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RESEARCH PAPER

A new rotating-disk sorptive extraction mode, with a copolymer of divinylbenzene and *N*-vinylpyrrolidone trapped in the cavity of the disk, used for determination of florfenicol residues in porcine plasma

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Abstract A novel extraction approach was developed based on rotating-disk sorptive extraction (RDSE). In this approach the rotating-disk extraction device consists of a Teflon disk, with a cavity that is loaded with a commercial sorbent phase selected according to the polarity of the analyte. To avoid leakage of the sorbent, the cavity is covered with a fiberglass filter and sealed with a Teflon ring. The proposed novel analytical RDSE technique was used in this study to determine florfenicol levels in plasma as a model analyte, or sample system, to describe the pharmacokinetics of a veterinary formulation. The sorbent used for this application was the copolymer of divinylbenzene and *N*-vinylpyrrolidone (Oasis HLB), which was selected because the florfenicol molecule contains both hydrophilic and lipophilic moieties. After the extraction, final determination of the analyte was performed by HPLC–DAD. Calibration plots and other analytical features were obtained after 90 min of extraction. The calibration plot was linear over the interval 0.4–16 μg mL^{-1} (n=6), with R^2= 0.9999. Recovery and repeatability were determined using a blank plasma sample spiked with 4.8 μg mL^{-1} florfenicol. A recovery of 91.5 %, with a relative standard deviation (RSD) of 8.8 %, was obtained when the extraction was evaluated using six different rotating-disk devices. Precision was also assessed, using the same disk (containing the same sorbent phase) for eight aliquots of the same sample. The RSD under these conditions was 10.2 %, clearly indicating that the sorptive phase could possibly be re-used. Accordingly, RDSE is a suitable sample preparation alternative to liquid–liquid extraction (LLE), solid-phase extraction (SPE), and stir-bar sorptive extraction (SBSE).

Keywords Rotating-disk sorptive extraction · Florfenicol · Oasis HLB · Plasma · Sample preparation

Introduction

One of the most important steps in sample preparation for determination of trace amounts of organic compounds is the extraction and/or separation of the analyte from the matrix. This increases the sensitivity of the chemical measurement, and minimizes the interference associated with the matrix. In this context, extraction techniques have been the focus of intensive research over the last 15 years, with the objective of achieving advances in automation, miniaturization, and simplification [1, 2].

Currently, the most-used replacement for the traditional liquid–liquid extraction (LLE) method for separating organic pollutants from liquid samples is solid-phase extraction (SPE) [3]. An important aspect of SPE performance is control of the flow rate during sample loading, because in this technique the flow of the sample is almost exclusively unidirectional. Flow that is too fast can result in low recovery, caused by breakthrough during the analyte retention step or inadequate elution during the elution step [3].

In addition, several "solvent-free" sample-preparation strategies for liquid samples have been developed, starting from solid-phase microextraction (SPME) [4], which are based on the use of appropriate polymeric sorbents for extraction and concentration of the target compounds. One of the

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most representative extraction techniques belonging to this group is stir-bar sorptive extraction (SBSE), which was introduced in 1999 [5] with the purpose of increasing sensitivity compared with SPME. The extraction device in SBSE contains a volume of extracting phase much higher than that used in SPME.

As an alternative to SBSE, rotating-disk sorptive extraction (RDSE) was introduced in 2009 [6]. The typical dispositive used in this technique is a Teflon disk containing an extraction phase with a high surface-area-to-volume ratio. The extraction device can be stirred at much higher velocity, without damaging the phase, than can be achieved with the stir bar used in SBSE, because the extraction phase is only in contact with the liquid sample. The higher rotation velocity of the extraction device reduces the boundary layer of water at the interface, speeding up the analyte mass transport.

In the standard version of RDSE, the sorbent phase is a polymer film adhered to one side of the Teflon disk. In this configuration, polydimethylsiloxane (PDMS) and octadecyl (C18) was used as the solid phase for extraction of low-polarity analytes (log P between 3 and 7) [6–11]. However, for more-polar analytes (log $P<3$), quantitative recoveries are not achievable with short extraction times [8]. We propose a second configuration for RDSE, consisting of a disk with a cavity (Fig. 1) that can be loaded with a commercial sorbent phase typically used in SPE and selected depending on analyte polarity. To avoid leakage of the sorbent from the cavity, the cavity is covered with a fiberglass filter and sealed with a Teflon ring.

