Pharmaceutical Research, Vol. 11, No. 11, 1994

Quantitative Analysis of a Drug in an Animal Feed Employing Sample Preparation by Supercritical Fluid Extraction

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Received February 2, 1993; accepted May 18, 1993

A pharmaceutical agent has been quantified in a rat feed matrix wherein sample preparation was achieved by supercritical fluid extraction. Spiking levels ranged from 0.0335% to 1.12%. Pure carbon dioxide, solid phase trapping on stainless steel with acetonitrile and liquid chromatographic assay yielded recoveries greater than 90% with relative standard deviations less than 5% in all cases except for the lowest spiking level. In this case recovery was 89.6% with an RSD of 9.6.

KEY WORDS: supercritical fluid extraction; atovaquone; rat feed matrix.

INTRODUCTION

Although supercritical fluid extraction (SFE) has been touted as a likely successor to many of the current soxhlet and liquid-solid extractions (1), to this point SFE has resided largely in the research laboratory. Some of the properties that have brought supercritical fluids to the forefront are their high diffusity and low viscosity, when compared to traditional extraction solvents. Another benefit is the ability to control the solvating power of the supercritical fluid by control of pressure and temperature (2). This paper illustrates the use of SFE in the quantitation of a drug substance in an animal feed matrix.

A search of the literature reveals few applications of SFE to pharmaceutical agents. Mulcahey (3) et al. showed the application of SFE for direct extraction of active ingredients from a liquid pharmaceutical matrix. The work involved the extraction of sulfamethoxazole and trimethoprim from Septra Infusion®, which is used for treatment of urinary tract infections. The drug was extracted in two ways. First the Septra Infusion® liquid was extracted directly using a modified extraction vessel, which was designed to bubble supercritical fluid through the liquid before exiting to the trap. This method yielded very little extract due to restrictor plugging caused by precipitation of sulfamethoxazole when the solution pH was lowered by introduction of CO_2. Second, a method of spiking the Septra Infusion® onto Celite was explored followed by extraction of the drug component from the dried Celite matrix. Nearly quantitative recoveries for both analytes by using the latter method were realized.

Startin (4) et al. used supercritical CO_2 to extract four veterinary drugs from freeze dried pig kidney: trimethoprim, hexestrol, diethylstilbestrol, and denestrol. Qualitative analysis was attempted by on-line SFE/SFC/MS/MS. It was concluded from this study that the method demonstrated great potential, but detection limits were not sufficient for this particular matrix.

Richter (5) et al. have discussed two applications involving modified CO_2 for extraction of pharmaceutical agents. The first application was the use of 10% chloroform modified CO_2 for the extraction of anti-histamine from a transdermal patch. In this matrix the active agent was suspended in a gel under an adhesive. Richter reported quantitative results and a relative standard deviation (RSD) of 2.7%. In a second application, sulpha drugs were extracted under various pressure and temperature conditions from spiked liver and pork samples. Incurred sulphamethazine was also extracted from pork and quantitative recoveries were reported. Liquid solvent trapping was employed in both instances.

Locke (6) et al. and Messer (7) et al. were among the first to apply SFE to an animal feed matrix. Locke extracted menadione (Vitamin K_3) spiked at the 1 mg/g level from rat feed and reported an average recovery of 90.5% with a 2.2% RSD. To achieve these recoveries Locke employed a 20 minute static extraction using 100% CO_2 held at 8000 psi and a temperature of 60°C. Trapping was achieved by allowing the supercritical CO_2 to decompress into a 6 in. × ¼ in. O.D. stainless still tube filled with silica gel. The silica gel was then washed with 10 mL of methylene chloride.

Messer reported on the applicability of supercritical CO_2 extraction as a quantitative method for recovery of 4′-trifluoromethyl-2-biphenyl carboxylic acid spiked into a rat feed matrix at a level of 1%. Off-line extraction with solid phase trapping and solvent rinsing was utilized. An optimized method for quantitative extraction of the pure drug was initially developed with high reproducibility. Three drug/rat feed matrices were examined. The as-received "crystalline matrix" yielded the poorest reproducibility suggestive of a heterogeneous matrix. A laboratory-prepared crystalline drug/feed matrix and a matrix prepared by spiking the animal feed with a solution of the drug gave ten-fold better RSD's then the "crystalline matrix".

As the technology of supercritical fluids matures, and the understanding of SFE deepens, the application of SFE for routine analysis will increase. We would like to report one such application. Using a commercially available instrument, quantitative supercritical fluid extraction of *trans*-2-[4-(chlorophenyl) cyclohexyl]-3-hydroxyl-1,2-naphthoquinone, shown in Figure 1, (atovaquone) from a rat feed can be performed on a routine basis. The concentration of drug in the feed ranged from 0.03% to over 1.0%. Supercritical fluid extraction was completed in approximately one hour, with recoveries of greater than 90% and RSD's generally less than 5%.

