration, we conducted the analysis of basic compounds requiring an alkaline mobile phase for successful separation. When a neutral mobile phase is used, basic compounds become positively charged, resulting in unwanted electrostatic and hydrogen bond interactions with the stationary phase in addition to hydrophobic interactions. On the other hand, these are eliminated by deprotonation of basic compounds in alkaline mobile phases, allowing the separation of basic compounds as free bases, which is attractive for routine analysis. Silica-based columns are reluctantly used because of their inherent instability to alkaline mobile phases. Herein, we demonstrate the analysis of the basic antidepressants clomipramine and imipramine (pK$_a$ of 9.3 and 9.5, respectively, shown in Fig. 5A), known as tricyclics. Since addiction to psychotropic drugs is a serious problem, the routine analysis of these substances in biological fluids is of significance. First, the effect of the mobile phase pH on the elution behavior of clomipramine using a CaCO$_3$–PMAcO packed column was investigated. Fig. 5B shows the chromatograms of clomipramine on a CaCO$_3$–PMAcO stationary phase eluted with a neutral or alkaline mobile phase. A sharper peak was obtained when using the alkaline mobile phase compared to the neutral mobile phase (1.4-fold number of theoretical plates; 0.7-fold peak asymmetry factor). Protonation of clomipramine in the neutral mobile phase resulted in the interaction with anionic carboxylates or carbonates, causing tailing and extension of the retention time. The suppression of clomipramine protonation eliminated these unwanted interactions, leading to a sharp chromatographic peak with simple retention behavior only governed by hydrophobic interactions.

![Fig. 5](https://example.com/fig5)

**Table 1** Recovery of basic drugs from a whole blood sample

<table>
<thead>
<tr>
<th>Basic drug</th>
<th>Level (ng mL$^{-1}$)</th>
<th>Recovery† (%,$n=3$)</th>
<th>RSD (%)</th>
<th>From peak area</th>
<th>Recovery† (%,$n=3$)</th>
<th>RSD (%)</th>
<th>From peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>5.0</td>
<td>96.4</td>
<td>0.2</td>
<td>95.6</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clomipramine</td>
<td>5.0</td>
<td>97.6</td>
<td>0.7</td>
<td>96.2</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Conclusions**

This work is to the best of our knowledge the first example of a biomimetic material in which the hierarchical structure and the polymer function are fused to enable effective control of substance distribution. By mimicking the formation process of...
biominerals, mesoporous CaCO₃ microspheres were fabricated and surface-modified with an alternating amphiphilic copolymer consisting of hydrophobic alkyl chains and anionic carboxylate groups. Because of the monodisperse and mesoporous structure modified with hydrophobic octadecene groups on its surface, this biomineral-inspired hybrid material was found to be suitable for application in HPLC. When applied as a HPLC column packing material, it showed reversed-phase retention behavior and high resistance against alkaline mobile phases. In a proof-of-concept application, whole blood spiked with basic antidepressants clomipramine and imipramine was successfully analyzed with an alkaline mobile phase, in which conventional ODS packings have significant stability limitations. Thus, the present work clearly demonstrates the potential of biomineral-inspired PMAcO-modified mesoporous CaCO₃ microspheres as a HPLC column packing material. By decreasing the particle size of the mesoporous CaCO₃ microspheres, the pore size distribution can be narrowed. Furthermore, since decreasing the particle diameter leads to an expansion of the specific surface area and an improvement of packing density, it is expected that a higher separation ability can be obtained by decreasing the particle diameter. Furthermore, it is expected that various retention modes can be imparted to this packing material with a variety of maleic acid-based alternating polymers. Therefore, we expect that this work opens a new route for biomimetic materials in separation applications.

