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Determination of Flurbiprofen and Ibuprofen in Dog Serum with Automated Sample Preparation

B. G. SNIDER ˣ, L. J. BEAUBIEN, D. J. SEARS, and P. D. RAHN

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Abstract □ Methods for the determination of flurbiprofen and ibuprofen in dog serum were developed using high-performance liquid chromatography and automated serum extraction. Sample extraction was automated by use of cartridges packed with a styrene–divinylbenzene macroreticular resin in a microprocessor-controlled centrifugal system. The average recoveries were 98.9% for flurbiprofen and 94.5% for ibuprofen. The limits of detection were ~0.04 μg/ml for flurbiprofen at 254 nm and 0.5 μg/ml for ibuprofen at 230 nm. The relative standard deviations for the determination of a laboratory standard between days was 2.4% (20 μg/ml) for flurbiprofen and 1.7% (13 μg/ml) for ibuprofen. Peak height ratios were linear with concentrations of 0.04–100 μg/ml for flurbiprofen and 1.0–50 μg/ml for ibuprofen. These methods are simple, rapid, sensitive, and specific. The use of an automated sample preparation procedure improved the between-day precision by a factor of two when compared to a manual extraction procedure. These methods were applied to bioavailability studies in dogs.

Keyphrases □ Flurbiprofen—determination in dog serum with automated sample preparation □ Ibuprofen—determination in dog serum with automated sample preparation □ Bioavailability—determination of flurbiprofen and ibuprofen in dog serum with automated sample preparation

Flurbiprofen [*dl*-2-(2-fluoro-4-biphenylyl)propionic acid] and ibuprofen [*dl*-2-(*p*-isobutylphenyl)propionic acid] are nonsteroidal anti-inflammatory drugs. Several gas chromatographic (GC) procedures were previously developed for flurbiprofen (1) and ibuprofen (2–4). Methods using high-performance liquid chromatography (HPLC) were previously developed for ibuprofen (5, 6) and similar compounds: indoprofen (7), ketoprofen (8), and naproxen (8). To reduce the amount of labor and time involved in performing assays for flurbiprofen or ibuprofen in serum, an HPLC procedure using an automated sample processor[1] was developed. The previously developed GC procedures use manual liquid–liquid sample preparations and derivatization prior to analysis. Sample extraction with the automated sample processor uses a liquid–solid extraction with a cartridge packed with a styrene–divinylbenzene macroreticular resin in a microprocessor-controlled centrifugal system, resulting in reduced analysis effort and improved assay precision.

[1] Prep I, Dupont Co, Wilmington, Del.

EXPERIMENTAL

Reagents and Materials—Reagents were of at least analytical reagent grade quality, and acetonitrile[2] was distilled-in-glass grade. Stock solutions of flurbiprofen[3] and ibuprofen[3] were prepared in pH 7.2 phosphate buffer (0.05 *M*).

Instrumentation—A variable-wavelength detector[4], a solvent pump[5], and an autoinjector[6] were used for the chromatographic analysis. Preparation of samples was performed with an automated sample processor.

Chromatographic Conditions—Chromatography took place on a 0.46-cm i.d. × 25-cm long column packed with octadecylsilane bonded to microparticulate silica[7] (10 μm). The precolumn, 4.2 cm × 0.3-cm i.d., was packed with octadecylsilane bonded to microparticulate silica[8] (30 μm). The mobile phase was acetonitrile–0.05 *M* acetic acid (40:60).

The flow rate was 2.0 ml/min, the column temperature was ambient, and the column back-pressure was ~1500 psi. The approximate retention times of flurbiprofen and ibuprofen were 14 and 19 min, respectively. Preliminary work was performed with the acetonitrile–water ratio at 50:50 and the flow rate at 1.2 ml/min. The mobile phase was filtered and deaerated by vacuum sonication prior to use.

Automated Extraction—The automated sample processor is designed to perform automatic extractions simultaneously of up to 12 liquid samples in 30 min or less. Centrifugal force is used to move solvents through an extraction resin bed. The system is composed of an inner rotor, which holds extraction columns, and a larger outer rotor, which holds the corresponding effluent and recovery cups. The extraction column is comprised of a sample reservoir and a resin bed. The rotor first spins clockwise to force the sample through the resin bed. A predetermined amount of wash solvent is forced through the resin bed and into an effluent cup. In this manner, unwanted components are removed from the column. The rotor direction is then reversed so that the extraction column is positioned over the recovery cup.

An aliquot of a second solvent elutes the component of interest and is collected in the recovery cup. If desired, the extract is then heated and blown to dryness. The dried extract is manually reconstituted and transferred to another instrument. Fifteen programs are currently available which vary timing, compartment temperature, and the option of sample evaporation.

