Organic Solvent-Free and Simple Method for Determining Cyromazine and its Metabolite, Melamine, in Cow’s Milk

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Abstract

This paper described an organic solvent-free, rapid, simple, and space-saving method of sample preparation followed by HPLC coupled photo-diode array (PDA) detector for simultaneous quantification of cyromazine (CYR) and its decycropropylated metabolite, melamine (MEL), in milk. The HPLC-PDA was performed on an Inertsil® HILIC column with an isocratic aqueous mobile phase. Analytes were extracted from the sample using water, and purified by MonoSpin®-C18, a centrifugal monolithic silica spin mini-columns, and quantified within 20 min. The method, performed under 100% aqueous conditions, obtained average recoveries for CYR and MEL in the range of 93.2–99.1% with relative standard deviations ≤ 2.8%. The quantitation limits were 8.5 ng/mL for CYR and 10 ng/mL for MEL, respectively. No organic solvents were used at any stage of the analysis.

Keywords: Cyromazine; Melamine; HPLC-PDA; Milk; Organic solvent-free

Introduction

Cyromazine (CYR) is a triazine insect growth regulator used as an insecticide. In veterinary medicine, CYR is used as an ectoparasiticide and is added to animal feed to prevent fly from the manure, so as to improve the hygiene control of animal housing environments. CYR can be decycropropylated to melamine (MEL) (Figure 1) in plants and food-producing animals. The biotransformed MEL has been isolated from laying hen, goat, sheep, cow, and their products [1].

The 2008 Chinese milk scandal broke as infants consumed formula containing MEL were falling sick with urinary calculus and kidney damage [2] and the melamine adulteration of a variety of food products has instantly become a global problem. MEL, a nitrogen-rich organic chemical, is added to milk to boost the protein levels, making them appear higher than they actually are. Chronic exposure may lead to reproductive damage, or bladder or kidney stones, which can lead to bladder cancer [3-6].

On October, 2008, FDA issued its Interim Safety and Risk Assessment of MEL in Food for Humans in consideration of the potential public health concerns from foods.

In applying an additional 10-fold safety factor and using the TDI (Tolerable Daily Intake) of 0.63 mg/kg b.w./day, the safety/risk assessment concluded that a maximum tolerance levels of MEL below 2.5 ppm in foods other than infant formula do not raise public health concerns [7].

In response to the global MEL problem, the United Nations food standards body, the Codex Alimentarius Commission, announced formal international limits for melamine allowed in food and animal feed during its meeting on July, 2010. The maximum amount of MEL allowed in foods and animal feed is 2.5 mg/kg, equal to the FDA limit. The international maximum levels help governments differentiate between low levels of unavoidable melamine occurrence that do not cause health problems, and deliberate adulteration—thereby protecting public health without unnecessary impediments to international trades [8].

Milk contains a good balance of protein, fat and carbohydrate, is an indispensable food because it is inexpensive and readily available. It becomes a raw material of every processed food.

To ensure that milk for human consumption is residue-free of CYR, the Codex has set a maximum residue limit (MRL) for CYR in milk at 10 ng/mL [9]. Strict and rapid monitoring the presences of CYR and MEL in milk is, therefore, an important means of guaranteeing the international food safety.

In response to the recent expansion in the internal food trade, the development of international harmonized methods to determine chemical residues in foods is essential to guarantee equitable international trade in these foods and protect health for consumers. Whether in industrial nations or developing countries, an internal harmonized method for residue monitoring in foods is urgently needed. The optimal harmonized method for the routine monitoring chemicals such as melamine in foods must be simple, small scale,
The Risk Associated with These Solvents Extends Beyond Direct Effects to Human Health by a Mechanism Without Organic Solvent Consumption. This article describes an ultra safe, idiot proof, and inexpensive method to strictly monitor CYR and MEL residues in milk using a 100% aqueous solution in sample preparation and HPLC separation without organic solvent consumption.

**Experimental**

**Reagents**

Standards of cyromazine (CYR) and melamine (MEL) and other chemicals were purchased from Wako Pure Chem. Ltd. (Osaka, Japan). Distilled water was of HPLC grade. 0.5 mol/L 1-octanesulfonic acid solutions were kept in a refrigerator (5°C). Standards and chemicals were greater than 99% purity.