**Experimental section**

**Materials and instruments**

Calcium chloride (CaCl₂), sodium carbonate (Na₂CO₃), 5 wt% aqueous sodium hypochlorite (NaClO) solution, tetrahydrofuran (THF) and HPLC-grade methanol were purchased from Kanto Chemical (Tokyo, Japan). Toluene, acetone, potassium hydroxide (KOH), trifluoroacetic acid (TFA), oleic acid, sodium tetraborate decahydrate (Na₂B₄O₇·10H₂O), sodium hydroxide (NaOH), naphthalene and clomipramine hydrochloride were purchased from Wako Pure Chemical (Osaka, Japan). Poly(sodium 4-sulfonate) (PSS, Mn: ~ 70 000) and poly(maleic anhydride-alt-1-octadecene) (PMAO, Mn: 30 000–50 000) were purchased from Sigma-Aldrich (St. Louis, MO). Benzene, methylbenzene, ethylbenzene, amylbenzene, butylbenzene, hexylbenzene, heptylbenzene, n-octylbenzene, nonylbenzene, decylbenzene and imipramine hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). A 3 μm fully porous ODS silica gel (CHEMOSORB 3-ODS-L) was purchased from Chemco Scientific (Osaka, Japan). Porcine whole blood with 0.3 wt% citric acid was purchased from Tokyo Shibaura Zouki CO (Tokyo, Japan). Ultrapure water (18.2 MΩ cm) was obtained from a PURELAB flex water purification system (ELGA, Veolia Water, Marlow, U.K.).

**Fabrication of CaCO₃ microspheres**

128 mL of 1 M CaCl₂ solution was added to 4.0 mL of 16 mM Na₂CO₃ solution containing 1.0 g L⁻¹ PSS. After mixing, the solution was stirred at 1500 rpm for 90 s with a discoid shape magnetic stirrer tip (diameter: 30.5 mm, and height: 12 mm) and then left for 24 h. After filtration, the organic components included in the products were extracted by immersion in 5 wt% NaClO aqueous solution for 48 h. The resultant microspheres were washed extensively with purified water and annealed at 400 °C, leading to bare CaCO₃ microspheres.

**Preparation of PMAcO by hydrolysis of PMAO**

315 mg of solid KOH was added into 10 mL of a solution of 1.0 g of PMAO in THF, followed by vigorous stirring for 2 hours at room temperature. After evaporating the solvent, the residue was dissolved in a small amount of acetone, and an excess amount of TFA was added. Aggregates were re-dissolved in 5 mL of acetone and slowly poured into 100 mL of water to precipitate the polymer. This purified polymer was collected by suction filtration with water washing and dried in a vacuum.

**Modification of PMAcO onto the surface of CaCO₃ microspheres**

100 mg of PMAcO was dissolved in 20 mL of THF and 30 mL of toluene. After addition of 1.0 g of bare CaCO₃ microspheres, stirring was vigorously continued for 2 hours at 60 °C. The collected “CaCO₃–PMAO” microspheres were washed with a small amount of THF and dried at 60 °C. Oleic acid-modified CaCO₃ microspheres were obtained by the same procedure. 100 mg of oleic acid was dissolved in 50 mL of toluene. After addition of 1.0 g of bare CaCO₃ microspheres, stirring was vigorously continued for 2 hours at 60 °C. The collected “CaCO₃–oleic acid” microspheres were washed with a small amount of THF and dried at 60 °C.