Assay Procedure—Blank serum spiked with 100 μl of flurbiprofen or ibuprofen solutions in 0.05 *M* phosphate buffer (pH 7.2) was used to prepare standards to obtain a calibration curve for each chromatographic run. One milliliter of blank or sample serum was pipetted into the car-

[2] Burdick & Jackson, Muskegon, Mich.
[3] The Upjohn Co, Kalamazoo, Mich.
[4] Perkin-Elmer LC-55B, Norwalk, Conn.
[5] Model 100A, Altex Scientific, Berkeley, Calif.
[6] WISP model 710, Waters Associates, Milford, Mass.
[7] RP-8, Rheodyne, Berkeley, Calif.
[8] Permaphase ODS, Dupont, Wilmington, Del.

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Table I—Effect of Mobile Phase pH and Percent Acetonitrile on the Retention Times of Flurbiprofen and Ibuprofen (1.0 ml/min)

Acetonitrile, %	pH	Retention Time of Flurbiprofen, min	Retention Time of Ibuprofen, min
60	3	6.3	7.8
55	3	6.9	8.7
50	3	9.6	12.6
	4	7.8	11.1
	5	5.4	8.7
40[a]	3	17.0	22.0

[a] At 2.0 ml/min.

tridges. Standard cartridges were spiked with the appropriate stock solution and were rotated to promote mixing. A 0.2-ml aliquot of 0.5 *M* H_2SO_4 was added to each cartridge, which then was rotated to promote mixing. The cartridges were loaded into the automated sample processor. Water and methanol were added to the appropriate reservoirs in amounts sufficient to wash each cartridge with 1.0 ml of water and elute each cartridge with 2.0 ml of methanol. The addition of the washing and eluting solvents and the evaporation of the methanol by blowing air at 50° over the aluminum recovery cups were performed by the automated sample processor. The dried contents of the recovery cups were dissolved in the mobile phase. To determine ibuprofen at 10 μg/ml, the cups were reconstituted with 1 ml of mobile phase containing 6.0 μg of flurbiprofen/ml as the internal standard, and 0.2-ml aliquots of the reconstituted samples were chromatographed. In the determination of flurbiprofen, mobile phase containing ibuprofen was used to reconstitute the samples.

Peak heights were measured for flurbiprofen and ibuprofen, and the peak heights for the analyte were divided by that of the internal standard. Linear least-squares fit of the peak height ratios was used to calculate the serum drug concentration.

Drug Administration—Male beagle dogs were fasted for 16 hr prior to dosing and for 4 hr after administration. Flurbiprofen and ibuprofen were dosed as solutions of sodium salts (pH 7) in a hard gelatin capsule. For ibuprofen, 4 ml of a 20 mg/ml solution was administered to each dog; for flurbiprofen, 4 ml of a 12.5 mg/ml solution was administered. Blood was collected in 10-ml evacuation tubes and allowed to clot. Separated serum was stored at −18° prior to analysis.

RESULTS AND DISCUSSION

HPLC—For the determination of flurbiprofen in the 0.04–2-μg/ml range, a mobile phase of acetonitrile–0.1 *M* acetic acid (50:50) was used with UV detection at 254 nm. The effect of the mobile phase pH and percent acetonitrile on the retention times of flurbiprofen and ibuprofen are shown in Table I. By using a low pH for the mobile phase, ion-pairing is not necessary for adequate retention of the two compounds. The relative retention times of some metabolites of flurbiprofen are shown in Table II, and all are well resolved from flurbiprofen as well as ibuprofen. The major metabolite in dogs is 4′-hydroxyflurbiprofen (9). At 254 nm, the molar absorbtivity of ibuprofen was only 1.2% that of flurbiprofen.

To determine flurbiprofen above 10 μg/ml, the ibuprofen concentration needed to provide equivalent responses became quite large, and problems occurred with low ibuprofen recovery with the manual extraction procedure. Therefore, UV absorbance was monitored at 230 nm to optimize ibuprofen sensitivity and still have adequate sensitivity for flurbiprofen. However, a peak that eluted at approximately the same retention time as ibuprofen was observed, and the mobile phase was changed to acetonitrile–0.05 *M* acetic acid in water (40:60) at 2.0 ml/min to separate this interfering peak from ibuprofen. A typical chromatogram of extracted blank serum and serum spiked with flurbiprofen and ibuprofen is shown in Fig. 1.