There is great interest in the veterinary drug industry in the development of effective analytical methods for measuring veterinary residues in plasma to describe the pharmacokinetics of the administered drug product. Such methods would facilitate more efficient selection of administration routes and dosage levels to achieve adequate chemotherapeutic concentrations and distribution of the product. Determination of drug concentrations in plasma is also an important indicator for formulation bioequivalence studies [12].

The development of new, greener analytical strategies for sample preparation is an opportunity to incorporate innovations into the current analytical chemistry of the veterinary drug industry. In this context, the proposed novel analytical technique for RDSE was used to determine florfenicol levels in plasma, as a model analyte or sample system (Fig. 2). Florfenicol is a broad-spectrum antibiotic, used in treatment of cattle, pigs, poultry, and fish because it is rapidly metabolized to florfenicol amine and has a relatively short withdrawal time [13]. Considering the amphiphilic structure of florfenicol, Oasis HLB was selected as the sorptive phase for this RDSE application. To describe the pharmacokinetics of absorption and elimination, it is necessary to determine the concentration of the drug in plasma. After the RDSE procedure, quantification can be performed by high-performance liquid chromatography (HPLC) with a UV detector, using a reversed phase [14].

Sample preparation techniques including liquid–liquid extraction (LLE) with ethyl acetate [15] and SPE with C18 phase [14] have previously been used for florfenicol extraction from plasma samples. SBSE has also been used for plasma samples, but for other analytes than florfenicol [16].

Experimental

Reagents

Nanopure water from a Barnstead water system (Dubuque, IA, USA) was used for all experiments. Florfenicol (100 % purity) was provided by Sigma-Aldrich (Milwaukee, WI, USA). A stock standard solution of the analyte, of 1000 μg mL^{-1}, was prepared in acetonitrile and was stable for at least two months at −18 °C. Intermediate standard

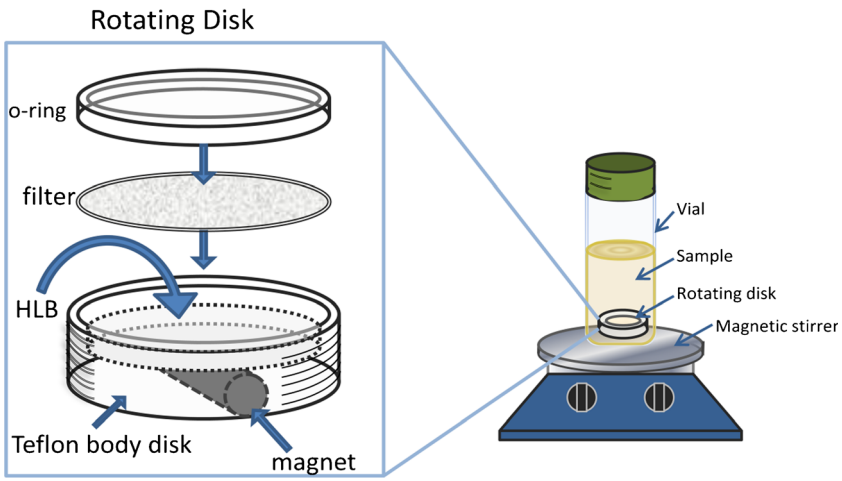

Fig. 1 Schematic diagram of the rotating disk used in this study

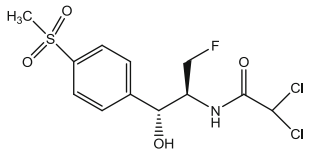

Fig. 2 Structure of florfenicol

solutions of 10 μg mL^{-1} were prepared by dilution with acetonitrile and were stable for at least one week at 4 °C. The powdered extraction sorbent Oasis HLB (30 μm particle size) was obtained from cartridges provided by Waters (Milford, MA, USA). Octadecyl (C18, 30 μm particle size) cartridges obtained from Waters were used for comparison. All solvents were HPLC grade and were obtained from Merck (Darmstadt, Germany).

Animals and experimental design

Five healthy 11-week-old hybrid pigs (land race×large white, Duroc) were used in this study. All five pigs were male and weighed 32±2 kg. Before use, they were housed in a collective pigpen for seven days. The animals were fed pelleted feed (florfenicol-free) and provided with drinking water. Animal experiments were performed in an approved ethical manner following the Guidelines of Good Clinical Practice [17].

