(5) R. Keay, C. F. Onochie, and D. Standfield, in "Nigerian Trees," vol. II, Federal Department of Forest Research, 1964, p. 140.
(6) A. Pelter and P. I. Amenechi, *J. Chem. Soc.*, **6**, 887, (1969).
(7) F. Okoli, "Chemical Constituents of the Roots of *L. cyanescens*," B. Pharm. Project, University of Nigeria, Nsukka, 1981.
(8) F. Delle Monache, F. Marletti, G. B. Martini-Bettolo, J. F. Del-Mello, G. de lima, *Lloydia*, **40**, 201 (1977).
(9) P. Ventura, M. Visconti, and G. Pifferi, *Boll. Chim. Pharm.* **117**, 217 (1978).
(10) E. Bombardelli, B. Gabetta, E. M. Martinelli, and G. Mustich, *Fitoterapia* **1**, 11 (1979).
(11) A. A. M. Habib, N. A. El-Sebakhy, and H. A. Kadry, *J. Pharm. Sci.*, **68**, 1221 (1979).

## ACKNOWLEDGMENTS

This work is supported by Senate Research Grant No. 00330/79 of the University of Nigeria, Nsukka, Nigeria.

The author is grateful to Drs. E. Bombardelli and M. Metawally for the gift of the reference compounds and to the World Health Organization for the award of a visiting scientist grant to Oxford University.

# Paired-Ion High-Performance Liquid Chromatographic Assay for Sulfinpyrazone in Plasma

**N. J. PATEL, D. O. KILDSIG, G. S. BANKER, P. R. MAYER, and M. A. GONZALEZ *x**

Received July 23, 1979, from the *Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907.* Accepted for publication February 18, 1982. *Present address: Key Pharmaceuticals, Inc., Miami, FL 33169.

**Abstract** □ A specific and sensitive liquid chromatographic method is reported for the assay of sulfinpyrazone in plasma utilizing ion pairing between the tetrabutylammonium cation and the sulfinpyrazone anion. The method is rapid in that conventional extraction procedures are avoided in favor of using disposable cartridges packed with an octadecylsilane bonded phase as a means of separating the drug from plasma. The samples were chromatographed on a $C_{18}$ reversed-phase column using a mobile phase consisting of 0.005 *M* tetrabutylammonium phosphate in methanol–water (56:44). The coefficient of variation obtained was 4.5% and the response was linear over a range of 0.2–80 μg/ml.

**Keyphrases** □ High-performance liquid chromatography—paired ion, assay for sulfinpyrazone in plasma □ Sulfinpyrazone—paired-ion high-performance liquid chromatographic assay in plasma

Sulfinyprazone has been used as a uricosuric agent for over 20 years. Recently, new interest in this drug has been generated by reports indicating that it may protect patients from sudden death after myocardial infarction (1, 2).

Several methods have been reported in the literature that can be used for the quantitative determination of sulfinpyrazone in biological fluids. A spectrophotometric method reported previously (3) involves a tedious double extraction and lacks sensitivity, since it is based on UV absorption. A high-performance liquid chromatographic (HPLC) method was described (4) using radiolabeled sulfinpyrazone as an internal standard which complicates the routine application of this method. An HPLC method using a microparticulate silica column also was published (5). This method requires that the sample be injected 2 mm inside the column, thus, making it necessary to periodically refill that portion of the column with fresh silica. Another method (6) involves a two-step extraction and lacks adequate sensitivity.

It was considered desirable to develop a simple, rapid, and sensitive method for sulfinpyrazone determination in biological fluids. The reversed-phase HPLC method described here is based on the formation of ion pairs between sulfinpyrazone anions and tetrabutylammonium cations. The reported method has the advantage of not involving any extraction step. The drug is isolated from plasma using disposable cartridges filled with octadecylsilane bonded phase packing ($C_{18}$ cartridge[1]).