Isolation of Flurbiprofen and Ibuprofen from Serum—Various

Table II—Relative Retention Times of Flurbiprofen, Ibuprofen, and Flurbiprofen Metabolites

Compound	Relative Retention Time, min
Flurbiprofen	1.0
Ibuprofen	1.3
2′-Hydroxyflurbiprofen	0.56
4′-Hydroxyflurbiprofen	0.50
3′-Hydroxyflurbiprofen	0.53

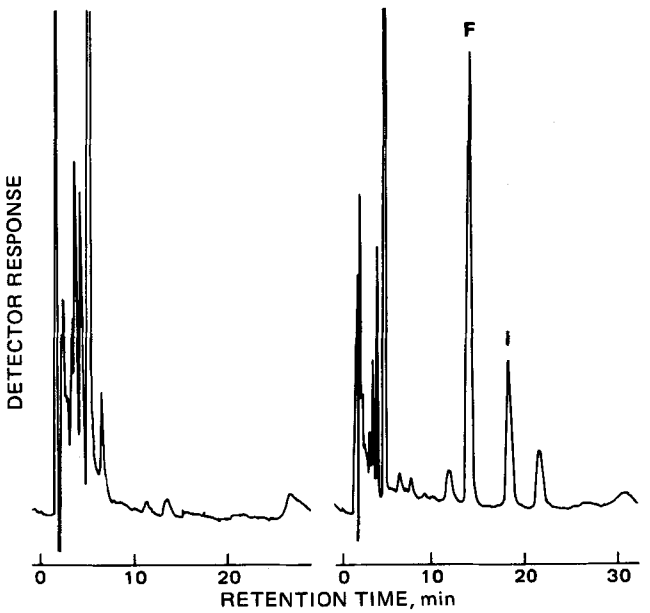

Figure 1—*HPLC chromatograms of drug serum extracts. Key: left, serum sample without administration of drug; and right, serum sample containing flurbiprofen (F) (6.0 μg/ml) and ibuprofen (I) (10 μg/ml).*

methods for the isolation of flurbiprofen and ibuprofen from serum were investigated. Precipitation of serum proteins with acetonitrile followed by analysis of the supernate was suggested as an alternative method for serum sample preparation (7). However, for flurbiprofen and ibuprofen, very low recoveries were obtained when this method was used, and the supernate contained a large amount of dissolved solids. Acidifying the serum before addition of acetonitrile improved recovery, but this approach was discontinued because of problems with injecting large amounts of acid. An extraction procedure developed previously (1) was used, except that toluene was substituted for benzene in the extraction, and the sample cleanup steps involving TLC were omitted. An automated sample extraction procedure was also developed and compared to the manual extraction procedure.

To optimize the automated extraction procedure, several different eluting solvents were investigated and the amounts of wash solvent, and eluting solvent, and the acid added were varied. These experiments attempted to reduce the amount of insoluble material acquired in extraction, to obtain quantitative recovery, and to minimize the number of extraneous peaks produced in the chromatogram. The more polar eluting solvents tended to yield cleaner reconstituted samples. The extent to which the serum was acidified influenced both flurbiprofen recovery and the number of extraneous peaks displayed in the chromatogram. Unacidified serum yielded 37% recovery. One late-eluting peak was eliminated by reducing the amount of acid used from 250 to 200 μl. For each extraction column, the use of 1.0 ml of water (wash solvent), 2.0 ml of methanol (eluting solvent), and 200 μl of 0.5 *M* H_2SO_4 produced the cleanest samples with the greatest recovery.

Assay Linearity, Precision, and Accuracy—Linearity of peak height ratios with the concentration of flurbiprofen and ibuprofen was investigated. Calibration curves prepared from serum spiked with the appropriate amounts of flurbiprofen or ibuprofen showed no significant deviation from linearity in the 0.04–100-μg/ml range for flurbiprofen and in the 1–200-μg/ml range for ibuprofen. Correlation coefficients for the fit of the experimental points to a line were between 0.998 and 1.000. The intercepts were not significantly different from zero in all cases, and no interferences from constituents in blank serum were found.

The extraction efficiency was determined over the same ranges as those for the linearity, and the average recoveries were 98.9 ± 3.3% and 94.5 ± 1.2% for flurbiprofen and ibuprofen, respectively.

To investigate the precision of the extraction process, six runs with six samples each of a pooled serum sample containing 20 μg of flurbiprofen/ml were prepared. The mean relative standard deviation of the extraction within the six samples was ~1.8%. The relative standard deviation for different runs of the automated sample processor was ~1.3%. Therefore, the extraction process was reproducible both within run and between run. This precision was obtained without adding the internal

