Valone is a chronic anticoagulant rodenticide that has come into wide use in China. Current literature lacks analytical methods for the determination of valone. In this paper, a sensitive and selective assay was established for the identification and quantification of valone in serum by liquid chromatography–tandem mass spectrometry. After addition of the internal standard, warfarin, serum samples were extracted with 10% methanol in acetonitrile and cleaned using Oasis HLB solid-phase extraction (SPE) cartridges. The compounds were separated on an Agilent SB C18 column with a mobile phase of methanol/acetic acid-ammonium acetate (5 mmol/L, pH 6.3) (75:25, v/v). Detection was performed by electrospray ionization ion trap mass spectrometry in the negative multiple reaction monitoring mode. The transition ions of m/z 229 \rightarrow 145 and m/z 307 \rightarrow 161 were selected for quantification of valone and the internal standard, respectively. The overall extraction efficiency was between 81.1% and 91.1%. The limit of quantification was 0.5 ng/mL. Regression analysis of the calibration data revealed good correlation (r^2 > 0.99) for valone. Intra- and interday precisions for quality-control samples were less than 7.8% and 12.8%, respectively. This method combines a rapid SPE procedure with an extremely fast chromatographic analysis, which is especially advantageous for clinical laboratories.

**Experimental**

**LC–MS–MS system**

Separations were performed on an Agilent 1100 series LC–MSD Trap SL system (Agilent Technologies, Waldbronn, Germany) with the LC–MSD Trap Software 4.2 (Bruker Daltonik GmbH), consisting of a quaternary pump (G1311A), a column thermostat (G1316A), a degasser unit (G1379A), an autosampler (G1313A), a diode-array detector (1315B), and an ion trap MS with an ESI interface.

**Solvents and materials**

We purchased HPLC-grade methanol, acetonitrile, acetic acid, and ammonium acetate from Merck (Darmstadt, Germany) and collected the standards of warfarin (> 99%, internal standard (IS)) and valone (> 99.5%) from Sigma (St. Louis, MO). Water was purified by a Milli-Q system (Millipore, Molsheim, France). Blank human serum was collected from healthy volunteers (three women, three men), aged 21 to 25 years.
years, from Ningbo University (Ningbo, China); all were in general good health. Samples A, B, and C were collected from three patients who were suspected for incidental ingestion of valone rodenticide. Table I lists the detailed information about samples A, B, and C. All blood samples were centrifuged in 5-mL polypropylene centrifuge tubes at once and the serum specimens were kept refrigerated until processing.

Preparation of standard stock solutions

We prepared the following stock solutions: 1.0 mg/mL valone in methanol and 1.0 mg/mL warfarin (IS) in methanol. The stock solutions were stored at 4 ºC in tightly closed bottles until use. Working solutions of valone at the concentrations of 0.5, 1.0, 3.0, 5.0, 15.0, 50.0, and 100.0 ng/mL were diluted from the stock solution with MeOH, whereas the IS concentrations were 50.0 ng/mL.

Preparation of spiked human serum samples

Appropriate amounts of the diluted standard solutions (1.0 mg/L) of valone were taken to a 2-mL polypropylene centrifuge tube, and evaporated to dryness under a gentle stream of nitrogen. Then the residues were reconstituted with 0.5 mL of blank human serum, which was thawed to room temperature in advance, to give final valone concentrations of 0.5, 1.0, 3.0, 5.0, 15.0, 50.0, and 100.0 ng/mL, and the IS concentrations were 50.0 ng/mL. In order to facilitate the residues’ complete dissolution, the tubes were immerged into a KQ 500DB ultrasonic cleaning bath (Kunshan Ultrasonic, Jiangsu, China) which contained a 5-cm depth of water, and ultrasonicated for 2 min. These sequences of spiked human serum solutions were considered as the matrix-matched calibration standards and three of them (3.0, 15.0, and 100.0 ng/mL) as quality control (QC) samples.

Preparation of sample

Sample preparation was as follows: 200-µL serum samples were thawed to room temperature in a 2-mL polypropylene centrifuge tube before analysis, and 1.0 mg/L IS (10.0 µL) was vortex mixed and extracted using 1.0 mL 10% methanol in acetonitrile for 5 min. After centrifugation for 5 min at 7800 rpm, the upper organic layer was transferred to a disposable glass centrifuge tube before analysis, and 1.0 mg/L IS (10.0 µL) was added to the extract was filtered though a 0.45-µm nylon syringe filter (Agilent Technologies, Palo Alto, CA), and a 20-µL aliquot was injected into the HPLC system.

LC

The separation was accomplished on an SB C18 column (30 mm × 2.1-mm i.d., 3-µm particle size, Agilent Technologies) using acetic acid/ammonium acetate (5 mmol/L, pH 6.3)/methanol (25:75, v/v) at a flow rate of 0.5 mL/min. The column temperature was held at 35 ºC, and the injection volume was 20.0 µL.

