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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SULFINPYRAZONE AND ITS METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic method is described for the quantitative analysis of sulfinpyrazone and its sulfone and *p*-hydroxy metabolites in plasma and urine. The method uses two different procedures for sample preparation: (1) a rapid and convenient procedure using a single extraction with 1-chlorobutane and subsequent back-extraction into sodium hydroxide solution for the analysis of sulfinpyrazone and its sulfone metabolite, and (2) a more time consuming procedure using triple extraction with ethylene dichloride, a buffer wash, and back extraction into the base for the additional analysis of the *p*-hydroxy metabolite. The lower limit of sensitivity for sulfinpyrazone is 50 ng/ml. Concentrations of sulfinpyrazone between 0.05 to 0.1 and 50 μg/ml were measured with an average coefficient of variation of 3.9%, ranging from 1.5 to 6.1%.

INTRODUCTION

Several methods have been reported for the analysis of the antiplatelet and uricosuric agent sulfinpyrazone in biological fluids (Table I). The original spectrophotometric assay [1, 2] involves solvent extraction from acidified plasma or urine with ethylene dichloride, a buffer wash, back extraction into sodium hydroxide solution, and subsequent measurement of the ultraviolet absorbance at 260 nm. This method has been found to suffer from both the lack of sensitivity and specificity inherent in spectrophotometric assays [3].

Three high-performance liquid chromatographic (HPLC) methods and one gas—liquid chromatographic (GLC) method have been described. Two HPLC methods have obvious disadvantages, one using radiolabelled sulfinpyrazone as an internal standard requiring the collection of the effluent solvent mixture and subsequent liquid scintillation counting [4], the other not having any internal

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TABLE I

COMPARISON OF METHODS FOR ANALYSIS OF SULFINPYRAZONE IN BIOLOGICAL FLUIDS

References	Analytical method	Sample	Sensitivity (μg/ml)	Reproducibility (coefficient of variation)	Comments
1,2,3	Spectrophotometric	Plasma, urine	Not given	Not given	Lacks specificity and sensitivity
4	HPLC	Serum	3 μg/ml	Not given	Uses radiolabelled internal standard
5	HPLC	Plasma, urine	0.2 μg/ml	3.8% (replicates of 0.2—80 μg/ml from plasma and urine)	No internal standard. Separate metabolites
6	HPLC	Plasma, urine	2 μg/ml	4.3% (calibration curves of 2—100 μg/ml from plasma)	Simple two-step extraction
7	GLC	Plasma	Not given	Not given (correlation coefficient 0.99 for calibration curve)	Several transfer or extraction steps. Derivatization. Time consuming
This paper	HPLC	Plasma, urine	0.05 μg/ml	3.9% (calibration curves of 0.1—50 μg/sample from plasma and urine)	Simple two-step extraction (or triple extraction). Measures simultaneously sulfinpyrazone and metabolites

standard [5]. The third HPLC method uses warfarin as an internal standard and is simple and specific but lacks sensitivity [6]. These methods have used 1-chlorobutane [4, 6] or a mixture of 1-chlorobutane and ethylene dichloride [5] as the extraction solvent, and an evaporation step [4], direct injection of the organic solvent [5], or back-extraction into sodium hydroxide solution [6]. The absorbance has been measured at 254 nm [4, 5] or 275 nm [6]. The GLC method is time consuming and involves several transfer or extraction steps, the use of benzene as the extraction solvent, evaporation, and derivatization before chromatography [7].

Sulfinpyrazone is both metabolized in the liver and excreted unchanged in the urine. The metabolites are *p*-hydroxy-sulfinpyrazone, 4-hydroxy-sulfinpyrazone, a sulfone metabolite of sulfinpyrazone, and glucuronides of sulfinpyrazone and the sulfone metabolite [8, 9]. Although one of the HPLC methods has demonstrated that the non-glucuronated metabolites are separated from sulfinpyrazone [5], no attempt has been made to develop an HPLC assay which measures sulfinpyrazone and its metabolites. This is an important consideration, however, for clinical pharmacological studies on sulfinpyrazone, since it has been suggested that its antiplatelet effect may involve a metabolite [10]. This paper describes a simple, specific, sensitive, and accurate HPLC assay for the simultaneous analysis of sulfinpyrazone and two of its metabolites in plasma and urine.

