Determination of Drug Residues in Urine of Dogs Receiving Anti-Cancer Chemotherapy by Liquid Chromatography–Electrospray Ionization-Tandem Mass Spectrometry: Is There An Environmental or Occupational Risk?

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Abstract

Cytotoxic drugs, previously used only in human medicine, are increasingly utilized for cancer treatment in veterinary practice. We developed and validated a liquid chromatography (LC)–electrospray ionization-tandem mass spectrometry (MS–MS) method to determine vincristine, vinblastine, cyclophosphamide, and doxorubicin in canine urine. Sample pretreatment consisted of liquid–liquid extraction, and LC separation was carried out on an RP C18 column employing a 0.5% formic acid/methanol gradient system. The analytes were detected in positive ion mode using the MS–MS scan mode. The mean recoveries in six different urine samples were between 64.2% and 86.9%. Limits of quantitation were 0.5 µg/L for vincristine and vinblastine, 1 µg/L for cyclophosphamide, and 5 µg/L for doxorubicin; limits of detection were approximately 0.25 µg/L for vincristine, vinblastine, and cyclophosphamide and 0.5 µg/L for doxorubicin. It could be demonstrated that all investigated drugs are found in urine of dogs undergoing chemotherapy. In samples from day 1 after chemotherapy, as much as 63 µg/L vincristine, 111 µg/L vinblastine, and 762 µg/L doxorubicin could be detected. Cyclophosphamide showed only minor concentrations on day 1, but up to 2583 µg/L could be found directly after chemotherapy. These initial data show that there might be a potential contamination risk when administering cytotoxics in veterinary medicine.

Introduction

Veterinary pharmaceuticals are used in large amounts worldwide. Unfortunately, there are only limited data with high uncertainty available on the sale and usage of these compounds in Denmark, Germany, the U.K., and the U.S. However, the European Federation of Animal Health estimates that approximately 8500 tons of antibiotics were used in human medicine and 4700 tons in veterinary medicine in the European Union (including Switzerland) in 1999 (1). Tetracyclines and sulfonamides represent the major classes of veterinary antibiotics prescribed most intensively worldwide (2,3). The use of large amounts of these compounds in intensive livestock farming led primarily to residues in liquid manure (4,5) and in topsoil (4,6).

In addition, veterinary pharmaceuticals are also used in the treatment of companion animals. As a result of improvements in nutrition and better prophylactic and therapeutic medical care, pet animals live to older ages. This, in combination with improved diagnostic capabilities has, as in human medicine, led to an increasing occurrence of neoplastic diseases in dogs and cats, which has resulted in a demand for cancer treatment options, including cytotoxic drugs (7). The increased utilization of chemotherapeutic agents in small animal medicine, however, introduces the aspect of potential occupational and/or environmental hazards caused by the residues of these drugs. The International Agency for Research on Cancer (IARC) has classified cytotoxic drugs according to their carcinogenicity in humans. Thus, cyclophosphamide (CPP) is classified as carcinogenic in humans, doxorubicin (DXR) as probably carcinogenic in humans, and vincristine (VIC) and vinblastine (VIB) as not classifiable as to carcinogenicity in humans. Nevertheless, all of these substances are teratogenic. In addition, cyclophosphamide and doxorubicin are mutagenic in several in vitro tests (8–12). Furthermore, it has been shown (13–15) that none of the drugs investigated here (or analogues) meet the criteria for being readily biodegradable (≥ 60% within 28 days).

In a number of studies in human medicine, routes of exposure to cytotoxic drugs in hospital personnel have been inves...
tigated (16–21). But, at present, there are no clinical studies investigating concentrations of cytostatic drugs including vincristine, vinblastine, cyclophosphamide, and doxorubicin (Figure 1) in canine body fluids such as serum and urine available. Therefore, it is unknown whether body fluids of pet animals treated with cytotoxic drugs exhibit a similar risk for hospital personnel and especially pet owners as in human medicine. Currently, guidelines on the prevention of occupational and environmental exposure to cytotoxics in veterinary medicine are based on data from human sources (22).

There are several methods, such as high-performance liquid chromatography with mass spectrometry (HPLC–MS) or tandem mass spectrometry (HPLC–MS–MS), for investigating various substance classes in different biological fluids (18,23–31). Yet, there is no multi-method available to selectively detect the four veterinary cytotoxics investigated here in the microgram-per-liter range. A multi-method is reasonable because patients often receive a combination therapy consisting of several chemotherapeutic agents.