MS

MS conditions were as follows: ESI source in the negative ion mode; capillary voltage 3.0 kV; capillary exit voltage –135 V; dry temperature 325 ºC; vaporizer temperature 325 ºC; high purity (99.999%); dry nitrogen gas 5.0 L/min; nitrogen nebulizer pressure of 60.0 psi; and dwell time 200 ms. LC–MS–MS experiments were performed using helium as collision gas at a pressure of 1.22 × 10–5 mbar and a collision energy setting adapted for each compound. To determine the product ions of valone and IS, the deprotonated ion [M–H]– at m/z 229 and 307 were detected in MRM mode with a transition of m/z 229 → 105 for valone, and m/z 307 → 161 for IS, respectively. Table II outlines the values set for both compounds.

Recovery and matrix effect

The extraction recoveries were assessed by comparing the peak area of valone under a stream of nitrogen on a heating block at 40 ºC in a disposable glass tube. Then the residues were dissolved with 2.0 mL methanol/water (80:20, v/v) by ultrasonication for 5 min. The solution was uploaded on an Oasis HLB (200 mg, Waters, Milford, MA) cartridge that first was conditioned with 2.0 mL dichloromethane, 2.0 mL methanol, and 2.0 mL water. The flow rate of the samples was 5.0 mL/min. The elution was used by 1.0 mL MeOH and 1.0 mL dichloromethane, then evaporated to dryness by a gentle stream of nitrogen, and the residues were reconstituted with 200 µL of methanol by ultrasonication for 5 min to facilitate dissolution. Prior to analysis, the extract was filtered through a 0.45-µm nylon syringe filter (Agilent Technologies, Palo Alto, CA), and a 20-µL aliquot was injected into the HPLC system.

Table I. Sample A, B, and C Information and the Result of Valone Concentration in Serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Coagulation Time (min)†</th>
<th>Concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>male</td>
<td>15</td>
<td>8.3</td>
<td>13.5</td>
</tr>
<tr>
<td>B</td>
<td>female</td>
<td>48</td>
<td>13.8</td>
<td>405.6</td>
</tr>
<tr>
<td>C</td>
<td>female</td>
<td>6</td>
<td>6.4</td>
<td>n.d.‡</td>
</tr>
</tbody>
</table>

† Determined by glasses test tube method (at room temperature).
‡ n.d.: less than 0.5 ng/mL.

Table II. The MRM Parameters for Valone and Warfarin (IS)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Valone</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor ion (m/z)</td>
<td>239</td>
<td>307</td>
</tr>
<tr>
<td>Product ion for detection and quantification (m/z)</td>
<td>145</td>
<td>161</td>
</tr>
<tr>
<td>Additional ions for confirmation (m/z)</td>
<td>172, 187, 214</td>
<td>250, 117</td>
</tr>
<tr>
<td>Width (m/z)</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Cutoff mass</td>
<td>73</td>
<td>84</td>
</tr>
<tr>
<td>CID (V)</td>
<td>1.50</td>
<td>1.15</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>5.22</td>
<td>3.43</td>
</tr>
</tbody>
</table>
those to which the analytes had been added post-extraction for five replicates. For an evaluation of matrix effect, the peak areas of extracted QC samples were compared to the peak areas of pure diluted substances.

**Precision and accuracy**

Accuracy was expressed as recovery, and precision was expressed as intra- and interday relative standard deviation (RSD). Intraday RSD was determined by five replicate analyses of the QC samples on one day. Interday RSD was determined by five replicate analyses of the QC samples on three different days within a 14-day period.

**Results and Discussion**

**LC–MS–MS method development**

Electrospray MS–MS was used to analyze valone and IS, as it is beneficial in developing a selective and sensitive method. The negative ion mass spectra and product ion mass spectra of valone and IS are shown in Figures 1 and 2, respectively. \([M-H]^-\) was the predominant ion in the MS spectra and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from \(m/z\) 229 → 145 for valone and from \(m/z\) 307 → 161 for IS, which were selected for quantification. The product ions of \(m/z\) 229 → 172 and \(m/z\) 229 → 187 were the qualitative ions for valone. LC–MS–MS–MRM is a powerful technique for the residues of poisons analyses because it provides sensitivity, selectivity, and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM parameters were optimized to maximize the response by direct infusion of 1.0 mg/mL of the analytes. The parameters listed in Table II are the result of optimization.

Typical MRM chromatograms obtained with a spiked serum sample containing 10.0 ng/mL valone and representative blank serum sample (six different sources) are shown in Figure 3. The MRM transitions show valone and IS, and demonstrate that there is little interference from endogenous substances. The apparent responses at the retention times of valone (\(t_R = 5.22\) min) and IS (\(t_R = 3.43\) min) were compared to the lower limit of quantification (10.0 ng/mL). The specificity of the method was examined by analyzing six extracts of blank human serum samples (Figure 3A) and an extract spiked only with the IS (Figure 3B). As shown in Figure 3A, no significant interference in the blank serum was seen from endogenous substances at.