**Apparatus**

The following apparatuses were used in the sample preparation:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Silica (type)</th>
<th>Trade name</th>
<th>Pore diameter (nm)</th>
<th>Pore volume (ml/g)</th>
<th>Surface area (m²/g)</th>
<th>Carbon load (%)</th>
<th>HPLC separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C1</td>
<td>WakoSil 5TMS</td>
<td>12</td>
<td>1.0</td>
<td>300</td>
<td>4</td>
<td>Not Separated (NS)</td>
</tr>
<tr>
<td>B</td>
<td>C4</td>
<td>MightySil RP-4 GP</td>
<td>12.5</td>
<td>1.1</td>
<td>350</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>C</td>
<td>C18</td>
<td>MightySil RP-18 GP Aqua</td>
<td>13.5</td>
<td>0.9</td>
<td>270</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>D</td>
<td>C18</td>
<td>InertSil ODS-4</td>
<td>10</td>
<td>1.05</td>
<td>450</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>E</td>
<td>Alkyl diol</td>
<td>InertSil HILIC (alkyl diol)</td>
<td>10</td>
<td>1.05</td>
<td>450</td>
<td>20</td>
<td>Completely separated</td>
</tr>
</tbody>
</table>

Table 1: Physical/chemical specifications of the reversed-phase columns used and chromatographic CYR and MEL separations obtained under the HPLC conditions examined.

**HPLC operating conditions**

The analytical column was an InertSil® HILIC (150 x 4.6 mm, 5 μm d.) column using a 0.05 mol/L 1-octanesulfonic acid mobile phase at a flow rate of 1.0 mL/min at 40°C. PDA detector (Shimadzu Scientific Instruments, Kyoto, Japan). Table 1 lists the particle physical specifications.

**Preparation of stock standards and working mixed solutions**

Stock standard solutions of CYR and MEL were prepared by spiking appropriate aliquots of the mixed standard solutions in blank milk samples. Calibration standards were used to construct calibration curves from which the concentrations of analytes in unknown monitoring samples are determined practically. QCs used

**Preparation of calibration standards and quality control samples**

For method validation studies, calibration standards and quality control samples (QCs), terms defined in the FDA guideline [18], were prepared by spiking appropriate aliquots of the mixed standard solution in blank milk samples. Calibration standards were used to construct calibration curves from which the concentrations of analytes in unknown monitoring samples are determined practically. QCs used
to evaluate the performance of the proposed method. In this study, the standards were prepared in the range of 20 – 1,000 ng/mL for CYR and 20 – 3,000 ng/mL, respectively. Three QC levels (QC1 = 30 ng/mL for CYR and 50 ng for MEL; QC2 = 100 ng/mL for both analytes; QC3 = 500 ng/mL for CYR and 250 ng/mL for MEL) were prepared.

**Method validation**

The performance of the developed method was validated in terms of some parameters from the international guidelines for bio-analytical procedure [18-23]. The quality parameters established were standard solution stability, linearity, accuracy, precision, sensitivity, specificity, selectivity, robustness, system suitability.

**Results and Discussion**

**Sample preparation**

An accurate 0.1 mL sample was taken into a micro-centrifuge tube and homogenized with 0.6 mL of water with a handheld ultrasonic-homogenizer for 30 s. After being homogenized, the capped tube was centrifuged at 10,000 g for 5 min. A 0.1 mL of supernatant liquid was poured to a MonoSpin-C18 and centrifuged at 3,500 g for 1 min. The eluate was injected into the HPLC system.

**Optimum HPLC conditions**

In order to achieve the separation with a 100% aqueous mobile phase, this study tested five types of non-polar sorbent columns. Table 1 lists the physical and chemical specifications. The author used two types of centrifugal monolithic silica spin mini-columns, MonoSpins (MonoSpin-C18 and SCX), for the further cleanup technique. The spin mini-column is a monolithic SPE column excellent for the small volume sample with easy and quick operation by centrifuge [23]. A 100% water was used as the eluent and the recoveries of CYR and MEL from these mini-columns were compared. Since the recoveries of the target compounds from the column vary with centrifugal acceleration (rotary speed), this study was also investigated an optimal acceleration (≥ 1,000 g) to recovery CYR and MEL from the spin mini-columns. A 100 µL portion of a mixed standard solution containing 0.5 µg of each compound was applied to the spin mini-column. The centrifugal time was standardized at 1 min. MonoSpin-C18 gave satisfactory recoveries (≥ 96%) and repeatabilities (RSD ≤ 2%, n=3) for CYR and MEL when the centrifugal acceleration was 3,500 g for 1 min. The MonoSpin-C18 was therefore used a centrifugal monolithic silica spin mini-column. The present procedure can realize small-scale extractions and easy purifications of CYR and MEL in a short time while completely eliminating the consumption of organic solvents. The procedure resulted in high recoveries and repeatabilities.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CYR</th>
<th>MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard stability*: At 25°C for 24 h</td>
<td>101.0 (0.6)</td>
<td>99.3 (0.9)</td>
</tr>
<tr>
<td>At 5°C for 10 days</td>
<td>98.6 (0.8)</td>
<td>100.6 (1.0)</td>
</tr>
<tr>
<td>Linearity (r²)</td>
<td>0.991</td>
<td>0.994</td>
</tr>
<tr>
<td>Accuracy²</td>
<td>93.2 – 97.8</td>
<td>94.5 – 99.1</td>
</tr>
<tr>
<td>Precision²</td>
<td>≤ 2.8</td>
<td>≤ 2.4</td>
</tr>
<tr>
<td>Sensitivity (QL*)</td>
<td>8.5</td>
<td>10</td>
</tr>
</tbody>
</table>