## EXPERIMENTAL

**Materials**—All reagents were analytical grade. All solutions were prepared using glass distilled water.

The high-performance liquid chromatographic system included a solvent pump[2] equipped with an injector[3], a fixed wavelength UV detector[4] set at 254 nm ($\lambda_{max}$ was 260 nm), a recorder[5], and in integrator[6].

The analytical column was 30 cm long with 4-mm i.d. It was packed with octadecylsilane-bonded silica (particle size 10 μm)[7].

**Mobile Phase**—The mobile phase consisted of 0.005 *M* tetrabutylammonium phosphate[8] (I) in methanol–water (56:44). It was prepared by adding sufficient glass-distilled water to a vial of I to make 440 ml. An aliquot of 560 ml of absolute methanol was added, the solution was stirred for 10 min, and the volume was adjusted to 1 liter with a 56% (v/v) methanol–water solution. The solution was then filtered using a 0.5-μm pore, inert filter[9]. The mobile phase was deoxygenated using a vacuum flask attached to a water aspirator. The flow rate of the mobile phase was 1 ml/min at ambient temperature.

**Preparation of Standards**—A stock solution of sulfinpyrazone[10] was prepared by dissolving 100 mg of the drug in 100 ml of 50% (v/v) methanol–water. Solutions of varying concentration were prepared by serial dilutions of the stock solution in 50% (v/v) methanol–water. The plasma samples for use in the preparation of calibration curves were prepared by adding 200 μl of the appropriate drug solution to 1.8 ml of rat plasma in a 15-ml conical centrifuge tube. The plasma samples were mixed by agitating for 30 sec on a vortex-type mixer[11], and a 1.0-ml aliquot was taken for analysis.

[1] $C_{18}$ Sep-pak, Waters Associates, Milford, Mass.
[2] Model 6000 A, Waters Associates, Milford, Mass.
[3] Model U6K, Waters Associates, Milford, Mass.
[4] Model 440, Waters Associates, Milford, Mass.
[5] Omniscribe, Houston Instruments, Austin, Tex.
[6] Shimadzu Chromatopac-E1A, Shimadzu Seisakusho Ltd., Kyoto, Japan.
[7] Bondapak $C_{18}$, Waters Associates, Milford, Mass.
[8] PIC Reagent-A, Waters Associates, Milford, Mass.
[9] Type FH, Millipore Corp., Bedford, Mass.
[10] Ciba-Geigy Corp., Summit, N.J.
[11] Maxi Mix Mixer, No. M-16715, Thermolyne Corp., Dubuque, Iowa.

0022-3549/82/1200-1413$01.00/0

**Sample Preparation**—Sulfinpyrazone was isolated from plasma using disposable $C_{18}$ cartridges. Plasma samples (1.0 ml) with concentrations ranging from 0.2 to 80 μg/ml were used. The cartridges were conditioned by initially passing 3 ml of absolute methanol and 3 ml of a 0.01 *M* aqueous (I) solution through the cartridge. All solvents and solutions were introduced into the cartridge using disposable 5-ml hypodermic syringes. An equal volume of 0.02 *M* I in 50% (v/v) methanol–water was added to the plasma samples, which were then agitated for 30 sec. Each plasma sample was then passed quantitatively through a cartridge, and the eluate was discarded. The cartridges were washed with 1 ml of a solution containing 0.01 *M* I in methanol–acetonitrile–water (15:15:70). The drug retained on the cartridge was then eluted with 1 ml of absolute methanol. A new cartridge was used for each sample.

**Assay**—A 20-μl aliquot of the methanol from the last step in the previous section was precisely measured and injected into the chromatograph using a microliter syringe[12].

**Reproducibility Study**—To study the reproducibility of the procedure, 0.6 ml of a 100-μg/ml solution of sulfinpyrazone was added to 5.4 ml of rat plasma to produce a drug dilution containing 10 μg/ml. Five 1-ml aliquots of this solution were then processed using the procedure described above.

