Rapid measurement of anticoagulant rodenticides in human blood and urine using online turbulent flow chromatography coupled with liquid chromatography–tandem mass spectrometry

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ABSTRACT

Anticoagulant rodenticides (ARs) are widely used to control rodents. A method based on online turbulent flow chromatography (TFC) combined with LC-MS/MS has been established for rapid quantitative determination of eight ARs in human blood and urine. This method, which does not require time-consuming pre-processing steps, renders it especially suited for use in emergency poisoning cases. Sample preparation, including extraction, centrifugation, and filtration, was followed by online clean-up using TFC. The total run-time was within 13.5 min, including online purification, chromatographic separation, and re-equilibration of the TFC system. The parameters for sample extraction, purification, separation, and detection in this study were optimized separately. The linear regression coefficients of matrix-matched calibration standard curves established for quantification were greater than 0.9976. The limit of quantification (LOQ) for the method were found to be 0.3–3.0 ng/mL in human blood and 0.06–0.6 ng/mL in urine. The recoveries of spiked target compounds at different concentrations in human blood and urine were 91.8–111.9% and 86.9–105.3%, respectively. Inter- and intra-day precision values were both less than 12.5%, and the matrix effects of human blood and urine samples for ARs were 75.3–108.6% and 102.7–130.0%, respectively. This method had successfully applied to the emergency detection of ARs in biological samples of poisoned patients.

KEYWORDS

anticoagulant rodenticides, poisoning, human blood, urine, turbulent flow chromatography

INTRODUCTION

Anticoagulant rodenticides (ARs) are used to control rodents. They act by restricting the synthesis of the plasma protein prothrombin in rodent livers, increasing the brittleness of capillary walls and causing visceral bleeding. This bleeding does not clot, and thus the affected mouse dies after several days [1].

Examples of frequently used ARs are bromadiolone, warfarin, coumatetralyl, coumachlor, chlorophacinone, difaphacinone, difethialone, and brodifacoum. ARs are divided into two categories based on their chemical structure: 4-hydroxycoumarin-containing ARs and indandione-containing ARs. Diphacinone and chlorophacinone belong to the second, while all the other ARs listed above belong to the first category.
ARs function by inhibiting the vitamin K epoxide reductase enzyme, causing vitamin K deficiency and an inactivation of coagulation factors (II, VII, IX, and X) [2]. The common clinical manifestations of AR poisoning are coagulopathy, bleeding, and death. Vitamin K1 is typically used clinically as an antidote to treat patients poisoned by ARs, subsequent to accidental or intended exposures (i.e., suicide attempts, or poisoning of others [3]. In this context, a rapid analytical method would be invaluable to confirm a diagnosis of AR poisoning in human patients.

Many methods have been previously used for detection of ARs, such as thin-layer chromatography (TLC) [4], capillary electrophoresis (CE) [5], high-performance liquid chromatography (HPLC) with ultraviolet and fluorescence detection [6, 7], gas chromatography-mass spectrometry (GC-MS) [8, 9], ion chromatography-mass chromatography (IC-MS) [10], and liquid chromatography-mass spectrometry (LC-MS) in SIM mode [11]. However, these methods all suffer from poor sensitivity and selectivity. Recently, however, the outstanding selectivity, speed, and sensitivity, liquid chromatography combined with electrospray ionization tandem mass spectrometry based on a triple quadrupole configuration has been used for the quantification of ARs in several complex matrices, such as blood [12, 13], urine [2], animal live [14, 15], human hair [16, 17], food [18, 19], sludge [20], and wastewater [21].

Various clean-up methods have been described for the isolation of ARs, such as liquid-liquid extraction [13], dispersive liquid-liquid microextraction [2], and solid-phase extraction (both standard [21] and QuEChERS (quick, easy, cheap, effective, rugged, safe) methods) [14, 18] and gel permeation chromatography [19]. Typically, these sample isolation and preparation procedures have been performed offline, resulting in labor- and time-intensive processes. In some cases, sample purification has been automated using turbulent flow chromatography (TFC), and this approach has offered a potential remedy for the disadvantages mentioned above [22].

