Using cell membrane chromatography and HPLC-TOF/MS method for *in vivo* study of active components from roots of *Aconitum carmichaeli*

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Abstract: An offline two-dimensional system combining a rat cardiac muscle cell membrane chromatography time-of-flight mass spectrometry (CMC-TOF/MS) with a high performance liquid chromatography time-of-flight mass spectrometry (HPLC-TOF/MS) was established for investigating the parent components and metabolites in rat urine samples after administration of the roots of *Aconitum carmichaeli*. On the basis of the analysis of the first dimension, retention components of the urine sample were collected into 30 fractions (one fraction per minute). Then offline analysis of the second dimension was carried out. 31 compounds including 24 parent alkaloids and 10 potential metabolites were identified from the dosed rat urine, and then binding affinities of different compounds on cell membranes were compared and influences of some functional groups on activity were estimated with the semi-quantification and curve fitting method. As a result, binding affinities decreased along with the process of deacylation, debenzoylation and demethylation, which may be related to the alleviation of toxicity in the procedure of herb processing or metabolism. Moreover, some minor components in rat urine (Sanguine, 14-benzoylneoline, Deoxyaconitine, etc.) exerted relatively strong affinity on cell membranes are worth exploring. The results delivered by the system suggest that the CMC can be applied to *in vivo* study.

Keywords: cell membrane chromatography; high performance liquid chromatography; time-of-flight mass spectrometry; two-dimensional system; *Aconitum carmichaeli*

1 Introduction

Cell membrane chromatography (CMC), a biological chromatographic technique proposed by He’ group in 1996 [1, 2], has been successfully applied to studying the interactions between ligands and membrane receptors [3-5]. The results obtained from CMC assay are consistent with those from radioligand binding assay (RLA) significantly [6, 7]. CMC has been proved to be an effective tool for screening of active components targeting membrane receptors from traditional Chinese medicines (TCMs) [8-12]. CMC, as an approach to recognition of active components, combined with other separation and identification techniques as well as pharmacological verification trials, will provide another way to rapidly discover target components from complex system [13-15]. CMC has already been applied to investigate the TCMs. In fact, the study of complex biological samples is also count for much. Under the environment of enzymes and body fluid, biotransformation will take place once the drugs or TCMs are absorbed by the body, leading to the changes of their pharmacological activity. Some active components are metabolized into low active, even inactive products, while some components of no obvious pharmacological effect *in vitro* will become active. Biological samples are extremely complicated, but CMC makes it convenient to study the components of TCMs and their metabolites in them.

We took rat urine samples after gavage administration of the roots of *Aconitum carmichaeli* Debx. (Fuzi, in Chinese) as an example to make use of CMC. Fuzi is one of the most useful herbal medicines, which has been widely used as an important ingredient in TCMs for their anti-inflammatory, analgesic and cardiotonic effects [16, 17]. C₁₀-diterpenoid aconite alkaloids in Fuzi traditionally can be divided into four major types according to the substitute at the C₆ or C₁₀ position: diester-diterpenoid alkaloids (DDAs), monoester-diterpenoid alkaloids (MDAs), alkylolamine-diterpenoid alkaloids (ADAs) and other C₁₀-diterpenoid alkaloids [18-20]. Acetyl functions at the C₆ position in the parent alkaloids, DDAs, are easily hydrolyzed to produce MDAs, and then, the benzoyl or anisoyl functions at the C₁₀ position is hydrolyzed to produce the final degradation products, ADAs (Figure 1). The detailed mechanism of the metabolism of these alkaloids has scarcely been reported. In rats, aconitine might be metabolized by CYP3A and CYP1A1/2 isoforms in liver microsome, and O-demethyla-
tion and N-deethylation were the main metabolic pathways [21].

Figure 1 Skeleton of C21-diterpenoid aconite alkaloids in Fuzi.