The pigs were weighed before administration of the drug, and the dose was adjusted accordingly. One single dose of 20 mg kg^{-1} bodyweight of florfenicol formulation (2 % oral Duflosan Veterquímica, Santiago, Chile) was administered orally. Blood samples were taken from each pig while it was immobilized in a restraining device, and were collected in Vacutainer tubes (Lithium Heparin) at 0.25, 0.5, 0.75, 1, 2, 4, 8, 12, 16, and 32 h after drug administration. Blank samples were taken from each pig just before drug administration. The samples were centrifuged for 10 min (1.5 G), and the plasma samples were stored frozen (−20 °C) until analysis. All samples were analyzed within two weeks after the experiments were performed.

Preparation of the rotating disks

The extraction device used in this study (Fig. 1) was a Teflon disk (1.5 cm diameter) containing an imbedded miniature magnetic stirring bar (Teflon-coated Micro Stir bar from VWR International). The disk has a cavity (0.44 cm^3) on one of its surfaces, into which 80 mg Oasis HLB sorbent was loaded. The cavity was covered with a fiberglass filter (1.4 cm diameter, mean pore size 3 μm) and sealed with a Teflon ring.

Instruments and apparatus

The rotating disk was driven using an MR 300 (Heidolph Instruments GmbH, Germany), a common laboratory magnetic stirrer. Quantification of florfenicol was performed using a LaChrom Elite HPLC System with an L-7400 UV detector (Hitachi, Tokyo, Japan) and a C8 HPLC column (250 mm×4.6 mm×5 μm, Waters). A LaChrom Elite HPLC System with an L-2450 diode-array detector (DAD) (Hitachi) was used for confirmation of florfenicol in samples.

Analytical procedure

Before use, the Oasis HLB sorbent packed in the rotating disk was conditioned by rotation for 10 min in a vial containing 10 mL methanol.

10 mL phosphate buffer (5 mmol L^{-1}, pH 3.2) was poured into a 25 mL EPA vial, and a 250 μL aliquot of porcine plasma sample was added. The previously conditioned disk was submerged in the sample and rotated at 1250 rpm for 90 min. After the extraction, the disk was cleaned with water for 30 s, dried using a lint-free tissue, and placed into a 15 mL vial that contained 8 mL acetone as the desorption solvent. The disk was stirred at 1250 rpm for a desorption time of 60 min. The solvent was then evaporated to dryness under an N_2 stream, and the final extract was re-dissolved in 2 mL mobile phase. This volume was filtered using a syringe containing a PVDF membrane (0.45 μm), and poured into a vial for injection into the HPLC instrument.

The mobile phase consisted of a mixture of acetonitrile and water at a ratio of 35:65 (*v*/*v*). The injection volume was 50 μL, the monitoring wavelength was 228 nm, the oven temperature was 25 °C, and the flow was 0.8 mL min^{-1}. The calibration curve was prepared using blank plasma at a range of concentrations from 0.4–16 μg mL^{-1} (n=6).

Spectrum match was used for confirmation of florfenicol in samples (HPLC–DAD).

Results and discussion

RDSE methods have previously been revealed to be applicable to water samples [6–11]. Furthermore, according to previous studies on SBSE and SPE [16, 18], the proposed RDSE technology could be extended to more complex liquid matrices, including plasma. RDSE does not require substantial control of the sample's passage through the extraction phase, as is mandatory in an SPE cartridge, because recirculation of the sample into the device maximizes its sorptive capacity, and the interface is continuously renewed during the extraction process. Furthermore, in RDSE, the extraction phase is only in contact with the liquid sample during the extraction process, whereas in SBSE, the extraction phase is in direct contact with the bottom of the sample vial, where the stir bar rotates, making the phase less durable.