EXPERIMENTAL

The extractions were performed on a Hewlett-Packard

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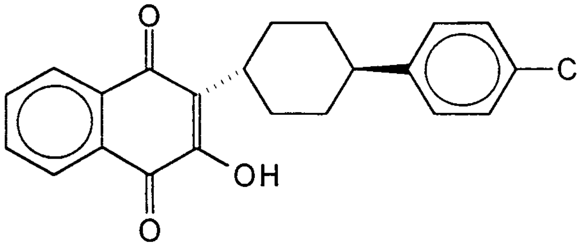

Figure 1. The structure of atovaquone.

(Avondale, PA) Model 7680A Supercritical Fluid Extractor. In brief, this extractor utilizes a cryogenically cooled dual head reciprocating pump, with an upper pressure limit of 381 bar and liquid flow rates of up to 4 mL/min. Unique to this instrument is the use of a computer controlled variable restrictor which independently controls both flow rate and pressure. Air Products and Chemicals Inc. (Allentown, PA) supplied SFC/SFE grade CO_2, and SFC grade 2% methanol modified CO_2 was supplied by Scott Specialty Gases (Plumsteadville, PA).

Analysis of the atovaquone extracts was carried out by liquid chromatography employing isocratic elution with a 4.6 × 250 mm Keystone Scientific (Bellefonte, PA) Hypersil ODS column (5 μm particle size and 120 Å pore size). The mobile phase was 780 mL/220 mL/5 mL of acetonitrile/water/phosphoric acid respectively. The flow rate was 2 mL/minute. Acetonitrile and water were HPLC grade from Fisher Scientific (Raleigh, NC). The phosphoric acid was 99.99% pure and obtained from Aldrich Chemical (Milwaukee, WI). The aqueous portion of the mobile phase was vacuum filtered using a 0.22 μm GS type filter from Millipore (Bedford, MA). A Hewlett-Packard (Avondale, PA.) 1050 isocratic pump was used, and connected to a Valco (Austin, TX.) model EQ-60 LC injector using a 10 μL loop. A Spectro Monitor III (Houston, TX) ultraviolet (UV) detector monitoring 254 or 220 nm and a Hewlett-Packard (Avondale, PA) model 3394A or model 3392A integrator were used. All atovaquone\rat feed samples and atovaquone standards were provided by Burroughs-Wellcome Company (Research Triangle Park, NC). HPLC quantitation was by peak area relative to external standards. Recoveries were calculated on the theoretical basis using the mass of spiked drug.

Additional sample preparation after supercritical fluid extraction was minimal. It was found that supercritical CO_2 also extracted components of the rat feed. These compounds produced a solid precipitate when the organic rinse solvent was diluted with water to achieve the appropriate strength for liquid chromatography. This precipitate was removed prior to drug analysis by syringe filtering. The filters used were Whatman® Puradisc 25 TF filters. These filters were 25 mm in diameter and made of PTFE membrane with polypropylene housing.

The current method of dosage verification for atovaquone is a liquid-solid extraction which uses approximately 1.5 grams of rat feed sample and 15 to 40 mL of acetonitrile as an extraction solvent. The specific amounts of extraction solvent used are dependent on the drug concentration in the matrix. The rat feed was weighed into a 50 mL polypropylene centrifuge tube, and the appropriate volume of acetonitrile was added. The sample was then placed on a horizontal shaker for 15 minutes and then centrifuged for 2 minutes. An aliquot of the supernatant was then diluted to the proper HPLC solvent strength and filtered prior to analysis.

RESULTS AND DISCUSSION

Extraction of atovaquone from an inert matrix was performed to determine the extraction profile of atovaquone in the absence of any matrix effects from the rat feed. Specifically, after increments of time, the dynamic extraction was interrupted, the trap was washed, and the resulting solution analyzed. After the completion of a several step combination static/dynamic extraction, the cumulative results could be evaluated. Through this extraction time versus percent recovery plot an approximation of both drug solubility in the supercritical fluid and drug extraction kinetics could be determined.