EXPERIMENTAL

Reagents and materials

Sulfinpyrazone (1,2-diphenyl-4-[2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione) and its metabolites, *p*-hydroxy-sulfinpyrazone (G 32642), 4-hydroxy-sulfinpyrazone (GP 52097), and sulfone metabolite of sulfinpyrazone (G 31442), were a gift from Ciba-Geigy (Basle, Switzerland). The internal standard, clofibrinic acid [2-(4-chlorophenoxy)-2-methylpropionic acid], was obtained from Sigma (St. Louis, Mo., U.S.A.). The acetonitrile was of "distilled in glass" quality and was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All glassware used during sample preparation was silanized. The silanizing agent, Prosil-28, was obtained from PCR Research Chemicals (Gainesville, Fla., U.S.A.). All other solvents and reagents were of analytical grade.

Sample preparation

A schematic representation of the procedure for the analysis of sulfinpyrazone and its sulfone metabolite is shown in Fig. 1. Plasma (1 ml) or urine (1 ml) is placed in a PTFE-lined screw-capped culture tube, and 60 μl of inter-

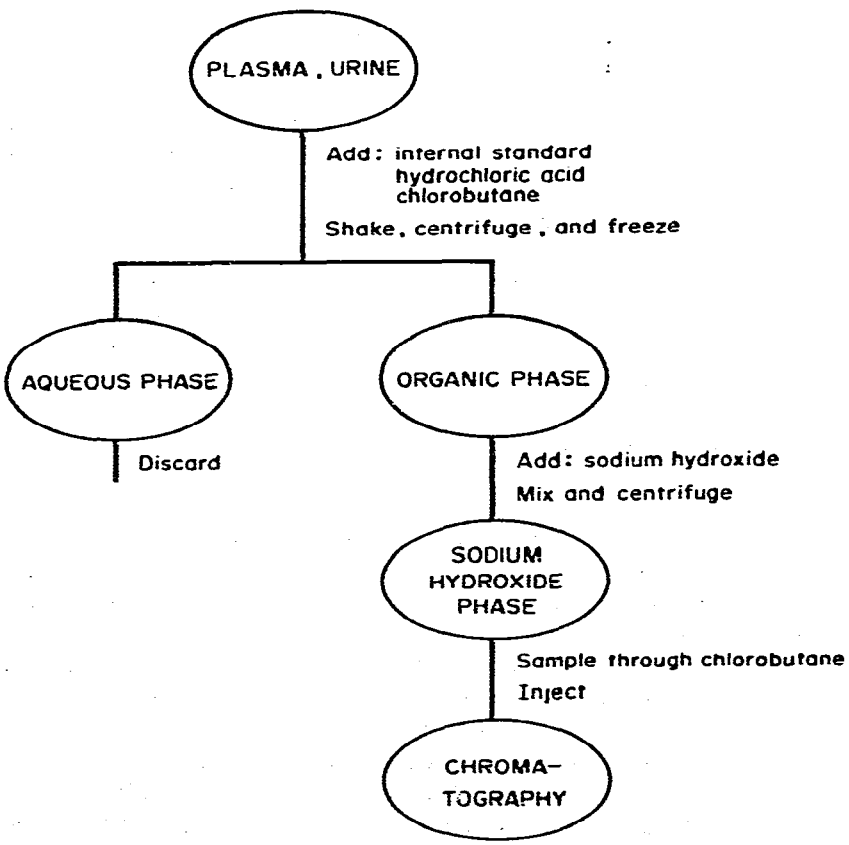

Fig. 1. Flow diagram of the sample preparation for the HPLC analysis of sulfinpyrazone and its sulfone metabolite.

nal standard solution (containing 60 μg of clofibrinic acid), 2 ml of 1 *N* HCl and 1 ml of 1-chlorobutane are added. The samples are extracted by mixing (using a Labquake automatic shaker) for 10 min, followed by centrifugation at 1000 *g* for 10 min to separate the aqueous and organic phases. The lower aqueous phase is frozen by immersing the tube in a dry ice—acetone bath, and the organic phase is poured into another tube, which has an elongated cone (capacity approximately 100 μl). Then 200 μl of 0.1 *N* NaOH are added, and the mixture is agitated in a Vortex mixer for 30 sec. After brief centrifugation, 100 μl of the aqueous phase are sampled from the elongated cone and injected into the chromatograph. When smaller volumes of plasma are used (0.1 and 0.5 ml), the same procedure is followed with two minor modifications, i.e., the volume of 1 *N* HCl added is reduced such that it remains twice that of the plasma sample, and the amount of internal standard added is reduced to 40 μg for 0.5 ml sample and to 20 μg for 0.1 ml sample.

For the additional analysis of *p*-hydroxy-sulfinpyrazone, 1 ml of plasma or urine is extracted three times with 1 ml of ethylene dichloride following the addition of the internal standard and hydrochloric acid solutions. The combined organic phase is washed by mixing with 1 ml McIlvaine's citric acid—phosphate buffer (citric acid 0.1 *M*, disodium phosphate 0.2 *M*; pH 5.0) for 5 min prior to back-extraction into the sodium hydroxide solution.

