SCREENING PROCEDURE FOR SIMULTANEOUS
DETERMINATION OF AZAPERONE, CARAZOLOL,
AND CHLORPROMAZINE IN ANIMAL URINE

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Abstract

A liquid chromatographic method coupled with UV-Vis and fluorescence detectors for the determination of azaperone, carazolol, and chlorpromazine residues in urine has been described. Analytes were isolated from biological samples with acetonitrile. The obtained extracts were applied to solid phase extraction Strata X-C 33 µm Polimeric Strong Cation columns. The analytes were eluted with alkalised acetonitrile. The whole procedure was validated according to European Decision 657/2002/EC. The detection limits were established at the level 2 µg/L for carazolol and 5 µg/L for azaperone and chlorpromazine. Detection capabilities for carazolol, azaperone, and chlorpromazine were 8.62, 18.9, and 6.53 µg/L, respectively. Recoveries were above 90%.

Key words: urine, neuroleptics, liquid chromatography.

Tranquilisers (e.g. azaperone) and β-adrenergic receptor blockers (e.g. carazolol) are often administered to reduce stress prior to handling, examining, or animal transport. Additionally, the anti-stress compounds administered to food-producing animals a few hours before slaughter, may give rise to residue problems. Eating meat containing high levels of these compounds can be harmful to consumers’ health. According to Commission Regulation (EU) 37/2010 of 22 December 2009, maximum residue levels (MRLs) have been established for azaperone in tissues taken from pigs, and for carazolol in tissues taken from pigs and cattle (4). Tranquilisers are also illicitly used in food-producing animals to enhance the feed conversion ratio by reducing animal activity. Phenothiazines (e.g. chlorpromazine) were used most frequently. Currently, their use is totally prohibited in food animals because of a possible genotoxic activity (2, 7).

Several analytical methods have been reported for the determination of tranquilisers and β-blockers in biological material. These methods involve liquid chromatography coupled with UV-Vis detection (10, 11, 14, 15), electrochemical detection (13), or FLD detectors (3, 14). Now, new analytical procedures utilise detection by mass spectrometry for screening or confirmatory purposes (1, 8, 9, 12). These procedures have been mostly used in the analysis of animal tissues (5, 6, 8, 16).

In contrast to methods focusing on animal tissues, we thought that there is a considerable need for a method that can be applied to animal urine. From the point of food safety and veterinary applications, such a method is important because of a possibility to control the use of azaperone, carazolol, or chlorpromazine not only at slaughterhouse level but also in urine samples taken directly from live animals on farms. This method also could be applicable for veterinary diagnostic toxicology or anti-doping control.

Therefore, a screening analytical procedure for the simultaneous determination of azaperone, carazolol, and chlorpromazine in urine by liquid chromatography coupled with UV-Vis and spectrofluorimetric detectors was developed.

Material and Methods

Reagents. All organic solvents were HPLC grade and all chemicals were analytical grade. Carazolol and azaperone were obtained from Dr Ehrenstorfer GmbH (Germany) and chlorpromazine was obtained from Riedel-de Haën (Germany). Ammonia solution (25%), sodium acetate, acetic acid, and sodium dihydrogen phosphate were from P.O.Ch. (Poland). Acetonitrile, methanol, and sodium hydroxide were from J.T. Baker (Germany). Strata X-C 33 µm Polymeric Strong Cation 500 mg/6 mL extraction columns were purchased from Phenomenex (Germany).

Standard solutions and buffers. Stock standard solutions (1 mg/mL) were prepared by weighing 10.0 ± 0.1 mg of standard substances and dissolving them in 10 ml of methanol (stable for one year at ~18°C). Working standard solutions and mixed standard solutions were prepared by diluting suitable
The blank samples were measured by adjusting 0.03 M sodium acetate solution to pH 6.0 with acetic acid and filtered through a 0.45 µm PTFE filter. A 0.05 M phosphate buffer with a pH 7.0 was prepared by dissolving 6.9 g of sodium dihydrogen phosphate monohydrate in water and the pH was adjusted to 7.0 with 10 M sodium hydroxide.

Preparation of urine samples. To optimise sample preparation and validate the whole procedure, urine was taken from adult cattle. The samples were centrifuged and then frozen at -20°C until the time of analysis.

Before validation, the blank samples were spiked with the working solution of carazolol at the level of 7.5, 15.0, or 22.5 µg/L, azaperone at 10.0, 20.0, and 40.0 µg/L, and chlorpromazine at 5.0, 10.0, and 20.0 µg/L. The spiking solutions were mixed with samples using vortex mixer and kept at ambient temperature for 10 min before analyses.