TFC enables chromatographic adsorption and size exclusion to be performed simultaneously, and is therefore especially suitable for processing samples containing low molecular-weight targets and high molecular-weight interferents [23]. TFC has been widely used to locate the analyte preparation step online with the HPLC step, enabling the separation of analytes prior to their introduction into a tandem mass spectrometer. Online TFC sample preparation methods are faster, simpler, and less labor-intensive than traditional, offline sample preparation methods, and have been successfully used (without additional sample cleanup) in the quantification of steroid hormones [24, 25], polyfluoroalkyl compounds [26, 27], mushroom toxins [28], nicotine [29], and clinical drugs [30] in human blood or urine samples.

Here, we present a fast, quantitative method for the analysis of eight ARs in human blood and urine based on a method that combines automated TFC with LC-MS/MS. The parameters for sample extraction, purification, separation, and detection were systematically optimized and the practicality of our method was determined by demonstrating its applicability to the detection of ARs in human blood and urine samples. We expect that this rapid, highly sensitive and operationally simple method for the quantification of ARs will have numerous important clinical and forensic applications.

EXPERIMENTAL

Reagents and materials

Water was purified using a Milli-Q system (Merck, Germany). Acetonitrile (ACN), and methanol (MeOH) were obtained from Merck. Isopropanol, acetone, formic acid (FA), and ammonium acetate (AA) were purchased from CNW (Germany). All of reagents and solvents were HPLC grades. Blank blood samples were provided by a local blood bank for scientific purposes, blank urine samples were collected from healthy volunteers in the authors’ laboratory.

Apparatus

A Thermo Scientific Transcend TLX-1 system (Thermo Fisher Scientific, Franklin, MA) controlled by Aria 1.6.2 software was used for online TFC cleanup and LC separation. The entire system was equipped with a PAL autosampler (CTC Analytics, Switzerland) and two Dionex U3000 RS pumps (Thermo Fisher Scientific, Germany), which were operated separately for loading and eluting samples. Ionic detections were performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, Santa Clara, CA, USA) with a heated electrospray ionization (HESI) ion source. Data acquisition and analysis were conducted with Xcalibur 3.0 software. The Turboflow Cyclone column (50 mm × 0.5 mm; 60 µm particle size; 60 Å pore size) that was used for online TFC cleanup was supplied by Thermo Fisher Scientific. For analytical measurements, a Hypersil Gold C18 column (100 × 2.1 mm; 1.9 µm particle diameter; Thermo Fisher Scientific) was used.

Sample treatment

A urine sample (0.50 mL) was treated with MeOH (0.50 mL) and subjected to vortex mixing for 1 min. The mixed sample was then centrifuged at 15,000 rpm (20,627 g) for 3 min. The resulting supernatant was then subject to online TFC cleanup, followed by LC-MS/MS analysis.

A whole blood sample (0.10 mL) was treated with ACN (0.90 mL) and subjected to vortex mixing for 1 min followed by sonication extraction for 10 min. The extract was then centrifuged at 15,000 rpm for 3 min. The resulting supernatant was then subject to online TFC cleanup, followed by LC-MS/MS analysis.

TFC method

Ten µL of the extract prepared according to the procedure described above was injected into a Turboflow Cyclone column. A MeOH/H2O (10:90, v/v) solvent mixture was used as the mobile phase and the flow rate was adjusted to 1.5 mL/min. At the same time, an analytical column was
equilibrated using the mobile phase of the eluting pump and at a flow rate of 0.30 mL/min. The analytes were delivered to the analytical column via a 200 μL solvent loop, which was pre-filled with ACN/H2O (70:30, v/v) through a six-port valve switching system. The elution flow-rate into the analytical column was set at 0.12 mL/min. Rinsing of the TFC column was performed using ACN and a mixture of ACN/isopropanol/acetone (40:40:20, v/v). The mobile phase of the analytical column contained a 4 mM AA aqueous solution and MeOH. The gradient of the TFC conditions used for sample purification, and for the subsequent separation of target compounds on the analytical column, is listed in Table 1 and the configuration system is shown in Fig. 1. The total time of the analysis, including the extraction, elution, and rinsing phases, was 13.5 min.