Chromatography

The high-performance liquid chromatograph consisted of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A high-pressure solvent delivery system which was equipped with a Model U6K injector and fitted with a Waters μBondapak C_{18} reversed-phase column (30 × 0.39 cm I.D.; particle size 10 μm). The absorbance was measured at 270 nm, with a 0.1 a.u.f.s. deflection, using a Waters Model 450 variable-wavelength detector. The mobile phase was the same as used in a recently published method [6], i.e., 0.1 *M* ammonium acetate in acetonitrile—water (30:70), adjusted to pH 5.0 with acetic acid. The flow-rate of the solvent mixture was 1 ml/min with a column input pressure of 55 atm (800 p.s.i.). Chromatograms were recorded on a Linear Instruments (Irvine, Calif., U.S.A.) Model 585 dual-pen recorder.

Calibration and accuracy

Calibration curves were constructed by adding known amounts of sulfinpyrazone and internal standard to control plasma or urine, and plotting the peak height ratio of sulfinpyrazone to internal standard against the amount of sulfinpyrazone added. In order to calibrate the method and determine its accuracy for each series of unknown samples, standards of 0.1, 0.5, 5, 10, 25, and 50 μg of sulfinpyrazone (in 100 μl of distilled water) were added to the control samples, which were assayed concurrently with the unknown samples. The mean normalized peak height ratios were used to calculate the amount of sulfinpyrazone in unknown samples, and the standard deviation of the normalized peak height ratios was used to determine the accuracy of the method over the range of sulfinpyrazone standards employed. The reproducibility of the method was also studied by submitting five replicate plasma samples containing 0.5, 5, 10, 25, and 50 μg of sulfinpyrazone to the entire procedure. Likewise, calibration

curves were constructed for the sulfone metabolite by adding standards of 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg to the same control samples. Replicate samples were also analyzed for the sulfone metabolite at these same concentrations. Calibration curves were also constructed for *p*-hydroxy-sulfinpyrazone by adding standards of 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg to control samples and using the ethylene dichloride extraction. The effect of sample size on the method was investigated by constructing additional calibration curves using 0.1 and 0.5 ml of plasma.

To estimate the recoveries for the method, the peak heights of analyzed samples containing known amounts of sulfinpyrazone, its metabolites, and the internal standard were compared to the respective peak heights obtained by injecting equal amounts directly into the chromatograph.

Application of the method to measure plasma concentrations

A healthy male volunteer received a single oral dose of 200 mg of sulfinpyrazone (one capsule, Anturane®, Ciba-Geigy). Samples of venous blood were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8 and 24 h after administration. The blood was collected in silanized culture tubes and anticoagulated with heparin (10 units of sodium heparin per ml of blood). After centrifugation, the plasma was transferred to glass vials and stored at −40° until analyzed.

RESULTS AND DISCUSSION

Since sulfinpyrazone is metabolized in the liver to three known metabolites, i.e., sulfone, *p*-hydroxy, and 4-hydroxy metabolites (and glucuronated products of sulfinpyrazone and its sulfone metabolite) [8, 9], and a metabolite may be involved in its antiplatelet effect [10], we were interested in developing an HPLC assay which would simultaneously measure sulfinpyrazone and its metabolites in biological fluids for subsequent clinical pharmacological studies. Preliminary experiments showed that sulfinpyrazone, and the sulfone and *p*-hydroxy metabolites all had absorption peaks between 255 and 270 nm. The 4-hydroxy metabolite, however, does not appreciably absorb ultraviolet light in this range. Since the latter is found in minimal concentrations in plasma, accounts for only less than 1% of the eliminated drug in man, and is therefore clearly the least important of the metabolites, its analysis was not pursued.

Although sulfinpyrazone has an absorption peak at 260 nm [1], the absorbance is measured at 270 nm in this method. This is because the internal standard, clofibrinic acid, has an absorption peak at about 280 nm (its major absorption peak, however, is at 226 nm). A compromise is therefore made between these two absorption peaks.