It is not yet clear whether these alkaloids and metabolites exhibit high or low toxicity, or they have additional biochemical activities. It cannot be excluded that some minor compounds may also exert pharmacological effects and therefore play a crucial role in clinical application [22]. DDAs, mainly, aconitine, hypaconitine and mesaconitine, are well known active and toxic ingredients, while MDAs show a much lower toxicity than that of DDAs. However, the pharmacological studies showed that MDAs and ADAs have significant active actions that would be the desired effects [23,24]. So these structural analogs, alkaloids sharing the common skeleton, may have similar pharmacological effects. CMC can be used for identification of active compounds from biological samples which contain both herbs and metabolites.

In this paper, we have successfully established an offline two-dimensional (2D) system, which combined a rat cardiac muscle/CMC- time-of-flight mass spectrometry (TOF/MS) with a high performance liquid chromatography time-of-flight mass spectrometry (HPLC-TOF/MS). This system was applied to investigate the parent drugs and metabolites in rat urine samples after administration of Fuzi extract. According to the total ions chromatogram (TIC) of CMC-TOF/MS system, components of urine retained by CMC were collected into 30 fractions. Then these samples were analyzed by HPLC-TOF/MS system. On the basis of semi-quantification of these samples, retention behaviors of each compound on CMC were simulated. Finally, relationship between the structure of compounds and affinity reflected by CMC was evaluated. To our knowledge, it is the first time that CMC was applied to in vivo studies.

2 Experimental

2.1 Chemicals and materials

The herbal medicine Fuzi was purchased from Dekang Drug Store (Shanghai, China) and authenticated by the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University (Shanghai, China). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany) and formic acid was obtained from Fluka (Buchs, Switzerland). Pure water was purchased from Hangzhou Wahaha Group Co., Ltd. (Shanghai, China). Macroporous silica gel (5 μm, 120 Å) and empty column (2.1 mm × 30 mm) were obtained from the Welch Materials Inc. (Shanghai, China). All other reagents and solvents were of analytical grade.

2.2 Preparation of Fuzi samples

Extracting solution of Fuzi was prepared using reflux water extraction method. 50 g of sample was placed into a 1000 mL round bottomed flask. 500 mL pure water (10 times their total weight) was added and then decocted to boil keeping for 2 h. The extracted solution was filtered through four layers gauze. To the filtrate, 1000 mL ethanol was added, at a filtrate/ethanol ratio of 1 : 2 (v/v) to precipitate the polysaccharide and protein. This sample was kept overnight at 4 °C and then filtered through two layers filter paper, and the filtrate was evaporated to dryness under reduced pressure with a rotary evaporator at 60 °C. The residue was dissolved in water to obtain an oral solution of Fuzi with a concentration of 5 g/mL (crude drugs). From the above oral solution, 0.2 mL was diluted and made up to 25 mL by addition of water. The diluted sample solution (FZ) was filtered through a 0.22 μm membrane before use. An aliquot of 5 μL of each sample was injected into the HPLC system.

2.3 Collection of urine samples

Four male Sprague-Dawley rats (200 - 250 g body weight) were provided by the Experimental Animal Center, Second Military Medical University (Shanghai, China). The animals were fasted for 12 h with free access to water before the experiments. Then they were housed in separate metabolic cages with free access to water. Blank urine (U0A, U0B, U0C and U0D) of each rat was collected during a 12 h collection period, after which the rats were given regular food and water for a week. Then the rats were fasted for 12 h with free access to water, and administered Fuzi extract orally at a single dose of 50 g/kg. Post dose urine (U1A, U1B, U1C and U1D) was collected from each rat by the 12 h urine collection procedure. The experiment was conducted in accordance with the National Institute of Health guidelines regarding the principles of animal care (2004).
2.4 Pretreatment of urine samples

All urine samples were centrifuged at 10800 rpm for 10 min and the supernatant obtained was stored at −20 °C until additional extraction and analysis. 100 µL of each blank urine sample was mixed into a 400 µL mixture of blank urine (U0M), and the same steps were conducted to make mixed dosed urine (UM). A volume of 800 µL of methanol was added to the urine sample (U0M and UM, separately), and the mixture was vortex-mixed for 2 min and then centrifuged at 10800 rpm for 10 min. Then the supernatant was evaporated to dryness under a stream of N2 gas. The residue and the mixture was vortex-mixed for 2 min and then diluted sample was injected into the HPLC system.