**Table 1.** The gradient program for TFC method coupled to HPLC.

| Pump 1 | Load pump | Pump 2 | Elute pump |
|--------|-----------|--------|------------|
| Injection volume | 10 μL | Analytical column | Hypersil GOLD (1.9 μm, 100 × 2.1 mm) |
| Purification column | Cyclone (0.5 × 50 mm), Thermo Scientific |
| Solvent A | ACT:IPA:ACN (1:2:2) |
| Solvent B | MeOH |
| Solvent C | Water |
| Solvent D | Acetonitrile |

| Step | Start | Sec | Flow | Grad | %A | %B | %C | %D | Tee | Loop | Comments | Flow | Grad | %A | %B |
|------|-------|-----|------|------|----|----|----|----|-----|------|----------|------|------|----|----|
| 1    | 0.0   | 60  | 1.5  | Step | 10 | 90 |     |     | Out | Loading | 0.3 | Step | 90 | 10 |
| 2    | 1.0   | 60  | 0.12 | Step | 30 | 70 |     |     | T   | In | Transferring | 0.2 | Step | 90 | 10 |
| 3    | 2.0   | 180 | 1.5  | Step | 100 |     |     |     | Out | Washing | 0.3 | Ramp | 40 | 60 |
| 4    | 5.0   | 60  | 1.5  | Step | 100 |     |     |     | Out | Washing | 0.3 | Ramp | 30 | 70 |
| 5    | 6.0   | 120 | 1.5  | Step | 100 |     |     |     | In  | Washing | 0.3 | Ramp | 10 | 90 |
| 6    | 8.0   | 120 | 1.5  | Step | 100 |     |     |     | Out | Filling the loop | 0.3 | Step | 10 | 90 |
| 7    | 10.0  | 12  | 1.5  | Step | 100 |     |     |     | Out | Equilibrating | 0.3 | Ramp | 90 | 10 |
| 8    | 10.2  | 138 | 1.5  | Step | 100 |     |     |     | Out | Equilibrating | 0.3 | Step | 90 | 10 |

Fig. 1. TFC on-line cleanup system modes
The data were acquired in selected reaction monitoring (SRM) mode and in negative electrospray ionization (ESI−) was used with the spray voltage set at −2.5 kV and the vaporizer temperature at 300 °C. Nitrogen was used as the sheath gas and as the auxiliary gas, with pressures of 45 and 15 arb, respectively. Argon was used as the collision gas, at a pressure of 10 psi. The dwell time was adjusted to 0.8 s. Table 2 details the MS parameters used for quantification of the eight ARs.

**Calibration**

AR standards (bromadiolone, warfarin, coumatetralyl, coumachlor, chlorophacinone, diphacinone, difethialone, and brodifacoum) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Single stock standards of 10.0 μg/mL of each AR were prepared in methanol and stored at −20 °C. Mixed solutions of working standards were prepared by diluting the standard stock solution with methanol immediately prior to use. Matrix-matched calibration standards were prepared by the addition of known amounts of stock standard solutions to appropriate volumes of AR-free blood or urine samples. The matrix-matched calibration standards were freshly prepared before use.

### RESULTS AND DISCUSSION

**Optimization of conditions for mass spectrometry**

To optimize MS parameters for the ARs, standard solutions (1 μg/mL) were continuously injected into the spectrometer via a flow injection pump at a flow rate of 10 μL/min. Deprotonated ions [M−H]− at m/z 291.0, 307.0, 339.0, 341.0, 373.0, 323.0, 327.0, and 537.0 for coumatetralyl, warfarin, diphacinone, coumachlor, chlorophacinone, brodifacoum, bromadiolone, and difethialone, respectively, were found by using Q1 full-scan mode. The flow injection of standard solutions (10 μL/min) was performed with the instrument set to product-ion scan mode, and the S-lens voltages and collision energies of different target compounds were also thus optimized. Two transitions per compound were selected for the SRM method, to comply with the EU requirements for confirmatory analysis (Commission Decision 2002/657/EC). The peak area of the most intense transition was used for quantitation, while the less intense transition was used to confirm the identity of each analyte.