Test samples were prepared by spiking 0.200 mL of a 1.00 mg/mL solution of atovaquone in methylene chloride onto a Celite bed inside the 1.5 mL extraction vessel. These samples were dried overnight at ambient conditions to allow for the evaporation of solvent. The atovaquone/Celite sample was then subjected to a five step extraction. For all steps supercritical CO_2 was used as the extraction solvent at a liquid flow rate of 2 mL/minute. The pressure was held at 350 bar with a chamber temperature of 50°C, which translates into a supercritical fluid density of 0.90 g/mL. The trap used was stainless steel spheres (100 μm) held at 5°C during the extraction step, and raised to 40°C during the rinsing of the trap. Temperature of the nozzle (i.e. variable restrictor) was held at 55°C for both the extraction and rinsing phases. The stainless steel trap was washed with 1 mL of acetonitrile after each step, with the exception of step 5, where the trap was rinsed with 3 mL of acetonitrile.

The five steps of the extraction were as follows. Step 1, equilibration (vessel pressurized), 2 minutes; dynamic (SF flows through the vessel) 2 minutes (vessel volumes sweeps = 2.7); steps 2 and 3 dynamic, 3 minutes (vessel volumes sweeps = 4.1); step 4 dynamic, 4 minutes (vessel volumes sweeps = 5.5); step 5 dynamic, 8 minutes (vessel volumes sweeps = 11.0). This gave a total dynamic extraction time of twenty minutes and 27.4 vessels volumes of supercritical CO_2.

As shown in Figure 2 the first step resulted in a recovery of 67.1% while, the second step showed a recovery of an additional 14.5%. Therefore over 80% of the atovaquone was extracted in the first five minutes of dynamic extraction. The third step of the extraction produced a recovery of 6.17%; while, the fourth and fifth steps of the extraction had a recovery of 3.17% and 3.51% recovery respectively. The total recovery for the extraction profile was 94.5%. This extraction profile indicated several important results. First, atovaquone shows a very high solubility in supercritical CO_2. Second, the recoveries versus extraction time are favorable. The third important observation is that the stainless steel trap is able to hold the analyte during the extraction process and to efficiently release the analyte upon application of the rinse solvent.

A second extraction profile was performed using 2% methanol modified CO_2 to determine if the recovery could be increased. This extraction was performed using the same

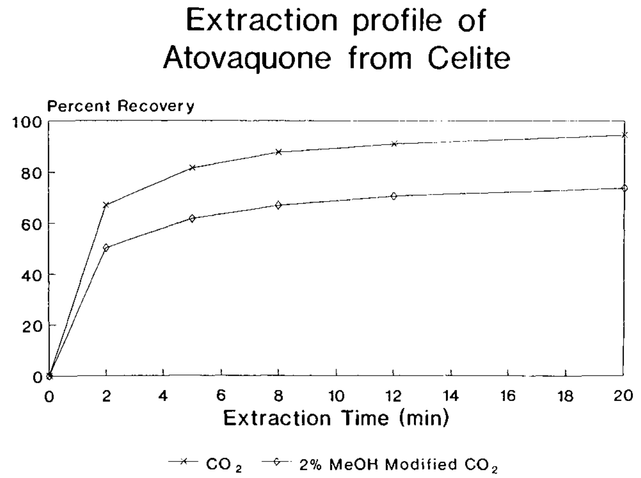

Figure 2. Extraction profile of atovaquone from Celite using 100% CO_2 and 2% methanol modified CO_2.

parameters as the previous profile with the exception of trap temperature. The trap temperature was raised to 70°C, to insure that the methanol (b. pt. = 67°C) appearing in the trap was in the gaseous phase rather than in the liquid phase. Mulcahey et al. (8) have shown that methanol in the liquid phase can reduce trapping efficiency of stainless steel beads.

Extraction recovery for the combined initial two steps was only 61.8% for the methanol modified CO_2, compared with over 80% for the extraction using 100% CO_2 (Fig. 2). Recoveries for steps 3–5 were 5.11%, 3.68%, and 3.15% respectively, which was similar to the CO_2 results. The lower recovery with methanol modified CO_2 is probably not due to lower solubility of the drug in the modified supercritical fluid. A more probable explanation is the loss of trapping efficiency due to the presence of liquid methanol on the stainless steel trap. Although the trap temperature was set for 70°C, this temperature is monitored at the heating jacket around the trap. The actual temperature inside the trap, after accounting for Joule-Thompson cooling, probably is low enough to allow for the presence of liquid methanol. The stainless steel trap is inert, and therefore offers no sorption mechanism to the analyte. In the early steps of the extraction, when the concentration of analyte in the trap is high, the mechanical loss of even a small amount of methanol from the trap can carry a substantial amount of analyte from the trap. In the later steps of the extraction, although methanol is still lost from the trap, the trap concentration of the analyte is much lower, thus the loss is substantially lower.