Extraction and clean up. A volume of 5.0 ±0.1 ml of sample was measured into a 50 ml plastic centrifuge tube and then 15 ml of acetonitrile was added. The content of the tube was mixed with vortex mixer for 1 min. The obtained solution was centrifuged at 3,500 rpm, at -20°C for 20 min and the supernatant was separated and transferred into the 50 ml tube with screw cap. To the tube with supernatant, 35 ml of 0.05 M phosphate buffer, pH 7.0, was added, mixed, and sonicated for 5 min. The Strata X-C SPE cartridge was conditioned with 10 ml of methanol and 10 ml of 0.05 M phosphate buffer, pH 7.0. After sonication, the sample extract was loaded to SPE cartridge. The bed of cartridge was rinsed with 10 ml of fresh re-distilled water, 10 ml methanol, and dried for 5 min under vacuum. Analytes were eluted twice with 4 ml of alkaline acetonitrile (95:5, v/v). The combined eluates were evaporated to dryness at ≤50°C under nitrogen. The dry residue was dissolved in 0.5 ml of LC mobile phase and transferred to a vial for analysis.

Liquid chromatography. The chromatographic system consisted of a Shimadzu Class VP Series high performance liquid chromatograph (Germany) equipped with a quaternary pump, degasser, autosampler, and column heater. The UV-Vis was set at 254 nm for chlorpromazine, while the FLD was set at λex = 245 nm and λem = 345 nm for detection of carazolol and azaperone. The both detectors were connected online. The CLASS-VP software controlled the LC system and processed the data. The chromatographic separation was performed with gradient elution (Table 1) on a C18 column (5 µm, 250 x 4.6 mm, Phenomenex, USA). A C18 guard cartridge (4 x 3 mm, Phenomenex) was used prior to the analytical one. The basic mobile phase for LC analyses consisted of acetonitrile and acetate buffer (0.03 M sodium acetate, pH 6.0). The injected volume was 50 µL and the separation of the analytes was accomplished with flow of 1 mL/min at 25°C.

Evaluation of the procedure. The whole procedure was validated according to recommendations of the European Decision 657/2002/EC (2). The validation was performed in terms of selectivity, specificity, accuracy, precision (repeatability and within-laboratory reproducibility), as well as calculation of decision limit (CCα) and detection capability (CCβ). Three-point matrix-matched calibration curves were tested assessing the recorded peak areas versus the corresponding concentrations of the analytes from the pig urine spiked samples. Accuracy (recovery) was evaluated at three concentrations levels: carazolol at 7.5, 15.0, and 22.5 µg/L, azaperone at 10.0, 20.0, and 40.0 µg/L, and chlorpromazine at 5.0, 10.0, and 20.0 µg/L (level of spiking samples was in accordance with suggestion of EURL in Wageningen (personal communication). The recoveries were calculated by comparing the measured concentrations to the spiked concentrations representing 100% recovery. Precision (repeatability and within-laboratory reproducibility) was determined by the repeated analysis (n=6) of urine sample spiked with carazolol at 7.5, 15.0, and 22.5 µg/L, azaperone at 10.0, 20.0, and 40.0 µg/L, and chlorpromazine at 5, 10, and 20 µg/L, from run-to-run during 1 d and 3 d, respectively. Precision was evaluated by calculating the relative standard deviation (RSD) of the results obtained for each level of the target compound.

Results

Typical chromatograms obtained from standard solution of neuroleptics at 100 ng/mL, the extract of blank bovine urine, and the extract of urine spiked with neuroleptics are shown in Fig. 1. For the separation we used the column Luna C18 (250 x 4.6 mm, 5 µm) with the basic mobile phase for LC analyses consisting acetonitrile and an acetate buffer (0.03 M sodium acetate, pH 6.0) in gradient mode (Table 1).

The whole procedure was validated according to European Decision 657/2002/EC and the validation parameters obtained for neuroleptics determinations in spiked urine samples are listed in Table 2.

Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Acetonitril (%)</th>
<th>Sodium acetate, pH=6 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>13</td>
<td>85</td>
<td>15</td>
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<tr>
<td>16</td>
<td>85</td>
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<tr>
<td>18</td>
<td>50</td>
<td>50</td>
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<tr>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>
Fig. 1. Typical chromatograms of: (a) standard solution of analytes (carazolol 0.15 µg/mL, azaperone 0.2 µg/mL, and chlorpromazine 0.1 µg/mL); (b) blank urine sample; (c) blank urine sample spiked with carazolol at the level of 15 µg/L, azaperone at the level of 20 µg/L, and chlorpromazine at the level of 10 µg/L.
Discussion

In many studies, the extraction of neuroleptics from biological material was done with acetonitrile (3, 5, 11-16). With these reports in mind, we started with acetonitrile to extract azaperone, carazolol, and chlorpromazine from urine.