The reversed-phase sorbent Oasis HLB is a macroporous copolymer made from a balanced ratio of divinylbenzene and

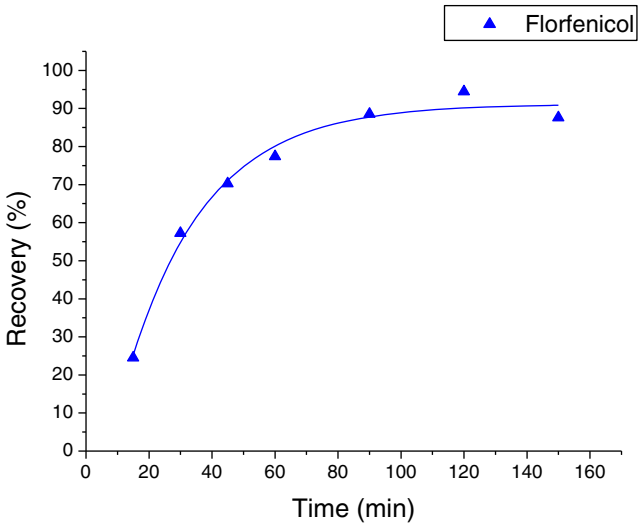

Fig. 3 Extraction time profile for florfenicol using RDSE

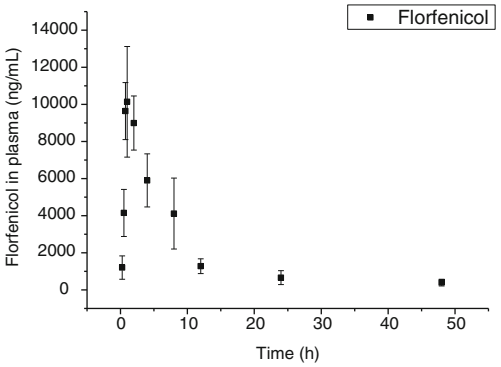

Fig. 5 Pharmacokinetics of florfenicol in pigs, obtained by RDSE for 2 % Duflosan (Veterquímica)

N-vinylpyrrolidone. This solid phase is currently one of the most commonly used sorbents for SPE extraction of drugs with different polarities [19–21], but has not been used for extraction of florfenicol. Oasis HLB is a copolymer with a high degree of crosslinking, a high porosity, and an open structure, enabling hydrophobic interactions (through π–π interactions between benzene rings and the less polar part of the analytes) and hydrophilic retention (via the formation of hydrogen bonds between the pyrrolidone moiety and the polar groups of the analyte). This adsorbent has been widely used in SPE systems, but use of this sorbent has not been reported in stir-bar sorptive extraction (SBSE) or in rotating-disk sorptive extraction (RDSE). RDSE with divinylbenzene-*N*-vinylpyrrolidone should be suitable for the extraction of a semipolar drug, for example florfenicol (log Kow 0.67), from porcine plasma, simplifying sample preparation and reducing the volume of solvent required.

An attempt to immobilize the solid-phase powder in the original rotating-disk configuration (without cavity), using a variety of adherents (double-sided tape, silicone, etc.), resulted in detachment of part of the phase during rotation of the disk. Thus the rotating device was designed, as shown in Fig. 1.

Florfenicol is a weak acid (pK_a 9.02), so higher recovery for extraction into Oasis HLB sorbent was obtained when the molecule was preferably neutral, at pH<9. However: between pH 4.5 and pH 9.0, different interfering signals appeared in the chromatograms, hindering proper identification and integration of the analyte's signal. Below pH 4.5, the analyte's signal in the chromatogram was not subject to interference. Therefore, a pH of 3.2 was selected for use in the RDSE experiments.

Use of matrix modifiers in microextraction techniques can enhance the efficiency of extraction of some analytes, depending on their polarity [10]. NaCl is a modifier that is often used because of the salting-out effect, which changes the ionic strength of the sample. For semipolar and polar analytes, the addition of salt usually increases the extraction efficiency by making the analytes insoluble in water and increasing their affinity for the apolar phase [9, 10]. The effect of adding 0–4 % NaCl was studied, and the addition of salt had a negative effect on the sensitivity of the signal. The interactions of the analyte with the divinylbenzene-*N*-vinylpyrrolidone polymer are probably not favored in a saline medium, for example PDMS. Consequently, no salt was added to the subsequent optimization experiments.