In the present paper, we provide a description of the methods developed for the extraction and determination of frequently used cytotoxic drugs in canine urine. First results of the residue measurement of vincristine, vinblastine, cyclophosphamide, and doxorubicin are shown, and potentially associated occupational and environmental risks are discussed.

Materials and Methods

Chemicals and reference substances

Vincristine, vinblastine, cyclophosphamide, and doxorubicin were purchased from Sigma (Munich, Germany). All other chemicals were of analytical or HPLC grade. Water was prepared in house with a MilliQ System (Millipore, Eschborn, Germany), and stock solutions of all standards by dissolving 1 mg of the drug in 1 mL methanol. The stock solutions were stored at –20°C and stable for at least two months. We prepared fresh working dilutions in 10 mM ammonium acetate in methanol on the day of use.

 Patients

Dogs diagnosed with lymphoma or mast cell tumor were included in the study. Patients with lymphoma (n = 7) were treated with a combination chemotherapy protocol consisting of intravenous administration of vincristine, cyclophosphamide, and doxorubicin with weekly treatment intervals for a duration of 12 weeks as described previously (32). Patients with mast cell tumors (n = 2) were treated with a protocol consisting of a combination of weekly intravenous vinblastine followed by oral cyclophosphamide for three consecutive days (33).

Sampling procedure

Urine samples were collected from dogs before and directly after receiving chemotherapy and on day 1 after chemotherapy. Urine samples were either voided or cystocentesis samples. The samples obtained before and after chemotherapy were immediately frozen and stored at –80°C prior to analysis. Because the patients left the hospital after receiving chemotherapy, the owners gathered voided samples on day 1 and refrigerated these until the next hospital visit, at which time they were subsequently frozen at –80°C.

Extraction procedure

In having a similar structure to tetracyclines, doxorubicin strongly absorbs a variety of materials (34). Therefore, all glassware used was heated for 2 h at 450°C, cooled, rinsed with 2.5 mL of a saturated methanolic EDTA solution, and air-dried prior to analysis (4).

One milliliter of canine urine was intensively stirred with 1.2 mL 1 M phosphate buffer (Na₂HPO₄, pH 9.5) in a 10-mL glass tube for 1 min. Six milliliters of dichloromethane (which shows substantially better extraction results when compared to ethyl acetate) was added, again intensively stirred for 3 min, and then kept in an automatic shaker for another 15 min. The sample was centrifuged for 10 min at 1000 × g, and the organic phase was transferred into a 25-mL flask. The extraction procedure was repeated, and the combined organic phases were evaporated to dryness under vacuum at 40°C. The dry residue was redissolved in 200 µL of 10 mM ammonium acetate in methanol. Prior to LC–MS–MS analysis, the samples were kept at 10°C in the autosampler.

HPLC–MS

MS was carried out using an LCQ ion trap with an electrospray ionization source (Thermo Finnigan, San Jose, CA). All cytotoxic drugs (10 ng/µL) were infused through an integrated syringe pump at a flow rate of 5 µL/min for tuning the MS and optimizing capillary temperature, isolation width, collision energy in MS–MS mode, sheath gas, and auxiliary gas flow rates. The source polarity was set to positive, and the spray needle voltage was 5 kV. Drying gas was nitrogen generated from pressurized air in an Ecoinert 2 ESP nitrogen generator (DWT, Gelsenkirchen, Germany). The optimized conditions were as follows: sheath gas flow set at 100 units, auxiliary gas turned off, and capillary temperature 150°C. Substance specific settings (isolation width, collision energy) are listed in Table I.

The HPLC system employed was a gradient system con-

![Figure 1. Molecular structures of vincristine, vinblastine, cyclophosphamide, and doxorubicin.](https://academic.oup.com/jat/article-abstract/34/3/142/766541/143)
sisting of a P4000 pump, an AS3000 autosampler (ThermoQuest, San Jose, CA), and a Hypersil Gold column (4.6 × 150 mm, 5 µm, Thermo Electron, Waltham, MA) operated at 23°C. The flow of 1 mL/min was split 1:10 for matrix reduction reasons before entrance into the MS. The mobile phase consisted of 0.5% formic acid in water with 1 mM ammonium acetate (solvent A), and methanol (solvent B). After 1 min 100% A, the gradient run was 0–60% B in 9 min and then held at 60% B for 2 min. At the end of each run, the column was rinsed for 3 min with 99% methanol and re-equilibrated for 9 min with solvent A. For optimal peak shape and baseline separation, it was necessary to choose initial conditions for the gradient of 100% aqueous phase. Although the manufacturers suggest running the column with at least 5% organic solvent, it shows good performance over years. All injections were performed employing the pushloop injection mode of the autosampler. Sample analysis was performed on the day of sample preparation. The injection volume was generally 8 µL. Urine samples containing cytotoxics in amounts beyond the linear calibration range were diluted with 10 mM ammonium acetate/methanol and re-injected. The autosampler was rinsed after each injection with 3 mL of 90% methanol/10% 10 mM oxalic acid in water.