2.5 Rat cardiac muscle/CMC module

Male Sprague-Dawley rats (200 - 250 g body weight) were from the Experimental Animal Center, Second Military Medical University (Shanghai, China). The rat cardiac muscle cell membrane was prepared as described [13, 25, 26]. After cervical dislocation, the heart of rat was harvested immediately and the heart atrium was removed. The cardiac muscular tissue was immerged into pre-cooled normal saline to wash out the bloodiness. After cutting into pieces in 10 mL pre-cooled normal saline, the tissue was put into a glass homogenizer for homogenizing. The homogenate was then centrifuged at 3000 × g for 10 min. 10 mL Tris-HCl (pH 7.4, 50 mM) was added to the pellet to produce a cell suspension, which was ruptured by sonication for 30 min immediately. The resulting suspension was vortex-mixed and clarified by centrifugation at 1000 × g for 10 min. The pellet was discarded, and the suspension was recovered by centrifugation at 12000 × g for 20 min. The pellet was resuspended in 10 mL phosphate buffer saline (pH 7.4, 50 mM) and cell membranes were collected by centrifugation at 12000 × g for 20 min. The pellet was resuspended in 5 mL distilled water. All the above operations were conducted under conditions at 4 °C.

Cell membrane stationary phase (CMSP) was prepared according to literature [1, 2]. Briefly, 0.15 g silica was activated at 120 °C for 30 min and used as a carrier. It was then homogenized with the cell membrane suspension, i.e., the mixture was slowly added to it under a vacuum and with agitation at 4 °C. The mixture obtained was packed into the column using a wet method to yield a CMSP column (2.1 mm × 30 mm, 5 µm). This CMC module was validated by negative and positive controls. They were solutions of salvianolic acid B (SAB), furosemide (FR), dobutamine (DB) and phenotolamine (PT), with concentration of 0.5 mg/mL, which were prepared separately by dissolving in normal saline. The life-span of this CMC column was about 5 days under a continuous usage.

2.6 CMC-TOF/MS system

The analysis of urine sample on cell membrane chromatography system was performed on an Agilent 1100 series HPLC system (Agilent Corp., Germany) consisting of a quaternary solvent delivery system, a thermostatically controlled column apartment and a diode array detector. Chromatographic separation was carried out on a rat cardiac muscle/CMC column (2.1 mm × 30 mm, 5 µm) at 37 °C. The mobile phase was 10 mM ammonium acetate solution at a flow rate of 0.2 mL/min. Then it totally flowed into the mass spectrometer source. The DAD wavelength was 230 nm for urine sample, 330 nm for FR and 280 nm for others.

Detection was performed by an Agilent orthogonal TOF/MS (Agilent Corp., USA) equipped with an ESI interface. The TOF/MS analysis was performed using full scan mode and mass range was set at m/z 100 - 1000 in positive ion mode. The conditions of ESI source were as follows: drying gas (N2) flow rate, 9 L/min; drying gas temperature, 350 °C; Nebulizer, 35 psig; capillary voltage, 4000 V; fragmentor voltage, 180 V; skimmer voltage, 60 V; octopole RF, 250 V. Tuning mix (G1969-85000, Agilent Corp., USA) was used for lock mass calibration in the assay. Eluate of the dosed urine (UM) on CMC system within 30 min was collected as one fraction per minute. In all, 30 fractions were collected and labeled as R1 - R30.

2.7 HPLC-TOF/MS system

The analysis of samples (FZ, U0M, UM, R1 - R30) was performed on an Agilent 1290 series HPLC system (Agilent Corp., USA) consisting of a binary solvent delivery system and a thermostatically controlled column apartment. Chromatographic separation was carried out on an Agilent ZORBAX SB-C18 column (4.6 mm × 150 mm, 5 µm) at 25 °C. The mobile phase consisted of 0.1% aqueous formic acid (v/v) (A) and acetonitrile (B), using a gradient elution of 5% - 25% B at 0 - 25 min, 25% - 45% B at 25 - 35 min, 45% - 45% B at 35 - 40 min. Afterwards, the column was rinsed for 5 min with 95% B, the B content lowered to 5% over 2 min, and the column was re-equilibrated for 10 min. The flow rate was kept at 0.8 mL/min, and a post column split was used to maintain a flow rate of 0.3 mL/min into the mass spectrometer source to obtain good nebulization efficiency.