**Characterization of PMAcO, CaCO₃ particles and modified CaCO₃ particles**

The morphologies of bare CaCO₃ and CaCO₃–PMAcO were observed using scanning electron microscopy (SEM, S-4700, Hitachi, Tokyo, Japan). Before observation, samples were coated with osmium using an osmium coater (HPC-1S, Vacuum Device, Ibaraki, Japan) for 15 s at 10 mA. For the observation of fractured CaCO₃–PMAcO, the sample was prepared by a thick coating with osmium using an osmium coater for 60 s at 10 mA, followed by crushing with a microspatula. The size of microspheres was measured using the image processing software ImageJ (National Institutes of Health). The Brunauer–Emmett–Teller (BET) surface area, pore volume and pore size distributions were determined by N₂ sorption (3Flex, Micromeritics, SHIMADZU, Kyoto, Japan). Before measurements, samples were degassed for 6 h at 160 °C. The crystal structures of the samples were confirmed by X-ray diffraction (XRD, MiniFlex II diffractometer, Rigaku, Tokyo, Japan) using CuKα radiation. Organic components of samples were also characterized using high throughput Fourier transform infrared spectroscopy (FT-IR, model-Alpha, Bruker, Germany). The contents of organic components of microspheres were measured by thermogravimetric analysis (TG, TG/DTA7200, SII, Chiba, Japan). Durability of PMAcO modification was evaluated by comparing the amounts of organic components in the 240–540 °C regime of the TG
Curves before and after washing with solvent. CaCO₃–PMAcO was washed 10 times with 10 mL (total 100 mL) of a mixed solvent of water and methanol (50/50, v/v) with suction filtration and dried in a vacuum. CaCO₃–oleic acid was evaluated according to the same procedure.

**Column packing**

A slurry of CaCO₃–PMAcO was packed into stainless steel columns (100 mm × 2.1 mm I.D., Chemco Scientific). The slurry of CaCO₃–PMAcO beads (0.7 g) in methanol (13 mL) was poured into a slurry reservoir (Chemco Scientific) connected to the stainless steel column. Methanol was flowed through the slurry reservoir using a HPLC pump (LC-6AD, SHIMADZU) for 30 min, followed by flowing methanol/water (50/50, v/v) for 60 min with a constant pressure of 35 MPa. CHEMOSORB 3-ODS-L was packed by the same procedure.

**Chromatographic analyses**

Chromatographic analyses were carried out using a Prominence-IC2030C (SHIMADZU) system equipped with a UV-Vis detector. All measurements were conducted at 25 °C with 1 μL injection volume. Elution behavior of all samples was monitored at 254 nm. All samples were dissolved in methanol and filtered through a 0.2 μm membrane filter. The pH of the 50 mM Na₂B₄O₇ buffer was adjusted to 10.8 with 100 mM NaOH aq. Chromatographic analyses were carried out using a Prominence-IC2030C (SHIMADZU) system equipped with a UV-Vis detector. All measurements were conducted at 25 °C with 1 μL injection volume. Elution behavior of all samples was monitored at 254 nm. All samples were dissolved in methanol and filtered through a 0.2 μm membrane filter. The pH of the 50 mM Na₂B₄O₇ buffer was adjusted to 10.8 with 100 mM NaOH aq.

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The following equations:

- Retention factor: \( R = \frac{(t_R - t_0)/t_0}{W_{S}} \), where \( t_0 \) and \( t_R \) are the retention times of solvent peak and the target analyte, respectively.

- Peak resolution: \( R_s = 2(t_{R2} - t_{R1})/(W_s + W_2) \), where \( t_R \) is the retention time (\( t_{R2} > t_{R1} \)), \( W_s \) is the peak width of the analyte, and \( R_s > 1.5 \) indicates complete separation.

- Theoretical plate: \( N = 16(t_R/W_s)^2 \)

- Peak asymmetry factor: \( A_s = b/a \), where \( a \) is the time from the leading edge of the peak to the peak midpoint, and \( b \) is the time from the peak midpoint to the tailing edge.

**Preparation of whole blood samples**

Porcine whole blood samples were pretreated using a protein precipitation method. To a 2 mL centrifuge tube, 200 μL of whole blood, 100 μL of basic drug mixture (50 μg mL⁻¹ imipramine, and 50 μg mL⁻¹ clomipramine in methanol) or 100 μL of methanol as a blank sample, and 700 μL of acetonitrile were added. These solutions were mixed using a vortex mixer for 10 minutes and centrifuged at 10 000 rpm for 10 minutes. The supernatant was filtered through a 0.2 μm membrane filter.

**Conflicts of interest**

There are no conflicts to declare.

**Notes and references**