![Figure 1. The MS spectra for IS (A) and valone (B).](image)

![Figure 2. The MS–MS spectra for IS (A) and valone (B). The diamond indicates the precursor ion.](image)

![Figure 3. MRM chromatograms of a blank serum in transition of \(m/z\) 229 → 145 for valone, and \(m/z\) 307 → 161 for IS (A); spiked at 10.0 ng/mL IS in transition of \(m/z\) 307 → 161 (B); spiked at 10.0 ng/mL valone in transition of \(m/z\) 229 → 145 (C).](image)
the retention time of the analyte.

Internal standards are especially useful for analyses in which the quantity of sample analyzed or the instrument response varies slightly from run to run for reasons that are difficult to control. However, it can be difficult to find an appropriate substance that will elute in a position on the chromatogram that does not interfere or merge with any of the natural components of the mixture. In this experiment, we first selected warfarin, another rodenticide which had a similar polarity and a similar chromatographic behavior to valone and was seldom used for rat killing, because of its insecticide resistance in China, as an IS. The results showed that warfarin was a suitable IS for this assay. Nevertheless, if both warfarin and valone coexisted in the samples, we would use an IS other than warfarin in order to obtain a more accurate result, which was the same as the overall IS assays when the analytes and the IS coexisted in the samples.

LC–MS–MS method validation

The assay was validated in accordance with Food and Drug Administration guidelines (18). The valone calibration curve was linear from 0.5 to 100.0 ng/mL, tested with a coefficient of determination \( r^2 = 0.993 \). The peak-area ratio \( (Y, \text{valone}/\text{IS}) \) was then used in conjunction with the calibration curve to derive the concentration \( (C, \text{ng/mL}) \) in serum. The regression equation was \( C = 117.22Y - 0.359 \). The mean RSD for the slope \( (a) \) and intercept \( (b) \) obtained from five consecutive calibration curves used for routine analysis during two weeks are 3.5% and 4.4%, respectively.

Recovery and precision data collected over a two-week period are summarized in Table III. The overall extraction efficiency was between 81.1% and 91.1% for valone. Intra- and interday accuracies for quality-control samples were between less than 7.8% and 12.8%, respectively.

We estimated the limit of quantification (LOQ) by analyzing a serum sample containing 3.0 ng/mL valone in a serum sample. The evaluation was by the criterion that the signal to noise ratio (S/N) should be >10, for quantification purposes. The LOQ was 0.5 ng/mL for valone.

We assessed the stability of valone in serum matrix; the QC samples were analyzed under different conditions. After three freeze-and-thaw cycles (room temperature to \(-20^\circ\text{C}\)) were performed at three concentrations (3.0, 15.0, 100.0 ng/mL), the percent bias was evaluated to be \(-4.3, -6.8, \) and \(-2.5\%\), respectively, in comparison with freshly prepared samples. Bench-top stability was done for 24 h and the percent bias, calculated by comparing with freshly prepared sample, was found to be \(-9.5, -6.7, \) and \(-8.2\%\), respectively. Autosampler stability was also done for 24 h, and the percent bias was found to be approximately \(-9.8, -7.3, \) and \(-5.9\%\), respectively.

Application to the real world samples

During our method validation, we obtained serum samples from three patients. As a result, valone in serum was detected. The concentrations of valone in serum were determined by the LC–MS–MS method. The results indicated that the concentrations were 0–405.6 ng/mL for valone, as shown in Table I. They also indicated that the concentrations of valone in serum were positive in correlation with the blood coagulation time. Generally, the coagulation time determined by glasses test tube method (at room temperature) was between 4 and 12 min for normal persons. Using this criterion, the hospital also evaluated the patients as to whether they were poisoned by anticoagulant or not. We found that low concentration of valone in serum may have a normal coagulation time; in fact, the poison still existed in the human body, so the poison residue monitoring was necessary to achieve the best therapeutic effects and the least toxicity for people.

Conclusions

The proposed method enables the identification and quantification of valone in both clinical and forensic specimens. This method combines a rapid SPE procedure with an extremely fast chromatographic analysis, which is especially advantageous for clinical laboratories.

Acknowledgment

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Table III. The Recoveries and Precisions of the Method for Spiked Samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Added (ng/mL)</th>
<th>Found (ng/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intraday (^d)</td>
<td>Interday (^d)</td>
</tr>
<tr>
<td>Valone</td>
<td>3.0</td>
<td>2.45 ± 0.19</td>
<td>81.6</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>13.67 ± 0.81</td>
<td>91.1</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>80.12 ± 2.31</td>
<td>81.1</td>
<td>2.9</td>
</tr>
</tbody>
</table>

\(^d\) Determined in one day.

\(^d^n = 5\).

\(^d^n = 5\) replicates x 3 days within a 14-day period.

References

3. T.M. Annesley and L.T. Clayton. Quantification of mycophenolic acid and glucuronide metabolite in human serum by

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