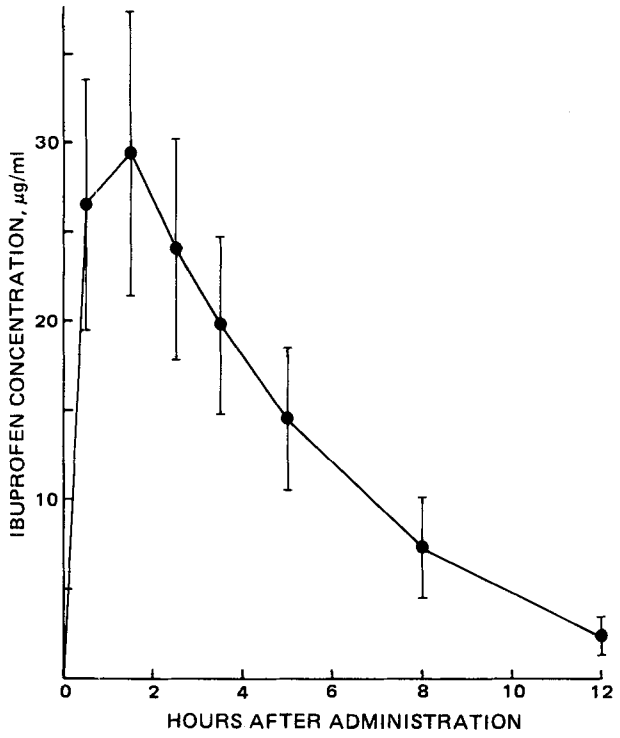

Figure 2—*Average (±SD) serum ibuprofen concentrations in male beagle dogs (n = 8, average weight 14.9 ± 2.2 kg) after single oral administration of 80 mg of ibuprofen as a solution of the sodium salt.*

standard prior to extraction, thus reducing the number of steps required for the assay.

The reproducibility of the entire assay was later demonstrated when a set of 300 samples was assayed in eight chromatographic runs of 16 hr each. The average of the laboratory standard (a pooled sample of serum) over the 8 days was 26.3 μg/ml, and the relative standard deviation was 2.4%. The relative standard deviation of standards in the 5–100 μg/ml concentration range was 3.2–0.8%. The relative standard deviation of the slope was 4%. The reproducibility of the extraction from day to day was a considerable improvement over the manual extraction procedure, which typically had a 6.0% *RSD* for the laboratory standard.

Assay Sensitivity—With a UV detector at 0.05 aufs, assay sensitivities based on a signal 2% of full scale were 1 and 2 μg/ml for flurbiprofen and ibuprofen, respectively, for a 0.06-ml injection from a reconstituted volume of 2 ml. When the dried extract was reconstituted in 1 ml and 0.2 ml was injected, the sensitivity was improved to 0.5 μg/ml for ibuprofen and to 0.2 μg/ml for flurbiprofen. Flurbiprofen can be determined at levels as low as 0.04 μg/ml if a 254-nm detector is used because absorbance is at a maximum at 248 nm and a single-wavelength detector at 254 nm has a higher signal to noise ratio than a variable-wavelength detector.

Assay Problems—Only a few problems occurred using the automated sample processor. Occasionally, the flow of samples through the cartridge was too slow, and water was left at the top of the cartridge at the end of analysis. This can result from insoluble material in the sample plugging the cartridge and from the cartridge drying out and flow becoming too slow. The first problem can be solved by filtering the sample, and placing an expiration date on the cartridge minimizes the second problem. The incidence of these problems was infrequent (~1%). The cartridges can be prescreened for suitability by placing 1.0 ml of water in them and checking to see if all of the water flowed through the cartridge in <1 min under the conditions typically used to force the serum–buffer mixture through the cartridge.

Another problem observed with ibuprofen was a change in recovery from 95 to 60% for one batch of cartridges when elution with 1 ml of methanol was used. The recovery between cartridges was still reproducible, with the assay variation being approximately the same for this lot as for other lots when 95% recovery was obtained. This finding is not surprising because of the reproducible manner in which the automated sample processor performs the extraction. For example, the relative standard deviation for the addition of eluting solvent to the cartridge was 1.5%. This situation is analogous to the use of a segmented flow system in which transfer of the sample through the various steps may not yield 100% recovery, but the recovery is reproducible from sample to sample. The reason for the dependence of recovery on the lot used involves the different elution properties of various lots of resin. If the standards are extracted with the same lot of cartridges as the samples, the change in recovery will not affect the quantitation unless the recovery becomes quite low. In this case, the detection limit and precision of the assay will be affected. The amount of methanol used for elution was changed to 2 ml/cartridge to minimize this problem, and recovery then increased from 60 to 86%.

Serum Levels in Dogs—The average serum concentration curve for the oral administration of 80 mg of ibuprofen is shown in Fig. 2. For ibuprofen, the average peak concentration was 29.4 μg/ml, the average time of individual peak occurrence was 1.5 hr, and the half-life calculated from the terminal slope was 2.8 hr. For flurbiprofen, the average peak concentration was 31.0 μg/ml, the average time of individual peak occurrence was 2.2 hr, and the half-life calculated from the terminal slope was 35 hr.

These methods are suitable for determining the pharmacokinetics of these two drugs and for evaluating drug availability from various dosage forms in dogs.

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