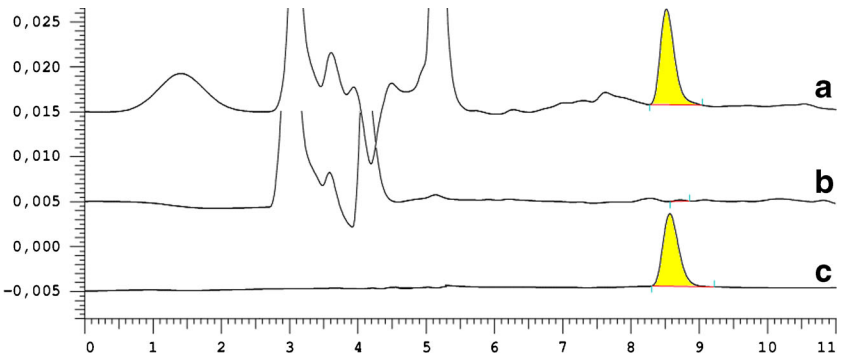

Fig. 4 (**a**) Chromatogram obtained after extraction by RDSE of a plasma sample from a pig treated with florfenicol (1 h after the oral administration of 2 % Duflosan). Chromatograms of (**b**) a blank plasma sample and (**c**) a florfenicol standard (equivalent to 4.8 μg mL^{-1} in plasma) are shown for reference. The signal of florfenicol was integrated, and is shown in *yellow*

Efficient stirring of the sample in contact with the extraction phase is mandatory to achieve partition equilibrium as rapidly as possible, because, in microextraction techniques, analyte mass transfer through the boundary layer in contact with the surface of the phase determines the rate of analyte extraction. Consequently, the rotating velocity of the disk was maintained at the maximum value allowed by the stirrer unit (1250 rpm).

The extraction time was determined under the established experimental conditions (Fig. 4). The samples were extracted by RDSE at different times, ranging from 15 to 150 min, and the extraction profile was obtained. Extraction time affects the amount of analyte concentrated in the Oasis HLB phase, as shown in Fig. 3. Extraction yield increases with extraction time until equilibrium is reached, after approximately 90 min.

As shown in Fig. 4, the chromatographic signal of florfenicol was clear and without interference when the proposed method was applied to a plasma sample under the selected conditions. For comparison, Fig. 4 also shows the chromatograms of a blank plasma sample and of a florfenicol standard.

Taking into account that C18 is the solid phase used in SPE extraction of florfenicol from plasma [14], this phase was also assessed in this new device for RDSE, by packing 80 mg C18 into the cavity of the extraction disk. The extraction capability of C18 was 20 % lower than that of Oasis HLB under the same extraction conditions.

A calibration plot and other analytical features were obtained after 90 min of extraction. The calibration plot was linear over the interval of 0.4–16 μg mL^{-1} (n=6), with R^2= 0.9999. Detection (LOD) and quantification (LOQ) limits of the method were determined using the 3.3σ and 10σ criteria [22], respectively. The LOD and LOQ were found to be 48.1 and 145.9 ng mL^{-1}, respectively.

Repeatability and recovery were determined using different extraction disks (n=6) at a concentration of 4.8 μg mL^{-1} florfenicol in the plasma. A recovery of 91.5 % and RSD of 8.8 % were obtained. The same study at concentrations of 0.4 and 8.0 μg mL^{-1} (n=6) obtained recoveries of 106.4 % and 94.6 %, and RSD 7.6 % and 10.0 %, respectively. Precision was also determined, by sequential use of the same extraction disk (containing the same sorbent phase) for different aliquots of the same sample at a concentration of 4.8 μg mL^{-1} florfenicol (n=8). The RSD under these conditions was 10.2 %, clearly indicating that the sorbent Oasis HLB could be re-used.

The pharmaceutical industry has developed diverse techniques to release drugs to animals in an optimum way. Consequently, pharmacokinetic studies are mandatory to establish whether release of the drug is optimum for the desired dose, or if it is necessary to use prolonged periods of drug release. In this context, the proposed method was used to determine the florfenicol concentration in porcine plasma in a pharmacokinetic study. Figure 5 shows the curve obtained after analysis of 50 samples (10 different time points after administration to five pigs). The pharmacokinetic data obtained from porcine plasma are consistent with the data reported in the literature [14, 23].

Conclusions

A microextraction method using RDSE was developed to extract florfenicol from the plasma of livestock. The method used the sorbent Oasis HLB, which is typically used in SPE. The proposed method is a good sample preparation alternative to LLE, SPE, and SBSE. The main disadvantage of this method is the relatively long extraction and elution processing times compared with those of SPE methods.

These positive results indicate that this method could also be extended to other complex liquid matrices of animal origin (urine, milk, fluids, etc). Even in animal tissue, where the maximum residue limits (LRMs) are established [13], analysis could be performed through a sample pretreatment enabling the analytes to be transferred to an aqueous phase for extraction and/or clean-up by RDSE.

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