**Method validation**

The method was validated for linearity, accuracy (recovery), precision, matrix interference, limit of detection (LOD), limit of quantitation (LOQ), selectivity, and stability of the analytes. Linearity studies were carried out with pooled control urines (obtained from dogs never having received chemotherapy or been in contact with cytotoxic drugs) spiked prior to extraction at the 0.5, 1, 5, 10, 50, 250, 1000, and 2500 µg/L level in replicates of three. However, because of the possibility of higher intersubject variability of urine in particular, we performed additional recovery experiments in five individual control urines at the 5, 50, and 500 µg/L levels. After spiking, the urine samples were allowed to equilibrate for 10 min at room temperature. Then, all samples were liquid–liquid extracted, processed as described earlier, and analyzed by HPLC–ESI-MS–MS. The accuracy was determined as percent recovery and was calculated as an average of all experiments carried out in the various control urines considering all spiking levels. Please note that all recovery data were obtained from samples that passed through the entire method. Thus, here recovery stands for overall process efficiency compensating for any matrix effects.

Furthermore, matrix effects were quantitatively evaluated by employing a post-extraction spike method under consideration of methods described recently (35,36). Briefly, blank matrix samples which have been carried through the sample preparation process were spiked at three different concentrations (0.25, 2, and 8 ng on column representing 6.25, 50, and 200 µg/L in urine) with the cytotoxics under investigation. Subsequently, the response of the cytotoxics in blank matrix sample was compared to that of the compounds in methanol/ammonium acetate.

The LOQ was based on triplicates of spiked urine samples from the linearity study and was determined to be the concentration analyzed with a relative standard deviation of less than 20%. The LOD was determined to be the concentration where the signal-to-noise ratio was at least 5:1.

Further, we carried out day-to-day variation experiments with two authentic samples for each substance under investigation including a low and a high concentration. The samples were split into five 1.2-mL portions, frozen, and processed on five individual days.

Peak identification was carried out according to Decision 2002/657/EU. The criteria for the confirmation of peak identity were as follows: retention time [relative standard deviation (RSD) < 1%] and at least two MS–MS ions specific for the standard spectrum. The maximum permitted tolerances for relative ion intensities were ± 20% for a relative intensity > 50%, ± 25% for a relative intensity between 20% and 50%, ± 30% for a relative intensity between 10% and 20%, and ± 50% for a relative intensity < 10%.

The stability of the cytotoxics in canine urine was assessed in eight authentic samples per substance stored after long-term storage (3 years at –80°C). In another four samples, freeze/thaw stability after three cycles was checked. Furthermore, the post-preparative stability was investigated in extracts kept in the autosampler tray for 24 h at 10°C.

**Results and Discussion**

We developed a new HPLC–ESI-MS–MS method permitting the multi-detection of vincristine, vinblastine, cyclophosphamide, and doxorubicin residues in canine urine. All validation data of the new method are given in Table II. The mean recoveries derived from all investigated urines were as follows: 71.3 ± 11.5 (VIC), 64.2 ± 12.8 (VIB), 86.9 ± 17.5 (CPP), 78.1 ± 20.0 (DXR). As shown in Table III, the day-to-day variation ranged from 14.7 to 28.9%. One reason for these variations may be the use of an ion-trap mass analyzer, which is well-known to generally produce higher variations in quantitative analysis when compared to triple-quadrupole systems (37,38).

In matrix calibration of the four drugs in urine showed linearity up to 100 ng per injection (r² ≥ 0.998). Quantification was obtained by comparing the peak areas of the sample with that of external calibration curves, and all data were corrected for
recovery. For the construction of calibration curves, standards with 0.5, 1, 5, 10, 50, 250, 1000, and 2500 µg/L were prepared independently in 10 mM ammonium acetate/methanol. With these standard solutions, a linear external calibration curve was prepared for the four cytotoxics with $r^2 > 0.992$.

Matrix effects in quantitative LC–MS–MS analysis of biological samples are well-known by many investigators (39). For vinblastine and vincristine, a linear signal suppression of approximately 29%, for cyclophosphamide of less than 15%, and for doxorubicin no suppression or enhancement was determined. With the exception of doxorubicin, these matrix effects correlate well with our recovery studies and were determined to be an overall process efficiency. Thus, it is reasonable to correct all values for recovery when applying this method.

