KEY WORDS: veterinary medicine; food hygiene; food control; drug residues; chlorpromazine; food-producing animals

Inspection of drug residues is of great importance in veterinary medicine. This is especially important in Slovene veterinary medicine where the use of certain drugs is prohibited for food-producing animals. This includes drugs containing chlorpromazine as an active compound.

A highly sensitive and selective analytical method has been introduced for the determination of chlorpromazine residues in the muscle tissue and urine of food-producing animals. It includes an appropriate extraction and sample clean-up, using solid phase extraction, and is then followed by the determination using high-performance liquid chromatography (HPLC) with UV detection. In spite of the relatively low recovery of the method, it enables a low detection limit and good selectivity.

ANALYTICAL PROCEDURE FOR THE DETERMINATION OF CHLORPROMAZINE RESIDUES IN MUSCLE TISSUE AND URINE OF FOOD-PRODUCING ANIMALS

N. KOŽUH ERŽEN

ANALITSKA METODA ZA UGOTAVLJANJE ZAOSTANKOV KLORPROMAZINA V MIŠIČNEM TKIVU IN URINU KLAVNIH ŽIVALI

Ključne besede: veterinarska medicina; higiena živil; nadzor nad živilim; zaostanki zdravil; klorpromazin; klavne živali

Nadzor zaostankov zdravil, posebno tistih, ki so v veterinarski medicini v Sloveniji prepovedana za uporabo pri živalih, namenjenih za prehrano ljudi, je izredno pomemben. Med taka spadajo tudi zdravila, ki kot aktivno učinkovino vsebujejo klorpromazin.

V ta namen smo za ugotavljanje vsebnosti zaostankov klorpromazina v mišičnem tkivu in urinu živali, namenjenih za prehrano ljudi, uvedli dovolj občutljivo in selektivno analitsko metodo. Ta je vključevala v prvi fazi ekstrakcijo zaostankov klorpromazina iz vzorcev mišičnega tkiva in urina ter čiščenje ekstraktov z...
ekstrakcijo tekoče-trdno, v drugi fazi pa njihovo ugotavljanje s tekočinsko kromatografijo visoke ločljivosti (HPLC) z UV zaznavanjem. Kljub razmeroma slabemu izkoristku smo s to analitsko metodo dosegli dobro ponovljivost, nizko mejo zaznavnosti in zadovoljivo selektivnost.

Introduction

In Slovene veterinary medicine the use of drugs containing chlorpromazine as an active compound has been prohibited for food-producing animals since 1999 (Off. Gazette RS no. 88/99) (1). Chlorpromazine was frequently used in veterinary practice to prevent stress in animals and to minimise death and injury during transport from the farm to the slaughterhouse. It was also used as a hypnotic agent. Chlorpromazine is used in human medicine in cardiovascular therapy, the treatment of heatstroke and in the production of hypothermia (2).

Chemically, chlorpromazine belongs to a group of phenothiazines. Figure 1 shows the molecular structure of chlorpromazine.

Figure 1: Molecular structure of chlorpromazine

Due to side effects such as hypersensibility, an obstructive type of jaundice and various dermatological reactions (3), the use of chlorpromazine for food-producing animals has been prohibited in veterinary medicine. The use of this drug has also been prohibited in the European Union since 1997 by Regulation (EC) No. 17/97 (4). Before that Directive, the maximum residue limit (MRL) of 20 ng/g for chlorpromazine in tissue had been adopted. The metabolism of chlorpromazine is relatively complex and the number of metabolites produced varies from species to species. The main metabolite is chlorpromazine sulphoxide (2). A long-term elimination of chlorpromazine and their metabolites from the body is not uncommon and they have been detected in human urine 6 to 18 months after the drug’s administration (5).