**Retention Time and Peak Height Correlation Study**—The peak height values obtained from 1-μg/ml plasma samples were used to investigate the correlation between peak heights and retention times. The peak height and retention time values generated during the development and optimization of the assay procedure were used. The latter involved changes in the methanol–water ratio, pH, and concentration of I.

## RESULTS AND DISCUSSION

Utilizing disposable $C_{18}$ cartridges adds great flexibility to any assay procedure. It must be kept in mind, however, that these cartridges are in essence miniature reversed-phase columns and must be treated accordingly. In the cartridge-conditioning step, methanol was passed through the cartridge as a cleaning solvent, but it served the dual purpose of also allowing drug diffusion from the serum to the packing material itself. An aqueous solution of I was used to condition the cartridge with the ion-pairing reagent. The tetrabutylammonium cations complex with sulfinpyrazone anions; the ion pair then has a greater affinity for the $C_{18}$ packing material than the sulfinpyrazone anion. This increased affinity allows the ion to be retained in the cartridge while the plasma water and its polar constituents pass through the cartridge.

Not all interfering substances in plasma or other biological fluids will be separated in the first step. Therefore, it is necessary to utilize a washing step to rid the cartridge of additional plasma constituents. This step is critical in that it represents a possible source of sample loss. An optimum recovery of 80% of the sulfinpyrazone with minimal plasma extraneous peaks was obtained by washing the cartridge with 1 ml of a solvent consisting of 0.01 *M* I in methanol–acetonitrile–water (15:15:70). After this washing step, sulfinpyrazone was eluted from the cartridge with 1 ml of absolute methanol. The methanol from this last step was then chromatographed.

The mobile phase used in this procedure also contained I. Sulfinpyrazone is too strong an organic acid (pKa 2.8) to be maintained in an undissociated state, as is desirable when using reversed-phase HPLC. If a mobile phase with a neutral pH is used, sulfinpyrazone will be completely ionized, and its retention volume on the column will rarely exceed the void volume. Therefore, it was advantageous to utilize a mobile phase which contained the tetrabutylammonium cation that could ion pair with sulfinpyrazone and increase its retention time. A typical chromatograph of blank plasma and plasma containing 20 μg/ml of the drug are depicted in Fig. 1. While the blank rat plasma contained a number of peaks which could not be removed by the disposable cartridge, none of these peaks had retention times that would interfere with sulfinpyrazone.

Calibration curves were prepared, and Table I summarizes the results obtained after chromatographing samples from plasma with different concentrations of sulfinpyrazone. Calibration curves could be prepared using either peak heights or areas under the peaks. The data for the 10-μg/ml point represents the mean of five determinations using five different cartridges. The coefficient of variation for this point was 4.5%. The reproducibility between cartridges was satisfactory, and the coefficient of variation could be further improved by utilizing the same cartridge, which is possible by washing the cartridge with three or four 1-ml aliquots of absolute methanol between samples. While in certain applications repeated use of a cartridge may not be desirable, it was found that used cartridges stored in methanol gave reproducible results for several separate assays. This feature greatly diminishes the cost of an assay.