The optimized retention time, precursor ions, and product ions for each analyte, as well as the S-lens voltages and collision energies used, are reported in Table 2.

**Table 2. Detection parameters of anticoagulant rodenticides**

| Analyte | RT (min) | Scan mode | Precursor ion (m/z) | Product ion (m/z) | S-lens (V) | Collison energy (eV) |
|---------|---------|-----------|---------------------|-------------------|------------|---------------------|
| Coumatetralyl | 5.90    | ESI−      | 291.0               | 141.0/247.0       | 120        | 28/23               |
| Warfarin | 6.00    | ESI−      | 307.0               | 161.0/250.0       | 120        | 21/23               |
| Coumachlor | 6.66   | ESI−      | 314.0               | 284.0/161.0       | 120        | 26/22               |
| Diphacinone | 6.86   | ESI−      | 339.0               | 167.0/116.0       | 120        | 25/48               |
| Chlorophacinone | 7.51  | ESI−      | 373.0               | 201.0/145.0       | 120        | 22/22               |
| Bromadiolone | 8.00   | ESI−      | 527.0               | 250.0/181.0       | 120        | 38/37               |
| Brodifacoum | 8.68   | ESI−      | 523.0               | 187.0/135.0       | 120        | 39/37               |
| Difethialone | 8.73   | ESI−      | 537.0               | 79.0/151.0        | 120        | 47/39               |

*Quantum ion.

As shown in Fig. 3, significantly lower peak responses were obtained for chlorophacinone, bromadiolone, brodifacoum, and difethialone in spiked urine samples that had been diluted with Milli-Q water but not subjected to any

**Optimization of conditions for chromatography**

A Hypersil Gold C18 (100 × 2.1 mm, 1.9 μm) column was successfully used for the chromatographic separation of ARs. Four mobile phases were compared for their effect on the retention characteristics, chromatographic peak shape and sensitivity of detection of analytes. These four phases were three aqueous solutions of AA (2, 4 and 10 mM, denoted A2, A4 and A10, respectively), and one solution of MeOH (denoted B). As illustrated in Fig. S1, similar strong peak responses for the eight ARs were achieved when the mobile phase was either A2 or A4, with the response obtained by use of A10 being much weaker. In addition, it was found that the inclusion of 0.2% FA in A4 significantly reduced the peak responses of five ARs (coumatetralyl, warfarin, coumachlor, diphacinone, and chlorophacinone). Based on these results, 4 mM AA (A) and MeOH (B) were used as the mobile phases, and these afforded good peak shapes for the eight ARs (Fig. 2).

**Sample preparation**

Biological sample matrices, such as whole blood and urine samples, are well known to contain high concentrations of interferents, such as proteins and phospholipids. Thus, the online TFC method used in this study functions as an effective means for removal of macromolecular interferents, and is thus a crucial step in the simple purification of samples. Simple purification has also been demonstrated to improve the service life of TFC columns and to reduce the interference of matrix effects on sample detection.

MeOH and ACN are commonly used solvents in the detection of ARs in blood or urine samples. To assess the suitability of these solvents for this study, their AR extraction and purification performances were investigated in blood samples (100 μL) and urine samples (500 μL) spiked with a mixed AR standard at 5 ng/mL. As showed in Fig. 3.
further purification than the peak response for these analytes in samples purified with MeOH or ACN. Similarly, larger peak areas for all the tested ARs (with the exception of coumatetralyl, warfarin, and diphacinone) were obtained when MeOH and ACN were used as solvents. Slightly greater peak responses were obtained for coumatetralyl, warfarin, and diphacinone in spiked urine samples when MeOH was used as the solvent. Based on these results, MeOH was used as the extraction solvent for urine samples in the final optimized method.