The monitoring of chlorpromazine in the tissue and urine of food-producing animals is very important due to the possibility of its illegal use. For this reason sensitive and selective analytical methods should be used for the determination of chlorpromazine residues. Several analytical methods for their determination in kidneys (6), various tissues (7, 8), urine (7, 8, 9) and body fluids (8, 12) are known. All published methods involve a suitable sample pre-treatment and appropriate high-performance liquid chromatographic (HPLC) detection.
The aim of our work was to develop a rapid and sensitive analytical method for the determination of chlorpromazine residues in the muscle tissue and urine of food-producing animals using high-performance liquid chromatography (HPLC) with UV detection. The method involved a suitable sample pre-treatment employing solid phase extraction. It was used for the routine monitoring of chlorpromazine residues in the muscle tissue and urine of food-producing animals.

**Materials and methods**

**Samples.**

Muscle tissue. Samples were homogenised and stored in plastic containers at -20 °C. 

Urine. Before the analysis, samples were filtered and stored in polypropylene tubes (protected from light) at -20 °C. 

Standard solutions. The stock solutions of chlorpromazine were prepared using a standard of chlorpromazine supplied by Sigma. The stock solution of chlorpromazine, in a concentration of 10 µg/ml, was prepared in methanol. Working standard solutions of chlorpromazine in concentrations from 2.5 to 200 ng/ml were prepared in a mobile phase and protected from light. They were stable for approximately one week.

**Reagents**

All chemicals were of analytical-reagent grade. The methanol, acetonitrile, tetrahydrofuran, toluene and sulphuric (VI) acid were all supplied by Merck. Orthophosphoric acid supplied by Carlo Erba, and triethylamine supplied by Fluka, were used for the preparation of the mobile phase. A 25 % ammonia solution and sodium chloride were supplied by Riedel de Haën. Supelco Sylon CT was used for deactivating the surface of the glassware.

**Instrumentation**

A Tehtnica Železniki mechanical shaker (VIBROMIX 313 EVT), a vortex (VIBROMIX 204 EV) from the same producer and an Iskra ultrasonic bath (UZ 2R) were employed for the extraction of samples. A Hettich centrifuge (ROTIXA/RP) was used for the centrifugation of samples. Extracts were evaporated using an Organomation N-EVAP No.111 evaporator. A Mettler Toledo pH meter (MI 229) was used for the adjustment of the pH of the mobile phase. A Supelco Vacuum Manifold, 500 mg of Varian Bond Elut C₈ and Merck LiChrolut C₁₈ endcapped solid-phase extraction (SPE) cartridges were introduced into the clean-up procedure and to enrich the drug in the urine and muscle tissue samples, respectively. The HPLC system (Thermo Separation Products) that consisted of a Spectra Systems P2000 pump, an AS300 auto injector and a Spectra Systems UV2000 UV detector was used for the determination of chlorpromazine. The 125 x 4.0 mm ID analytical
column, packed with LiChrospher 60 RP-select B (5 µm particle size), was supplied by Merck.

**Methods**

Extraction of chlorpromazine from samples. Acetonitrile was used for the extraction of chlorpromazine residues from the muscle tissue and urine. The 5.0 ml urine and 5.0 g muscle tissue samples were extracted with 20 ml of acetonitrile. The samples were then centrifuged and the extracts were cleaned using the appropriate SPE cartridges. An analytical procedure for determination of chlorpromazine residues in the muscle tissue and urine is presented in Figure 2.

Figure 2: Analytical methods for the determination of chlorpromazine residues in muscle tissue and urine