Detection was performed by an Agilent 6538 UHD Accurate-Mass Q-TOF/MS (Agilent Corp., USA) equipped with an ESI interface. The TOF/MS analysis was performed using full scan mode and mass range was set at m/z 100 - 1000 in positive ion mode. The conditions of ESI source were as follows: drying gas (N2) flow rate, 11 L/min; drying gas temperature, 350 °C; Nebulizer, 45 psig; capillary voltage, 4000 V; fragmentor voltage, dynamic adjustment from 120 to 385 V; skimmer voltage, 45 V; octopole RF, 250 V. All the data were processed by
Agilent MassHunter Software Ver. B.02.00. Tuning mix (G1969-85000, Agilent Corp., USA) was used for lock mass calibration in our assay.

Parent components and metabolites were identified according to the analysis of FZ, U0M and U1M. Then each compound was extracted from R1-R6 using the extract ion chromatography (EIC) mode.

3 Results and discussion

3.1 Validation of CMC module

Salvianolic acid B (antioxidants) and furosemide (diuretic) were used as negative controls and dobutamine (β1 adrenoceptor agonists) and phentolamine (α adrenoreceptor blocker) were used as positive controls to validate the specification of this CMC module. Their retention behavior was compared and the results are shown in Figure 2. SAB and FR could not be retained on rat cardiac muscle/CMC module. However, DB and PT could be retained obviously, and their retention time was 5.77 min and 10.17 min, respectively. It indicated that this CMC module could selectively retain the components that act on α adrenoreceptor or β adrenoceptor. This CMC module could be used for screening such components from TCM preliminarily.

![Figure 2 Chromatograms of the negative controls and positive controls on the rat cardiac muscle/CMC module. A, SAB; B, FR; C, DB; D, PT.](http://www.j-pharm-anal.com)

3.2 Collection of fractions

At present, various two-dimensional systems have been established, which combined CMC as the first dimension and HPLC-MS or GC-MS as the second dimension [10-15]. On the basis of the analysis of the first dimension, retention components were collected into many fractions. Then offline or online analysis of the second dimension was carried out. UV detectors are often used as the detection methods of the CMC systems. However, they may not be suitable for the biological samples for the concentrations of components are too low or some compounds are of weak UV absorption. It is difficult to detect the DDAs in vivo by UV detector because of the low level of concentration and rapid biodegradation [23], and the degradation products show weaker UV absorbance than the parent alkaloids. However, specific detection by TOF/MS techniques could provide determination of these alkaloids.

We used both UV and TOF/MS to determine the urine samples with the purpose of collecting all the retained components. The ultraviolet spectrum and the total ions chromatogram (TIC) of the dosed urine (U1M) are shown in Figure 3A and 3B respectively. Compared with the ultraviolet spectrum, the TIC indicated more information due to the high sensitivity of the TOF/MS detector. There were many compounds retained by the CMC within 30 min which...
were demonstrated in the TIC, while it was almost a straight line in the ultraviolet spectrum after 5 min. If we only collected and analyzed the components within 5 min, we might lose much information, because the binding affinity shows a positive correlation with the retention time according to the principle of cell membrane chromatography. With the purpose of comprehensive two-dimensional chromatography, the more fractions collected within 30 min, the more information would be obtained. Fractions were collected each minute, totally 30 fractions. Taking into account the dead volume of the system and the time delayed of the eluent from detection to collection, collection was delayed for 0.5 min, i.e. fraction 0.5 min to 1.5 min was collected and labeled as R₁, fraction 1.5 min to 2.5 min was collected and labeled as R₂, and so on.

![Figure 3](http://www.j-pharm-anal.com)

**Figure 3** The ultraviolet spectrum and the TIC of the dosed urine (U₁₄) on the cell membrane chromatography system. A, HPLC-DAD spectrum of U₁₄ monitored at 230 nm; B, HPLC-TOF/MS TIC of U₁₄.