For the human blood sample, significantly lower peak responses were obtained for coumatetralyl, diphacinone, chlorophacinone, bromadiolone, brodifacoum, and difethialone in spiked blood samples that had been diluted with Milli-Q water but had not been subjected to any further purification than the peak response for these analytes in samples purified with MeOH and ACN (Fig. 3). For blood samples containing brodifacoum and difethialone, the peak responses were significantly greater when ACN was used as the solvent. Based on these results, ACN was used as the extraction solvent for human blood samples in the final optimized method.

**Optimization of conditions for online TFC**

The online TFC procedure comprised five steps: sampling, purification, column regeneration, elution, and LC separation. Several parameters of this procedure were optimized, namely TFC column selection, loading solution, eluting solvent, transfer flow rate, transfer time, and injection volume. An AR-free urine sample spiked at 10 ng/mL with a mixture AR standard was used for the optimization of the online TFC procedure.

**Selection of online TFC column.** An ideal TFC column allows maximum retention and subsequent satisfactory elution of analytes, while avoiding carryover between samples. To select the optimal online TFC cleanup column, four types of TFC column (Cyclone (50 x 0.5 mm), Cyclone-P (50 x 0.5 mm), C18-XL (50 x 0.5 mm), and MCX (50 x 0.5 mm)) were investigated. A mixed standard solution of ARs was injected into the four TFC columns under the optimal LC conditions, and the peak shapes and intensities of the ARs were compared. As shown in Fig. 4, the peak intensities of the eight ARs associated with the Cyclone column were better than those obtained when using the other columns, and it was thus used for further experimentation.

![Fig. 2. The selected reaction monitoring (SRM) chromatograms of ARs](image)

![Fig. 3. The optimization of extraction solvent for whole blood and urine sample](image)
Loading solvent. The loading solvent can influence the retention capability of target compounds on TFC columns. A mixture of H₂O and MeOH was selected as the loading co-solvent in this study. The effect of a 0–40% proportion of MeOH in this co-solvent on the retention characteristics of ARs in the Cyclone column was investigated. The flow rate of the loading solution was set to 1.5 mL/min, which was sufficient to cause turbulence. As shown in Fig. 1, with 0–10% MeOH in the loading solution no significant difference was observed in the peak intensities of the ARs, indicating that the analytes had adsorbed onto the TFC column under these conditions. In contrast, with >10% MeOH the peak intensities of five of the ARs (i.e., coumatetralyl, warfarin, diphacinone, coumachlor, and chlorophacinone) were decreased. Based on these results, an H₂O/MeOH (90:10, v/v) co-solvent was selected as the loading solvent. Doping the loading solution with a small amount of organic phase was found to facilitate the removal of unwanted impurities (i.e., residual matrix components and macromolecules) from the extract.

Eluting solution. An eluting solution with a high concentration of the organic component achieved satisfactory recoveries of all analytes on the Cyclone column but failed to produce sharp peaks on the analytical column. In response, the eluting solvent was optimized to simultaneously achieve the desired quantitative elution and peak sharpness of the targets. Various mixtures of ACN and H₂O were used as the eluting solvent. Fig. 5 reports the recoveries of the eight ARs as the proportion of ACN was varied from 20 to 90%. With a decreasing concentration of ACN, the recovery of the analytes deteriorated. It was found that a 70% ACN solution in water was enough to completely elute the targets from the Cyclone column. Therefore, ACN/H₂O (70:30, v/v) was chosen as the eluting solvent.

Transfer flow rate. In the transfer step of coupled methods, analytes are transferred from the TFC column to the analytical column. A too-low transfer flow-rate may lead to peak broadening and longer retention, while a high transfer flow rate accelerates analyte transfer, but may increase the column pressure. In the optimization experiment, transfer flow rates of 0.04, 0.06, 0.08, 0.10, 0.12, and 0.14 mL/min were compared over a transfer time of 60 s. The peak intensities of the eight ARs under different transfer flow rates are shown in Fig. S3. With a transfer flow rate of 0.06 mL/min and transfer time of 60 s, the peak intensities of the ARs were very small, indicating an incomplete transfer. With an increase in the transfer flow rate, the peak areas increased. However, when the rate was 0.14 mL/min, the pressure of the analytical column nearly reached its maximum design limit. Based on these results, the transfer flow rate was set at 0.12 mL/min for subsequent experiments.