*The chromatographic peak area (%) after completion of the store time, data are expressed as averages (n=5, for each compound); relative standard deviation (RSD) in parentheses (%).

**Table 2: The main method validation data.**
present method can provide the quantitation and identification of CYR and MEL.

**Method validation**

**Main quality parameters:** Table 2 summarizes the values obtained for the main parameters. The stability of a working mixed standard solution (1,000 ng/mL of each compound) was evaluated at room temperature, 25°C, for 24 h and 5°C for 10 days, respectively. After completion of the storage time, the stability was tested by comparing the HPLC response with that of freshly prepared solution. The resulting stabilities were well within the international acceptance criteria [21]. The correlation coefficients for CYR in the range of 20-1,000 ng/mL and MEL in the range of 20-3,000 ng/mL were 0.991 and 0.994, respectively. The resulting line showed a significant linearity for individual compound (P <0.01). The accuracy and precision are well within the acceptance criteria [19-21, 23]. The QL for CYR (9 ng/mL) was lower than the MRL of 10 ng/mL [10]. The other validation findings are as follows:

**Specificity and selectivity:** The application of the proposed procedure to 10 blank milk samples from different species (Holstein-Friesian and Jersey) demonstrated that no interference peak was presented around the retention times for CYR and MEL in any of the sample examined.

The present HPLC-PDA system easily confirmed the peak identity of target compound. CYR and MEL were identified in a milk sample by their retention times and absorption spectra. The CYR and MEL spectra obtained from the milk sample were practically identical to those of the standards. Because of the complete separations and the high absorbance of CYR and MEL, PDA detection at trace levels is fully available. It is, therefore, instructive to demonstrate purification effectiveness of the sample preparation. The system did not require the use of MS, which is very expensive and is not available in a lot of laboratories for routine analysis, particularly in developing countries.

**Robustness:** Some HPLC parameters were performed using a spiked (100 ng/mL of each compound) milk sample obtained under the established procedure.

Changes of ±5% units of the flow rate (1.0 mL/min) and the column temperature (40°C) were determined. The effect on the peak areas and the validations in the retention times were evaluated. Changes of ± 5% of the flow rate and the column temperature had no effect on the peak areas, whereas the variations in the retention times were obtained with the flow rate and the column temperature. Normal retention times for CYR and MEL were 6.23 and 3.08 min, respectively. At ±5% of the flow rate, the two retention times were decreased, ranging between 1.6 and 5.7% and at -5%, the times were increased ranging between 5.5 and 8.0%. By changing the column temperature by +5%, decreasing retention times obtained were 1.9-7.5%, however, no significant variations were observed with -5%. During these studies, all the target compounds were separated.

**System suitability:** The system-suitability evaluation is an essential parameter of HPLC determination, and it ascertains the strictness of the system used. The suitability was evaluated as the relative standard deviations of peak areas and retention times calculated for 20 replicate injections of a spiked milk sample (100 ng/mL of each compound). The values for CYR and MEL were estimated to be <0.5% for peak areas and <0.8% for retention times, respectively.

**Cost and time performance:** The total time and budget required for the analysis of a single sample was <20 min and approximately US $6.1 as of March 5, 2012, respectively. For sequential analyses, a batch of 24 samples could be analyzed in 4 h. These findings became term required for an international harmonized residue analysis. The organic solvent-free and short analytical time not only increased the sample throughput for analysis but also positively affected the cost and environmental/human impacts.

**Application to residue monitoring in commercial samples**

Milk was purchased from a number of convenience stores in Osaka, Japan, used as real milk samples and analyzed using the proposed method. No samples contained detectable concentrations of CYR and MEL. The resulting chromatograms were free from interference.

**Conclusion**

An organic solvent-free method does not use organic solvents for simultaneous determination of CYR and MEL in milk has been successfully developed and validated. The present procedure provided an easy-to-use, rapid, space-saving, and non-use of organic solvents and resulted in high recovery and repeatability with considerable saving of analysis time/cost. The procedure may be proposed as an international harmonized analytical method for the routine monitoring of CYR and MEL in milk.

**References**

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