### 3.3 Identification of components

The typical base peak chromatograms (BPCs) of the Fuzi extract (FZ), the dosed urine (U₁₄) and the blank urine (U₀₄) are shown in Figure 4A, 4B and 4C respectively. According to our previous work [27], 24 parent components and 10 potential metabolites were identified. The identification results are shown in Table 1. This provides a foundation for further identification of active compounds from biological samples.

### 3.4 Simulation of elution curves

A total of 1020 (34 × 30) extracted ion chromatograms (EICs) were obtained by extracting 34 compounds from 30 collected fractions (R₁₋R₃₀). According to the Q-TOF semi-quantitative analysis of the fractions, the peak areas of target compounds in EICs were recorded. Then 34 scatter plots, with normalized peak area on the vertical axis and serial number of collected fraction on the horizontal axis, were finished. Actually, the serial number of fraction represents the retention time of fraction on CMC column, i.e., the retention time of corresponding constituent, so the scatter graph of a compound is the simulated elution curve of it on the CMC system.

Take the component C₁₆ (Benzoylaconitine, [M + H]+ m/z 604.3122) as an example. EICs were obtained by extracting m/z 604.3122 (± 5 ppm) from 30 collected fractions. Consequently, the compound C₁₆ can be detected in the collected fractions R₁₋R₁₅, and had the maximum peak area in fraction R₁₀ (Figure 5A). It cannot be detected in fractions R₁₋R₅ and R₁₆₋R₃₀, so the peak areas of C₁₆ were regarded as 0 in them. Then the simulated elution curve of C₁₆ on the CMC system was obtained by plotting the normalized peak area versus serial number of collected fraction (Figure 5B). Table 2 gives a concise and intuitive description of retention behavior of the 34 compounds on the CMC system. Peaks on CMC column were broadened from Start time to End time, and most of them were tailing peaks.
Figure 4  The typical base peak chromatograms (BPCs) of the Fuzi extract (FZ), the dosed urine (U1wa) and the blank urine (U0wa).  
A, BPC of FZ; B, BPC of U1wa; C, BPC of U0wa.

Figure 5  Process of simulating the scatter plot of C16 (Benzoylaconitine) on the CMC system.  
A, EICs of U1-U6 by extracting m/z 604.3122 (± 5 ppm); B, simulated elution curve of C16.
Table 1 Identification of the parent compounds and the potential metabolites from the dosed rat urine