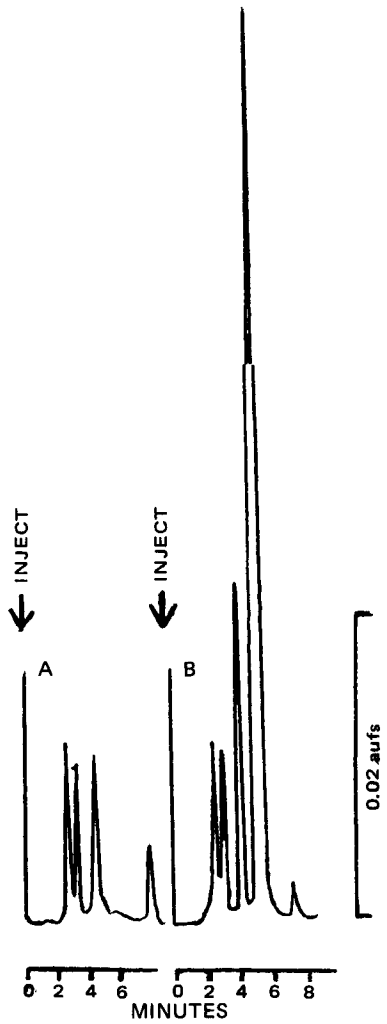


**Figure 1**—*Recorder tracings of representative chromatograms after 20-μl injections. Key: (A) plasma blank; (B) plasma spiked with sulfinpyrazone (20 μg/ml). Peak I is sulfinpyrazone.*

Since the drug is retained on the $C_{18}$ cartridge as an ion pair, it is conceivable that at high drug concentrations there could be insufficient tetrabutylammonium cations to interact quantitatively with the sulfinpyrazone anions. This would result in a deviation from linearity at the higher concentrations of a standard curve. This was found to be the case if plasma concentrations >80 μg/ml were processed undiluted through the procedure. The linearity, however, was good from 1 to 80 μg/ml resulting in a correlation coefficient of 0.9992 when peak heights were used and 0.9990 for peak areas. The sulfinpyrazone–tetrabutylammonium complex appeared to be very sensitive to methanol concentrations in the

**Table I—Values of Plasma Calibration Curve Points**

| Concentration, μg/ml | Peak Height[a], mm | Peak Area[b], μV sec × $10^{-4}$ |
|---|---|---|
| 0.2 | 4.0 | 1.43 |
| 0.6 | 8.5 | 2.84 |
| 1.0 | 14.0 | 5.18 |
| 6.0 | 67.7 | 27.21 |
| 10.0 | 112.0 | 45.70 |
| 20.0 | 199.0 | 82.60 |
| 40.0 | 440.0 | 188.06 |
| 80.0 | 844.0 | 362.30 |

[a] Peak heights are normalized for 0.05 aufs. [b] Units of area are expressed as microvolts per sec.

[12] Pressure-Lock Series B-110, Precision Sampling, Baton Rouge, La.

Table II—Observed and Predicted Values of Slopes

| Retention Time, min | Observed Slope | Predicted Slope |
|---|---|---|
| 5.2 | 91.81 | 90.27 |
| 7.8 | 70.47 | 68.58 |
| 8.0 | 61.69 | 59.68 |

mobile phase, such that changes in retention times occurred with slight changes in methanol concentration. Such changes in retention time would influence the peak heights, thus, peak areas were found to be preferable in the preparation of standard curves.

Peak heights can be used without constantly repeating standard curves, if a correction is made for changes in peak height due to changes in retention time. It is well established that an inverse relationship exists between peak heights and retention time. Different retention times were produced by varying pH, methanol concentration, and I concentration to study this relationship. The flow rate of the mobile phase, however, was always the same. There is a good correlation between the peak heights and the reciprocal of the retention time ($r = 0.9954$), and it is independent of the reason for the change in retention time. Because of the linearity of the response, it should be possible to predict a slope for a new standard curve from a single concentration point. This would preclude the necessity of repeating an entire standard curve when the retention time of sulfinpyrazone changes, although a three-point standard curve would reinforce that reliability. Slopes were predicted from peak heights obtained from a 1-μg/ml drug solution run at different retention times. These values are listed in Table II. Entire standard curves were then run at different retention times and slopes were calculated by linear regression analysis. The latter values are also listed in Table II. The maximum difference between the observed and predicted values of the slope was 2.7%.

It is evident from the results that paired-ion reversed-phase HPLC is a good method for assaying sulfinpyrazone in plasma. Using disposable $C_{18}$ cartridges further simplifies the analysis, since the entire separation of drug from plasma can be accomplished in 2 min. This isolation method also has the advantage of not requiring large volumes of expensive organic solvents as do conventional extraction procedures. While the reported calibration curve used 0.2 μg/ml as the lowest concentration, the sensitivity could easily be increased by increasing the injection volume. This sensitivity is adequate for pharmacokinetic studies or therapeutic drug level monitoring.