Preliminary experiments showed that the recoveries of analytes from urine samples were more than 90%, suggesting that acetonitrile extraction is a reliable method for sample preparation.

Sorbent performance plays a vital role in SPE method development because it has different effect on analyte recovery, precision, cleanliness of extracts, and sample preparation time.

With the increasing challenges, the conventional silica base SPE technology was improved by the development of new modified polymeric sorbents (3). In this study, to improve selectivity, recovery, and precision, a clean-up procedure of acetonitrile urine extracts, as well as the determination of azaperone, carazolol, and chlorpromazine was optimised using various polymer SPE sorbents. As it was found, the SPE mixed-mode polymeric cation exchanger (Strata X-C 33 µ Polymeric Strong Cation 500 mg/6 mL) coupled with basic elution (acetonitrile:ammonia solution 25%) gave acceptable and reproducible recoveries of analytes.

Lastly, we studied the influence of the loading pH on the extraction yields obtained with an SPE column containing a polymer sorbent. Using spiked blank samples, we made sure that no matrix interferences appeared after pH modifications. Loading at phosphate buffer (pH 7.0) is the best, as the molecules are not protonated and this increases affinity for the column. The yield records after solid-liquid extraction followed by purification on a Strata X-C column were between 95%-105%.

In many studies, the chromatographic conditions, especially the analytical column and the composition of mobile phase, were optimised through several trials to achieve the desired sensitivity, separation, run time, and symmetric peak shapes for carazolol, azaperone, or chlorpromazine (7, 14). In the presented procedure, Luna C18 column (5 µm, 250 x 4.6 mm) was selected, as it produced the satisfactory separation, peak shape, and a shorter analytical run time than the other columns. A sodium acetate buffer combination with acetonitrile resulted in the sensitive signals with optimised gradient elution.

Most analytical procedures utilise UV and fluorescence detectors for separate determination of carazolol, azaperone, or chlorpromazine (3, 10, 11, 14, 15). In our laboratory, we used two detection systems (UV and FLD) for simultaneous qualitative and quantitative analysis of the tested compounds. We decided to use both detectors at one analytical run because of the possibility of interferences from matrices compounds, especially with carazolol that has a very short retention time. The use of fluorescence detection eliminates interferences from biological compounds.

The validation was preformed according to the recommendations of the Commission Decision 2002/657/EC. The obtained results were repeatable and reproducible with acceptable recoveries from spiked urine samples. The developed procedure is simple and validation results indicate that it can be used for the control of urine samples taken from live animals after application of tranquillisers or β-blocker. As it was found in our preliminary studies, the developed procedure is applicable not only for bovine urine but also for urine samples taken from pigs or other animals (12). In our residue control programme, the confirmations of positive results are performed by analytical procedure utilising liquid chromatography with tandem mass spectrometry.

Table 2
Validation results obtained for tranquilisers and β-blocker in bovine urine

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Analytes</th>
<th>Azaperone</th>
<th>Carazolol</th>
<th>Chlorpromazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear regression equation (y=ax+b)</td>
<td>Azaperone</td>
<td>y = 871.14x + 8,117.83</td>
<td>Carazolol</td>
<td>y = 82,084x + 114,354</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>Azaperone</td>
<td>0.9852</td>
<td>Carazolol</td>
<td>0.9991</td>
</tr>
<tr>
<td>Decision limit (CCα) (µg/L)</td>
<td>Azaperone</td>
<td>13.34</td>
<td>Carazolol</td>
<td>8.17</td>
</tr>
<tr>
<td>Detection capability (CCβ) (µg/L)</td>
<td>Azaperone</td>
<td>18.09</td>
<td>Carazolol</td>
<td>8.62</td>
</tr>
<tr>
<td>Limit of detection (µg/L)</td>
<td>Azaperone</td>
<td>5</td>
<td>Carazolol</td>
<td>2</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>Azaperone</td>
<td>90.4-107.2</td>
<td>Carazolol</td>
<td>96.3-103.8</td>
</tr>
<tr>
<td>Repeatability (%)</td>
<td>Azaperone</td>
<td>6.3-8.0</td>
<td>Carazolol</td>
<td>1.5-3.4</td>
</tr>
<tr>
<td>In-laboratory reproducibility (%)</td>
<td>Azaperone</td>
<td>7.8-12.0</td>
<td>Carazolol</td>
<td>2.1-5.3</td>
</tr>
</tbody>
</table>

References