Injection volume. The injection volume of the sample can potentially affect the clean-up and recovery of analytes. Sample injection volumes of 10, 20, 50, and 100 μL were selected for optimization, and it was found that the peak intensities of the eight ARs decreased by 15.2–64.5% with injection volumes of 20, 50, and 100 μL. Thus, a sample injection volume of 10 μL was used for subsequent experiments.

Method validation

The method was validated in human blood and urine matrices by evaluating the parameters of selectivity, matrix effect, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision [24, 31]. The LODs and LOQs of the target compounds were defined as signal-to-noise (S/N) ratios of 3 and 10, respectively. The level of the spiked sample used for evaluating the parameters of LODs and LOQs was chosen based on the spiked sample concentration closest to these limits.

Selectivity. The bioanalytical method should be selective and specific for an analyte and not affected by endogenous interfering or co-eluting compounds in the biological matrix. Selectivity tests for endogenous matrix compounds were conducted using blank blood samples and blank urine samples. No interfering endogenous molecules were observed at the retention time for ARs.
Matrix effect. Matrix effects occur when molecules derived from the sample matrix co-elute with the target compounds and interfere with ionization processes in the mass spectrometer. This co-elution can lead to suppression or enhancement of the analyte signal and significantly affects the accuracy of the quantitation.

In this study, matrix effects were defined as the percentage ratios of the calibration curve slopes of matrix-matched and solvent-based standards. The differences in slope percentage ratio were designated as signal enhancement when >100%, and signal suppression when <100%. In our experiments, the matrix effects of blood and urine samples for the ARs were found to be 75.3–108.6% and 102.7–130.0%, respectively. Slight signal suppression was observed in the urine matrix for coumatetralyl, warfarin, and diphacinone. The matrix effects of other ARs were 90–110%.

These results indicated that there was no significant matrix suppression or enhancement for the five tested compounds in the urine samples. In contrast, signal enhancements were observed for all ARs in human blood, with that for coumatetralyl being 130%. The matrix effects of the ARs in the two matrices are shown in Table 3. The blood or urine matrix effects on quantitation of ARs after online TFC cleanup treatment were judged acceptable for quantitation of analytes in complex biological matrices. For additional quality assurance, a matrix-matched external standard method was also used to evaluate the samples, to compensate for the influence of the measured matrix effect.

Linearity, LOD, LOQ. Our method showed good linearity in the concentration range studied for all ARs, with correlation coefficients (R²) greater than 0.9976, as showed in Table 3. If the concentration of ARs was out of the linear range, the sample should be diluted with blank matrix before injecting. The LODs and LOQs obtained for the ARs in human blood samples were 0.1–1.0 and 0.3–3.0 ng/mL, respectively. The LODs and LOQs obtained for the ARs in urine samples were 0.02–0.2 and 0.06–0.6 ng/mL, respectively. Data describing the linearity and correlation coefficients of the ARs are presented in Table 3. The fact that LODs and LOQs are matrix-dependent means that matrix-matched calibrations should be performed for the quantitative analysis of samples.

Accuracy and precision. The recoveries for two matrices were evaluated using samples spiked at 5, 20, and 50 ng/mL for blood and at 1.0, 4.0, and 10 ng/mL for urine. The recoveries of ARs in human blood and urine were 80.5–110.3% and 81.6–112.8%, respectively. The intra-day precision of the method was obtained from the analysis of six replicates on the same day, and the inter-day precision was obtained by analysis of three replicates per day on each of three consecutive days. The relative standard deviations (RSD) derived from these results were used to express the intra- and inter-day precision.