## REFERENCES

(1) Anturane Reinfarction Trial Research Group, *N. Engl. J. Med.*, **298**, 289 (1978).

(2) *Idem.*, **302**, 250 (1980).

(3) J. J. Burns, T. F. Yu, A. Ritterband, J. M. Perel, A. B. Gutman, and B. B. Brodie, *J. Pharmacol. Exp. Ther.*, **119**, 418 (1957).

(4) T. Inaba, M. E. Besley, and E. J. Chow, *J. Chromatogr.*, **104**, 165 (1975).

(5) J. Lecaillon and C. Souppart, *ibid.*, **121**, 227 (1976).

(6) L. T. Wong, G. Solomonraj, and B. H. Thomas, *ibid.*, **150**, 521 (1978).

## ACKNOWLEDGMENTS

Presented at the APhA Academy of Pharmaceutical Sciences meeting, Anaheim Calif., April 1979.

# Protein Binding of Caffeine in Young and Elderly Males

**JAMES BLANCHARD**

Received January 18, 1982, from the *Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, AZ 85721.* Accepted for publication February 22, 1982.

**Abstract** □ The plasma protein binding of caffeine in young and elderly males was evaluated using an ultrafiltration technique. In spite of a significantly lower plasma albumin concentration in the elderly subjects the observed percent bound (~35%) was essentially identical in both subject groups. The binding of caffeine to human plasma albumin (4.5% w/v) *in vitro* was also examined using ultracentrifugation and it was observed to be bound to the extent of 37.8%. In both the plasma and albumin binding studies the free fraction remained constant over the range of concentrations examined. Although there was no apparent correlation between the percent bound and the albumin concentration in the plasma of either subject group the close agreement between the degree of binding of caffeine to albumin and human plasma indicates that albumin is likely the major plasma binding protein for caffeine.

**Keyphrases** □ Caffeine—protein binding in young and elderly males, ultrafiltration □ Protein binding—caffeine in young and elderly males, ultrafiltration □ Ultrafiltration—protein binding of caffeine in young and elderly males

It is well known that drug–protein interactions can influence drug pharmacokinetics (1). Since plasma albumin concentration decreases and globulin concentration increases with aging (2, 3), and because a great many drugs are bound reversibly to plasma albumin, the potential importance of age-related changes in protein binding is clear. Of the relatively few studies that have examined the effect of age on protein binding, significant reductions in binding with aging have been reported for meperidine, phenylbutazone, phenytoin, and warfarin, while for phenobarbital, benzylpenicillin, diazepam, desmethyldiazepam, salicylate and sulfadiazine, no alterations in the extent of binding were observed (4). While some studies of caffeine binding *in vitro* (5) and *in vivo* (6, 7) have been reported, no specific examination of possible alterations in its binding characteristics with aging has been made. Since the pharmacological effect of caffeine is probably best related to its unbound fraction (as is true for most drugs), knowledge of any age-related binding differences could prove valuable in helping to interpret the pharmacokinetics of this widely consumed agent, which has received increased attention recently due to its possible role in the treatment of premature apnea (8). The goal of this study was, therefore, to compare the plasma protein binding of caffeine in young and elderly subjects.

## EXPERIMENTAL

**Subject Selection**—Ten healthy, young adult male volunteers ranging in age from 18.8 to 30.0 years and eight healthy, active elderly male volunteers aged 66.0–78.2 years were studied. All subjects were given a physical examination, electrocardiogram, and the following laboratory tests: plasma urea, electrolytes, creatinine, bilirubin, aspartate aminotransferase, alkaline phosphatase, total protein, albumin, creatinine clearance, and complete blood count. In addition, all subjects had a normal health history and were not taking any medication at the time of the study.

0022-3549/82/1200-1415$01.00/0