<table>
<thead>
<tr>
<th>MUSCLE TISSUE</th>
<th>URINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 g of muscle tissue</td>
<td>5.0 ml of urine</td>
</tr>
<tr>
<td>addition of 20.0 ml of acetonitrile</td>
<td>addition of 20.0 ml of acetonitrile</td>
</tr>
<tr>
<td>sonification for 2 min and shaking for 30 min at 350 rpm at room temperature protected from light</td>
<td>shaking for 30 min at 350 rpm at room temperature protected from light</td>
</tr>
<tr>
<td>centrifugation for 10 min at 3000 rpm and 20 °C</td>
<td>centrifugation for 10 min at 3000 rpm and 20 °C</td>
</tr>
<tr>
<td>addition of 40 ml 10 % NaCl solution to 10.0 ml of supernatant</td>
<td>addition of 20 ml of redistilled water to 10.0 ml of supernatant</td>
</tr>
<tr>
<td>pass through the LiChrolut C18 cartridge previously activated with methanol and water</td>
<td>pass through the Bond Elut C8 cartridge previously activated with diluted ammonia solution (pH 8.0) and 1.0 ml of toluene</td>
</tr>
<tr>
<td>elution with 2.0 ml of acidic acetonitrile</td>
<td>elution with 3.0 ml of 4.0 % (V/V) solution of ammonia in methanol</td>
</tr>
<tr>
<td>evaporation under a gentle stream of nitrogen at 40 °C to dryness</td>
<td>evaporation under a gentle stream of nitrogen at 40 °C to dryness</td>
</tr>
<tr>
<td>dissolution of residues in 1.0 ml of mobile phase</td>
<td>dissolution of residues in 1.0 ml of mobile phase</td>
</tr>
<tr>
<td>injection of 150 µl of extract into the HPLC system</td>
<td>injection of 150 µl of extract into the HPLC system</td>
</tr>
</tbody>
</table>

HPLC measurements. High-performance liquid chromatography (HPLC) with UV detection at 255 nm was used for the determination of chlorpromazine. The mobile phase employed for the chromatographic separation was composed of a 0.05 M phosphate buffer with a pH of 4.5, and tetrahydrofuran and acetonitrile in a volume rate of 60 : 5 : 35 (by Arneth (7)). 150 µl of the extract was injected into the
HPLC system and the chromatographic separation was performed isocratically at a flow rate of 1 ml/min and a column temperature of 40 °C.

System suitability (SST) was tested before each set of measurements by considering the following parameters:

- theoretical plates (N): min. 1000
- capacity factor (k'): min. 1.0
- tailing factor on the 10 % of the peak height (T): max. 2.0
- RSD % of the retention time: max. 2.5 %

Results and discussion

A sensitive and selective analytical method has been developed for the determination of chlorpromazine residues in the muscle tissue and urine of food-producing animals. An extraction of chlorpromazine from the samples, a clean-up of extracts and a determination by high-performance liquid chromatography (HPLC) with UV detection were employed in the analytical procedure. The optimal solid phase extraction procedure was used to achieve a better selectivity of the method. The clean-up procedure and the enrichment of chlorpromazine residues in the muscle tissues and the urine were introduced, and slightly modified, on the basis of the methods published by Keukens and Aerts (6) and Arneth (7), respectively. Figure 2 in the Material and Methods section schematically shows both analytical procedures.

Chlorpromazine is a very polar substance and it tends to be adsorbed to fats. Due to this, the muscle tissue extracts were diluted with 10 % NaCl in the volume ratio of 1 : 4 prior to the solid phase extraction and the chlorpromazine was eluted from the separation cartridges using an acidic eluent (1 ml of 0.05 M H₂SO₄ in 100 ml of acetonitrile). In this case, the recoveries were generally acceptable but very hard to reproduce.

The clean-up and enrichment of the chlorpromazine residues in the urine samples was more convenient in alkaline conditions.

Appropriate chromatographic conditions were selected for the qualitative and quantitative determination of chlorpromazine in the muscle tissue and urine by high-performance liquid chromatography. The absorbance of chlorpromazine was tested in a wavelength range from 190 nm to 380 nm, using a standard solution of chlorpromazine in the concentration of 10 µg/ml. The maximum absorption was at 255 nm. The optimal chromatographic separations and the highest sensitivities were achieved using the Merck LiChrospher 60 RP-select B (150 x 4.0 mm ID; 5 µm particle size) column and a mobile phase composing of a 0.05 M phosphate buffer with a pH value of 4.5, tetrahydrofuran and acetonitrile in a volume ratio of 60 : 5 : 35 (7). The detection limit (LOD) was tested at the optimal chromatographic conditions, by injections of chlorpromazine standard solutions in concentrations below 200 ng/ml prepared in the mobile phase. LOD was 2.5 ng/ml and the linearity of the method, with the correlation coefficient > 0.999, was in the range from 2.5 to 200 ng/ml.
Typical chromatograms of a chlorpromazine standard solution in mobile phase at a concentration of 25 ng/ml, of both blank urine and muscle tissue samples and of both urine and muscle tissue samples spiked with 25 ng/ml and 20 ng/g of chlorpromazine, respectively, are presented in Figure 3.