The investigation of authentic samples after a storage period of 3 years at –80°C revealed amounts for CPP and DXR in good accordance, whereas for VIC and VIB, slightly increased amounts could be detected. All compounds were stable even after three freeze/thaw cycles. CPP, VIC, and VIB remained stable in extracts after 24 h storage in the autosampler, whereas the amounts of DXR decreased by 27%. Thus, samples containing DXR must be measured on the day of sample preparation.

The LOQs were 0.5 µg/L for vincristine and vinblastine, 1 µg/L for cyclophosphamide, and 5 µg/L for doxorubicin in urine, and the LODs were approximately 0.25 µg/L for vincristine, vinblastine, and cyclophosphamide and 0.5 µg/L for doxorubicin. The different LODs and LOQs may be due to the different behavior of the three substance classes during electrospraying. As recently shown for colchicine, alkaloids tend to easily protonate leading to high signals and thus display very low LOQs (40). In Figure 2A–2C, LC–MS–MS chromatograms and MS–MS spectra of a standard sample and a urine sample spiked with all four cytotoxics are shown at a concentration of 50 µg/L. All compounds under investigation can be confirmed unequivocally with two (vincristine, doxorubicin) or three (vincristine, cyclophosphamide) characteristic MS–MS ions according to Decision 2002/657/EU (41).

The MS–MS analyses of vincristine and vinblastine were carried out with the doubly charged ions. Tests performed during method development showed an approximately 4–5 times higher signal intensity for these ions. For this reason the precursor masses were $m/z$ 413.5 (vincristine) and $m/z$ 406.5 (vinblastine) (Table I), representing the molecular masses of 825.0 g/mol for vincristine and 811.0 g/mol for vinblastine. Even the fragmentation led to doubly charged MS–MS ions, thus resulting in MS–MS spectra shown in Figure 2B–2C.

### Table II. Validation Data

<table>
<thead>
<tr>
<th></th>
<th>VIC</th>
<th>VIB</th>
<th>CPP</th>
<th>DXR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>0.5–2500 µg/L</td>
<td>0.5–2500 µg/L</td>
<td>1.0–2500 µg/L</td>
<td>5.0–2500 µg/L</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.9996</td>
<td>0.9980</td>
<td>0.9998</td>
<td>0.9993</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>0.25 µg/L</td>
<td>0.25 µg/L</td>
<td>0.25 µg/L</td>
<td>0.5 µg/L</td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>0.5 µg/L</td>
<td>0.5 µg/L</td>
<td>1.0 µg/L</td>
<td>1.0 µg/L</td>
</tr>
<tr>
<td>Accuracy*</td>
<td>71.3%</td>
<td>64.2%</td>
<td>86.9%</td>
<td>78.1%</td>
</tr>
<tr>
<td>Precision†</td>
<td>2.0–14.8%</td>
<td>0.3–12.8%</td>
<td>1.6–13.9%</td>
<td>6.6–18.0%</td>
</tr>
</tbody>
</table>

* Given as overall process efficiency values derived from all recovery data.
† Given as relative standard deviation (RSD); ranges derived from linearity study.

Figure 2. Chromatograms and mass spectra of compounds under investigation: ion chromatograms of vincristine (VIC), vinblastine (VIB), cyclophosphamide (CPP), and doxorubicin (DXR) in a spiked urine sample (50 µg/L corresponding to approximately 2 ng on column) with LC–MS–MS (A); corresponding MS–MS spectra of these compounds (B); and MS–MS spectra obtained from a standard solution (250 µg/L corresponding to 2 ng on column) (C).
We successfully demonstrated the applicability of the new method in urine of nine dogs receiving chemotherapy. The amounts found differ between substances because of the varied standard dosages of the four different drugs administered, which amount to 200 mg/m² body surface area (BSA) (CPP), 30 mg/m² BSA (DXR), 2 mg/m² BSA (VIB), and 0.7 mg/m² BSA (VIC), but surely also because of their different pharmacokinetics (Figure 3).