The intra- and inter-day results for recovery and precision are listed in Table 4. The intra-day RSDs of the method for human blood and urine samples were 1.9–12.5% and...
| Toxins          | Spike level (ng/mL) | Recovery (%) | RSD (%) | Intraday (n = 6) | Interday (n = 3) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Intraday (n = 6) | Interday (n = 3) | Recovery (%) | RSD (%) |
|----------------|---------------------|-------------|---------|-----------------|-----------------|-------------|---------|-------------|---------|----------------|-----------------|-------------|---------|
| Coumatetralyl  | 5                   | 94.7        | 4.7     | 96.6            | 5.4             | 1.0         | 7.6     | 99.2        | 7.6     | 99.5           | 2.2             | 95.4        | 1.9     |
|                | 20                  | 102.6       | 6.2     | 97.3            | 5.3             | 4.0         | 3.9     | 95.8        | 3.9     | 95.4           | 1.9             | 97.1        | 1.0     |
|                | 50                  | 92.4        | 2.3     | 98.9            | 7.7             | 10.0        | 2.6     | 97.2        | 2.6     | 97.1           | 1.0             | 97.8        | 1.8     |
| Warfarin       | 5                   | 91.8        | 3.5     | 97.2            | 6.1             | 1.0         | 4.6     | 95.7        | 4.6     | 97.8           | 1.8             | 93.7        | 1.4     |
|                | 20                  | 103.8       | 4.0     | 102.4           | 5.9             | 4.0         | 2.0     | 93.6        | 2.0     | 93.7           | 1.4             | 100.2       | 1.2     |
|                | 50                  | 95.8        | 2.2     | 96.0            | 6.8             | 10.0        | 3.2     | 98.3        | 3.2     | 100.2          | 1.2             | 92.6        | 0.9     |
| Coumachlor     | 5                   | 96.1        | 3.2     | 98.2            | 4.0             | 1.0         | 2.3     | 93.1        | 2.3     | 92.6           | 0.9             | 97.0        | 0.7     |
|                | 20                  | 102.8       | 3.1     | 100.2           | 3.6             | 4.0         | 1.6     | 97.5        | 1.6     | 97.0           | 0.7             | 100.5       | 1.4     |
|                | 50                  | 95.8        | 3.1     | 94.7            | 4.8             | 10.0        | 1.8     | 99.7        | 1.8     | 100.5          | 1.4             | 92.1        | 3.9     |
| Diphacinone    | 5                   | 102.0       | 4.8     | 99.1            | 3.3             | 1.0         | 7.3     | 87.9        | 7.3     | 92.1           | 3.9             | 105.1       | 8.9     |
|                | 20                  | 99.7        | 2.2     | 103.5           | 4.5             | 4.0         | 5.2     | 98.2        | 5.2     | 105.1          | 8.9             | 95.9        | 1.9     |
|                | 50                  | 95.6        | 3.6     | 100.9           | 2.5             | 10.0        | 3.0     | 102.6       | 3.0     | 106.3          | 3.8             | 103.4       | 6.1     |
| Chlorophacinone| 5                   | 95.8        | 5.1     | 97.5            | 5.4             | 1.0         | 3.3     | 89.5        | 3.3     | 89.5           | 1.9             | 101.9       | 5.2     |
|                | 20                  | 103.5       | 1.9     | 103.4           | 6.3             | 4.0         | 3.9     | 103.4       | 3.9     | 103.4          | 6.1             | 104.7       | 2.3     |
|                | 50                  | 93.1        | 2.4     | 96.5            | 5.8             | 10.0        | 4.0     | 98.4        | 4.0     | 101.9          | 5.2             | 102.3       | 2.4     |
| Bromadiolone   | 5                   | 95.9        | 9.7     | 100.8           | 4.3             | 1.0         | 8.6     | 102.0       | 8.6     | 104.7          | 2.3             | 103.2       | 0.8     |
|                | 20                  | 104.0       | 3.9     | 105.7           | 4.9             | 4.0         | 3.3     | 102.5       | 3.3     | 102.3          | 2.4             | 101.9       | 5.2     |
|                | 50                  | 102.7       | 1.6     | 98.6            | 6.4             | 10.0        | 3.0     | 100.9       | 3.0     | 103.2          | 0.8             | 105.1       | 3.3     |
| Brodifacoum    | 5                   | 93.8        | 12.5    | 97.9            | 7.2             | 1.0         | 9.3     | 105.0       | 9.3     | 101.6          | 3.3             | 99.5        | 2.2     |
|                | 20                  | 106.2       | 7.5     | 96.9            | 2.6             | 4.0         | 9.2     | 96.5        | 9.2     | 100.8          | 3.8             | 86.9        | 2.3     |
|                | 50                  | 93.6        | 2.7     | 92.4            | 4.6             | 10.0        | 8.0     | 89.6        | 8.0     | 86.9           | 2.3             | 102.6       | 7.3     |
| Difethialone   | 5                   | 94.6        | 11.1    | 103.4           | 9.7             | 1.0         | 8.8     | 101.9       | 8.8     | 102.6          | 7.3             | 106.7       | 4.4     |
|                | 20                  | 111.9       | 8.7     | 104.8           | 2.4             | 4.0         | 8.8     | 102.6       | 8.8     | 102.6          | 7.3             | 106.7       | 4.4     |
|                | 50                  | 103.8       | 5.8     | 99.9            | 5.9             | 10.0        | 9.7     | 97.8        | 9.7     | 106.7          | 4.4             | 106.7       | 4.4     |