The relatively good selectivity of the method for determining chlorpromazine residues in muscle tissue and in urine can be seen from Figure 3.

To determine the recovery of the method, blank samples of both urine and of muscle tissue were first spiked with chlorpromazine in a concentration of 25 ng/ml and 20 ng/g, respectively, and then also in a concentration of 10 ng/ml or 10 ng/g, respectively. Table 1 represents the recovery and precision of the determination of chlorpromazine residues in the urine and muscle tissue samples.
Table 1: Recovery and precision of the determination of chlorpromazine residues in the urine and muscle tissue samples (addition of 10 ng/ml and 25 ng/ml or 10 ng/g and 20 ng/g, respectively)

<table>
<thead>
<tr>
<th></th>
<th>urine</th>
<th>muscle tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>found (ng)</td>
<td>recovery (%)</td>
</tr>
<tr>
<td>addition of 10 ng/ml</td>
<td>8.32</td>
<td>83.2</td>
</tr>
<tr>
<td></td>
<td>7.87</td>
<td>78.7</td>
</tr>
<tr>
<td>addition of 25 ng/ml</td>
<td>8.40</td>
<td>84.0</td>
</tr>
<tr>
<td></td>
<td>9.05</td>
<td>90.5</td>
</tr>
<tr>
<td>addition of 10 ng/g</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>addition of 20 ng/g</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>mean (%)</td>
<td>84.1</td>
<td>mean (%)</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>5.8</td>
<td>RSD (%)</td>
</tr>
</tbody>
</table>

= measurement was not performed

From Table 1 it is evident that the mean recovery of the chlorpromazine added to the muscle tissue in a concentration of 10 ng/g was 37.2 % (RSD = 14.3 %; n = 6), and when added in a concentration of 20 ng/g it was 38.9 % (RSD = 15.3 %; n = 6). We tried to improve the relatively low recovery and the poor reproducibility of the parallel determinations with some modifications to the extraction and clean-up procedures. Various separation cartridges and types of eluent with varying pH values were employed. The relatively low recoveries were probably caused by adsorption of the chlorpromazine to fats and also by the strong polar interactions of the chlorpromazine with any available silanol groups of the separation cartridges. Our observations are comparable with those published by Keukens and Aerts (6).

Better reproducibility and higher recoveries were achieved with the urine samples. The mean recovery of the chlorpromazine added to the urine sample in a concentration of 10 ng/ml was 84.1 % (RSD = 5.83 %; n = 4) and 73.1 % (RSD = 21.5 %; n = 5) when the chlorpromazine was added in a concentration of 25 ng/ml.

Because of the poor reproducibility and low recovery of the method, especially in the muscle tissue, the samples, which were protected from light, were analysed daily with two parallel determinations of the spiked samples with chlorpromazine in concentrations of 10 and 20 ng/g or 10 and 25 ng/ml.

The limit of detection (LOD) and the limit of quantification (LOQ) were also determined. The LOD of chlorpromazine residues in the muscle tissue was 2.5 ng/g and in the urine 1.3 ng/ml. The LOQ was 10 ng/ml in the urine and 50 ng/g in the muscle tissue.

Although the limit of quantification for chlorpromazine residues in the muscle tissue is 50 ng/g, both analytical methods have been introduced for the routine monitoring of chlorpromazine residues in the tissue and urine of food-producing animals. According to the European legislation (Council Directive 96/23/EC) (13) all suspicious samples have to be confirmed using liquid chromatography with the detection performed by a mass selective detector (LC-MSD).
Chlorpromazine is characterised by its concentration in the kidney and its elimination from the body by urine. Literature data indicates (5, 6) that the kidney is a good target-organ for the monitoring of chlorpromazine residues. Chlorpromazine’s adsorption to fat is not expected in the kidney and for this reason, the recovery will probably be higher there than in the muscle tissue.

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References