The next phase of this study was to establish the reproducibility of supercritical CO_2 extraction of pure atovaquone on Celite. Reproducibility was established using triplicate extractions and the parameters previously stated. These extractions had an equilibration time of 2 minutes followed by a 25 minute dynamic extraction. During this period, the 1.5 mL vessel was swept 34.3 times. The trap was rinsed with two-1 mL aliquots of acetonitrile. Triplicate extraction yielded an average recovery of 100.3% and a relative standard deviation (RSD) of 5.8%. On the basis of these results these parameters were used for the extraction of atovaquone from rat feed.

Extraction of Atovaquone from Rat Feed

The investigation of extraction of atovaquone from rat feed began with the extraction of blank rat feed. Assay of previous atovaquone extracts had used UV detection at 220 nm. The extraction of blank rat feed, however, showed an interference at this wavelength. In order to minimize the effect of this interference in the extract, the monitored wavelength was changed to 254 nm.

The study involved extraction of atovaquone at six levels, ranging from 0.0335% to 1.12%, of drug in the rat feed. Sample sizes for extraction were either 250 mg or 500 mg depending on the spiking level. Initially a 250 mg sample of 1.12% rat feed was extracted in triplicate. Extraction parameters were as previously described for the reproducibility study. Because of the larger amount of the drug extracted relative to earlier studies, the trap rinse solvent volume was increased from 2 mL to 4 mL (i.e. 1 mL aliquots) of acetonitrile. The average from this set of triplicate extractions was 98.7% recovery and a RSD of 2.3%. The next concentration of drug investigated was 0.838%. For five replicates, the average recovery of atovaquone was 102.5% with a RSD = 3.2%.

Triplicate extractions were also carried out on atovaquone from rat feed spiked at levels of 0.239%, 0.171%, and 0.0484%. Recoveries and reproducibilities at all these levels were excellent as shown in Figure 3.

The final drug level investigated was at 0.0335%. In the initial attempt a large reduction in recovery was obtained (i.e. only 82.4% recovery with a RSD of 4.2%). A second set of triplicate extractions was performed, using identical parameters to the first set of extractions. The average recovery of 96.8% with a RSD of 4.6%. Combining the two sets of extractions (n = 6), the average recovery was 89.6% and the RSD increased to 9.6%.

The industry method of dosage verification for atovaquone is a liquid-solid extraction employing 1.5 gram rat feed sample and acetonitrile. Typical extraction of 0.02% atova-

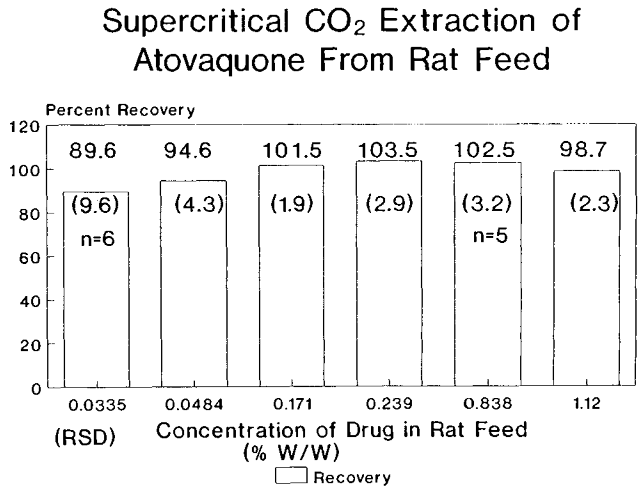

Figure 3. Average recovery for atovaquone from rat feed matrix over a range of concentrations. Liquid CO_2 flow rate was 2 mL/min, chamber temperature was 50°C. The trap was stainless steel beads, rinsed with acetonitrile.

quone from a rat feed yielded a recovery of 83.3% and a relative standard deviation of 1.1%, with nine replicate extractions. When the concentration of drug in the matrix was increased to 2.0%., liquid-solid extraction yielded recoveries of 94.9% and relative standard deviations of 2.1%, with eleven replicate extractions.

In summary, this study clearly (Figure 3) demonstrated the ability of supercritical CO_2 to quantitatively extract *trans*-2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone, (atovaquone) from a rat feed matrix. Triplicate extractions show recoveries over 95% and relative standard deviations of less than 5%. Comparing SFE results of the drug from Celite and from the rat feed indicated that the rat feed mixture is probably not binding with the atovaquone. Quantitative extraction can be achieved in approximately one hour including the rinse procedure. Supercritical fluid extraction results are comparable to conventional liquid-solid extraction results even though samples sizes were considerably less in the SFE case (e.g. 1500 mg versus 250 or 500 mg).

ACKNOWLEDGMENTS

We would like to thank Hewlett-Packard for the loan of their extractor and both Air Products and Chemicals Inc. and Scott Specialty Gases for supplying the supercritical fluids. We would also like to thank Burroughs Wellcome Company for their financial support.

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