Fig. 6. The SRM chromatograms of ARs in human blood (A) and urine (B) from one poisoned patient.
1.0–9.7%, respectively. The inter-day RSDs of the method for human blood and urine samples were 2.4–8.7% and 0.7–8.9%, respectively. Both intra- and inter-day precision studies afforded satisfactory results for all ARs, with intra- and inter-day RSDs <12.5% for blood and urine samples.

**Application case**

To verify the practicality of the newly developed method, it was used to detect ARs in human blood and urine samples obtained from actual poisoning cases.

Case 1: Three patients presented to hospital with symptoms of subcutaneous tissue hemorrhage after dinner at the same restaurant. Blood and urine samples were obtained from these individuals, and then analyzed. The presence of two ARs (bromadiolone and warfarin) was detected in the samples. In the blood samples of the three patients, the concentrations of bromadiolone were 426, 495, and 558 ng/mL, and the concentrations of warfarin were 0.310, 0.315, and 1.33 ng/mL. In the urine samples of the three patients, the concentrations of bromadiolone were 5.58, 5.42, and 4.76 ng/mL, and the concentrations of warfarin were 0.274, 0.288, and 0.166 ng/mL. The chromatograms of the ARs in the blood and urine samples of one of the poisoned patients are shown in Fig. 6. The three patients were immediately administered vitamin K1 as part of detoxification treatment at the hospital.

Case 2: A man presented to hospital with hematuria, oral hemorrhage, and hematochezia, which are all clinical symptoms of poisoning. A blood sample was collected and transferred to our laboratory to be evaluated for the presence of ARs. A high concentration of brodifacoum (172 ng/mL) was detected in the blood sample. The patient’s bleeding was treated with vitamin K1.

**CONCLUSIONS**

A new analytical method for quantitation of ARs in human blood and urine was developed, based on a simple pretreatment followed by online TFC-LC-MS/MS. The method was optimized for the analysis of eight ARs, with a simple online TFC-based extraction procedure used, rather than time-consuming manual cleanup steps.

This method enables the entire analysis of a blood or urine sample from a patient with suspected ARs, comprising pretreatment, detection and result analysis, to be performed in less than half an hour. The parameters of linearity, recovery, precision, LOD and LOQ were optimized using blood and urine samples, and the resulting values confirmed the validity of the method. The method greatly reduces pretreatment costs and shortens sample detection times while simultaneously yielding more reliable qualitative and quantitative results. The successful application of our method to actual sample testing illustrates the utility of this new analytical approach.

**Conflict of interest:** Li Fang and Fengmei Qiu contributed equally to this work and should be considered as co-first authors. The authors declare no conflicts of interest.

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**SUPPLEMENTARY MATERIAL**

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