| ID  | RT (min) | [M+H]+ m/z | Formula | Identification                     |
|-----|----------|-------------|---------|------------------------------------|
| C1  | 4.862    | 394.2593    | C14H26NO4 | Karakolidine                      |
| C2  | 5.495    | 394.2593    | C14H26NO4 | Chuanfumine                       |
| C3  | 8.093    | 424.2699    | C16H28NO4 | Senbusine                          |
| C4  | 8.442    | 486.2703    | C18H30NO4 | Mesaconine                         |
| C5  | 8.861    | 364.2488    | C14H24NO3 | 16-β-hydroxyacrylopetaline       |
| C6  | 9.228    | 424.2699    | C16H28NO4 | Senbusine                          |
| C7  | 9.861    | 408.2750    | C14H24NO3 | Isotalatizidine                   |
| C8  | 10.898   | 358.2382    | C12H20NO3 | Songorine                          |
| C9  | 12.787   | 454.2850    | C14H26NO4 | Fuziline                           |
| C10 | 13.846   | 438.2856    | C14H24NO3 | NeoLine                            |
| C11 | 16.443   | 422.2906    | C12H20NO3 | Talatizamine                       |
| C12 | 18.976   | 424.2648    | C14H26NO4 | Chasmanine                         |
| C13 | 21.104   | 464.3012    | C16H30NO4 | 14-acetyltaletatizidine            |
| C14 | 21.683   | 606.2908    | C18H28NO5 | 14-benzyl-10-OH-mesaconine         |
| C15 | 26.562   | 590.2965    | C16H28NO4 | Benzoylmesaconine                  |
| C16 | 28.909   | 604.3122    | C18H30NO4 | Benzoylaconitine                   |
| C17 | 30.077   | 574.3016    | C14H24NO3 | Benzoylpyaconitine                 |
| C18 | 31.376   | 558.3067    | C14H22NO3 | 14-benzyl-3,13-deoxyaconin         |
| C19 | 31.594   | 646.3227    | C16H30NO4 | Aconitine                          |
| C20 | 31.856   | 588.3173    | C14H24NO3 | Benzoyldeoxyaconitine             |
| C21 | 32.991   | 542.3118    | C12H22NO3 | 14-benzoylneoline                  |
| C22 | 35.131   | 616.3122    | C14H26NO4 | Hypaconitine                       |
| C23 | 35.633   | 600.3173    | C16H30NO4 | 13-deoxyhypaconitine              |
| C24 | 37.172   | 630.3278    | C18H32NO4 | Deoxyaconitine                     |
| M1  | 6.237    | 424.2699    | C14H26NO4 | Demethylated metabolite of neoline |
| M2  | 6.816    | 394.2593    | C14H24NO3 | Demethylated metabolite of isotalatizidine |
| M3  | 8.541    | 394.2593    | C14H24NO3 | Demethylated metabolite of isotalatizidine |
| M4  | 8.999    | 336.2175    | C12H20NO2 | Unknown                            |
| M5  | 12.470   | 424.2699    | C14H26NO4 | Isomer of demethylated metabolite of neoline |
| M6  | 13.267   | 408.2750    | C14H26NO4 | Demethylated metabolite of talatizamine |
| M7  | 15.821   | 450.2856    | C14H24NO3 | Demethylated metabolite of 14-acetyltalatizidine |
| M8  | 28.418   | 574.3016    | C14H22NO3 | Demethylated metabolite of benzoyldeoxyaconitine |
| M9  | 32.195   | 588.2809    | C14H24NO3 | Unknown                            |
| M10 | 35.142   | 618.2914    | C14H24NO3 | Demethylated metabolite of mesaconitine |

Table 2 Retention behavior of the 34 compounds on the CMC system

| ID  | Start (min) | RT_{sec}* | End (min) | ID  | Start (min) | RT_{sec}* | End (min) |
|-----|-------------|-----------|-----------|-----|-------------|-----------|-----------|
| C1  | 4           | 5         | 30        | C18 | 21          | 23        | 25        |
| C2  | 10          | 12        | 27        | C19 | 14          | 15        | 21        |
| C3  | 6           | 7         | 20        | C20 | 17          | 18        | 21        |
| C4  | 4           | 5         | 11        | C21 | 24          | 25        | 30        |
| C5  | 13          | 17        | 30        | C22 | 19          | 22        | 27        |
| C6  | 9           | 10        | 15        | C23 | 23          | 24        | 26        |
| C7  | 11          | 14        | 23        | C24 | 22          | 25        | 30        |
| C8  | 25          | 25        | 30        | M1  | 3           | 5         | 14        |
| C9  | 7           | 8         | 25        | M2  | 6           | 7         | 25        |
| C10 | 9           | 11        | 24        | M3  | 9           | 10        | 13        |
| C11 | 22          | 24        | 30        | M4  | 6           | 7         | 18        |
| C12 | 22          | 23        | 25        | M5  | 7           | 8         | 15        |
| C13 | 22          | 24        | 30        | M6  | 17          | 19        | 30        |
| C14 | 4           | 5         | 8         | M7  | 14          | 16        | 23        |
| C15 | 6           | 7         | 16        | M8  | 10          | 12        | 14        |
| C16 | 8           | 10        | 15        | M9  | 11          | 13        | 14        |
| C17 | 14          | 16        | 26        | M10 | 20          | 21        | 25        |

* Retention time was supposed to be the number of fraction which had the maximum peak area.
3.5 Evaluation of structure-activity relationship

According to the principle of cell membrane chromatography, longer retention time indicates stronger binding affinity. As shown in Table 2, different components had different retention time. For instance, the components C8, C21 and C24 had the maximum retention time of about 25 min, while the C1, C4, C14 and M1 had the minimum retention time of about 5 min. These differences may attribute to the diversity of their structures. So the relationship between the structure of compounds and binding affinity reflected by CMC—in other words, pharmacology activity—was evaluated.