Cyclophosphamide and doxorubicin can be found in substantial amounts immediately after administration: 2583 µg/L cyclophosphamide and 3631 µg/L doxorubicin. Because of the intensive hepatic metabolism of cyclophosphamide (42), only traces (< 8 µg/L) of the substance are detectable on day 1. In the case of doxorubicin, up to 762 µg/L were found on day 1. Vincristine and vinblastine showed, as expected, similar behavior with lower values (up to 305 µg/L for vincristine and 142 µg/L for vinblastine) immediately after administration if compared to cyclophosphamide and doxorubicin. Furthermore, vincristine and vinblastine were found in all day 1 samples (Figure 3). Urine sampling directly after treatment took place during the first 1–2 h following the completion treatment. Therefore, these variations in the exact time point of these urine samplings may explain the ranges of residue concentrations measured here. A variation in residue concentrations on the day following chemotherapeutic treatment may be due to urine sampling by the pet owner outside of the clinic, which bears certain limitations. However, this investigation aimed at demonstrating the presence or absence of residues under routine conditions in which canine chemotherapy patients are not hospitalized but return home after treatment.

Previous studies in human medicine mostly analyzed only one of the substances investigated here on the basis of their frequency of use. Human urine has mainly been investigated in association with exposure studies in health care personnel (17–19,43). The different exposure routes in human medicine include dermal exposure during preparation, administration, and decanting of patients’ urine or cleaning of toilets used by cancer patients. The first to investigate mutagenicity of urine from nurses was Falck et al. (44). Sottani et al. (45) analyzed cyclophosphamide in urine samples of hospital personnel.

Table III. Day-to-Day Variation of the LC–MS–MS Method in Urine Samples Containing the Four Cytostatics Under Investigation

<table>
<thead>
<tr>
<th>Sample (No.)</th>
<th>n</th>
<th>Conc. (µg/L)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>4.3 ± 0.8</td>
<td>17.8</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>16.7 ± 3.2</td>
<td>18.8</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>5</td>
<td>4.1 ± 0.9</td>
<td>21.9</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>263.1 ± 67.3</td>
<td>25.6</td>
</tr>
<tr>
<td>Vinblastine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>5</td>
<td>7.3 ± 1.1</td>
<td>15.3</td>
</tr>
<tr>
<td>79</td>
<td>5</td>
<td>82.9 ± 12.2</td>
<td>14.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>12.6 ± 3.6</td>
<td>28.9</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>318.9 ± 74.3</td>
<td>23.3</td>
</tr>
</tbody>
</table>

Figure 3. Measurement of drug residues in urine of dogs receiving anticancer chemotherapy (number of measurements in parentheses on x-axes): 6 dogs/18 treatments (A), 2 dogs/7 treatments (B), 6 dogs/10 treatments (C), and 6 dogs/16 treatments (D). Box-Whisker-Plot was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Unfortunately, it was not possible to obtain urine samples at every time point for each dog. A long-term study was recently published (50).
before and after work. The amounts of cyclophosphamide measured ranged from 0.1 to 1.9 µg/L. By way of the previously mentioned pathways, the cytotoxic drugs also gain access to the environment (i.e., wastewater and sewage treatment plants) (46) and finally reach rivers (47–49).

The authors acknowledge that the use of chemotherapy in human medicine exceeds the use in veterinary oncology by far. But whereas in human medicine the entrance routes of chemotherapeutic agents are known and well-understood as pointed out earlier, in veterinary medicine, dogs treated with cytotoxics may exhibit a hitherto unknown way of exposure. After treatment, owners take their pets home from the hospital. Here, the close environment and surrounding people (family members, friends) may get into close contact with the dog, possibly including its body fluids. In the present study, it could be shown that there might be a potential contamination risk with cytotoxics by dog urine for a certain time period following chemotherapeutic treatment (Figure 3).

Because these pharmaceuticals are mutagenic, teratogenic, and partially carcinogenic according to the IARC, exposure to these compounds should be avoided. The IARC assesses any detectable levels of cytotoxics as a hazard; therefore, measurable cytotoxic residues in canine urine may be seen critically. Thus, a sensitive method for the determination of cytotoxic drugs utilized in veterinary medicine is necessary. The method presented here allows the simultaneous determination of vincristine, vinblastine, cyclophosphamide, and doxorubicin with low LOQs and LODs. This enables investigation of the presence or absence of hazards associated with chemotherapy use in companion animals as well as further development and improvement of guidelines (22) for preventing occupational and environmental exposure to cytotoxic drugs in veterinary medicine.

Conclusions

We have demonstrated that LC–ESI-MS–MS combined with an appropriate sample cleanup is the method of choice for the simultaneous measurement of vincristine, vinblastine, cyclophosphamide, and doxorubicin in urine in the microliter-per-gram range. The application of this method makes it possible to detect significant amounts of these drugs belonging to three different substance classes of cytotoxics in urine from dogs treated with chemotherapy. Investigations are currently underway to determine any possible hazards of environmental contamination as well as potential risks for people in contact with the treated animals.

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References