Figure 6 Simulated elution curves of certain compounds on the CMC system. A, C19 (Aconitine); B, C16 (Benzoylaconitine); C, C22 (Hypaconitine); D, C17 (Benzoylhyponaconitine); E, C15 (Benzoylmesaconine); F, C4 (Mesaconine); G, C7 (Isotalatizidine); H, M2; I, M3; J, C10 (Neoline); K, M1; L, M5.

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3.5.1 Effect of deacylation and debenzylation

When the DDAs became MDAs, i.e., acetyl functions at the Cα position were hydrolyzed, the retention time on CMC system reduced. For example, compound C19 (Aconitine, RT = 16 min) (Figure 6A). Its hydrolysis product C16 (Benzoyl-aconitine) is a MDA, which had the retention time of 10 min (Figure 6B). That means the affinity decreased with the deacylation. It is the same with C22 (Hypaconitine, RT = 22 min) and C17 (Benzoylhypaconitine, RT = 16 min) (Figure 6C and 6D). In a similar way, when the benzoyl functions at the Cα position of MDAs were hydrolyzed to produce the ADAs, the binding affinity weakened. For example, when Cl5 (Benzoylmesaconine) is broken down to form C4 (Mesaconine), the retention time on CMC system reduced from 7 to 5 min (Figure 6E and 6F). As suggested above, the affinity decreased with the deacylation and debenzylation, which could partly explain the fact that MDAs show lower toxicity than the parent DDAs.

3.5.2 Effect of demethylation

Simulated elution curves of C7 (Isotatalatizidine), M2 and M3 are shown in Figure 6G, 6H and 6I respectively. M2 and M3 were considered to be the probable metabolites of C7 because the methyl functions at the Cα or Cβ position of the parent compounds might degrade to form the metabolites. The retention time of C7 was 14 min, which was longer than that of M2 (7 min) and M3 (10 min). Similarly, M1 and M5 were considered to be the potential metabolites of Cl0 (Neoline), and their retention time was 5 min, 8 min and 11 min, respectively (Figure 6K, 6L and 6J). M6 was supposed to be the potential metabolite of C11 (Talatizamine) and their retention time was 19 min and 24 min, respectively. All the retention time of the parent compounds was longer than that of the corresponding demethylation products. To sum up, the affinity decreased as the process of demethylation. All the above results are listed in Table 3.

4 Conclusion

The establishment of an off-line two-dimensionalsystem, which combined a rat cardiac muscle/CMC-TOF/MS with an HPLC-TOF/MS, is characterized by high sensitivity and convenience, and can be used in the case of investigating the parent compounds and metabolites in urine samples. By using the semi-quantification and curve fitting method, binding affinities of different compounds on cell membranes were compared and influence of some functional groups on activity were estimated. It showed that some minor components, such as C8 (Songorine), C21 (14-benzyloxyneoline) and C24 (Deoxyaconitine), exerted stronger affinities than the known aconitine and hypaconitine. Further research is needed to see whether these compounds can exhibit activity or toxicity and, therefore, play a key role in Fuzi. This work also demonstrates, for the first time, that the cell membrane chromatography can be applied to in vivo study.

Table 3 Relationship between structures and retention time of certain compounds

| Group ID | RT in min | Differences in structure |
|----------|-----------|-------------------------|
| 1        | C19       | Cα=O-acetyl             |
|          | C16       | Cα=OH                   |
| 2        | C22       | Cα=O-acetyl             |
|          | C17       | Cα=OH                   |
| 3        | C15       | Cα=O-benzoyl            |
|          | C4        | Cα=OH                   |
| 4        | C7        | Cα=O-methyl             |
|          | M2        | Cα=OH                   |
|          | M3        | Cα=O-methyl             |
| 5        | Cl0       | Cα=O-methyl             |
|          | M1        | Cα=OH                   |
|          | M5        | Cα=O-methyl             |
| 6        | C11       | Cα=O-methyl             |
|          | M6        | Cα=OH                   |

* Retention time was estimated based on the simulated elution curves.

The same parts of structure are not listed in each group.

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