The reported methods for the quantitative analysis of sulfinpyrazone in biological fluids have used as the extraction solvent either ethylene dichloride [1, 2], 1-chlorobutane [4, 6], or a mixture of these two solvents [5], while one method uses benzene [7]. Preliminary experiments demonstrated two significant differences between ethylene dichloride and 1-chlorobutane as extraction solvents, i.e., 1-chlorobutane does not appreciably extract *p*-hydroxy-sulfinpyrazone from acidified plasma while ethylene dichloride does, and ethylene dichloride has to be washed with buffer before subsequent back-extraction into

the base, which 1-chlorobutane does not, to eliminate interfering peaks. This therefore resulted in the development of two different procedures for sample preparation: (1) a rapid and convenient procedure using a single extraction with 1-chlorobutane and subsequent back-extraction into sodium hydroxide solution for the analysis of sulfinpyrazone and its sulfone metabolite, and (2) a more time consuming procedure using triple extraction with ethylene dichloride and a buffer wash, when it is also desired to determine the concentrations of the *p*-hydroxy metabolite. The total recoveries of the three compounds using the former procedure range between 50 and 65%, while the total recoveries of the four compounds using the latter procedure range between 70 and 80%. It should be noted that the injection of the small volume of base (i.e., 100 μl) onto the column did not result in premature deterioration of the column.

Figs. 2 and 3 show chromatograms for blank plasma and plasma containing added amounts of the four compounds of interest using the two different procedures for sample preparation; Fig. 2 shows chromatograms following the

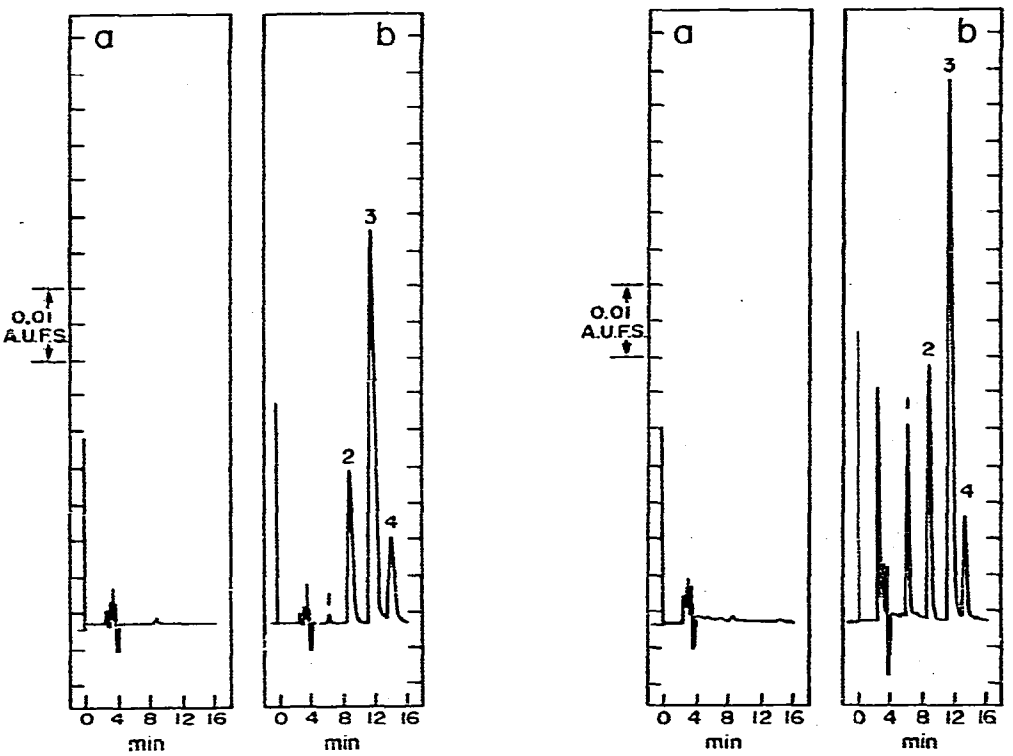

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing added amounts of (1) *p*-hydroxy-sulfinpyrazone, (2) the internal standard (clofibrinic acid), (3) sulfinpyrazone, and (4) the sulfone metabolite of sulfinpyrazone following the sample preparation using the single extraction with 1-chlorobutane. For visual clarity, only one tracing of the dual pen recording is shown.

Fig. 3. Chromatograms of (a) control plasma and (b) plasma containing the same added compounds and in the same amounts as in Fig. 2 following the sample preparation using the triple extraction with ethylene dichloride.

single extraction with 1-chlorobutane, Fig. 3 shows chromatograms following the triple extraction with ethylene dichloride and the buffer wash. Note the markedly increased recovery of *p*-hydroxy-sulfinpyrazone, as well as of the other compounds on the latter figure.

Estimates of accuracy for the method are shown in Table II. The average normalized peak height ratio for sulfinpyrazone obtained from calibration curves from plasma (different volumes) and urine had a mean coefficient of variation of 3.9% for a total of 14 such calibration curves using different volumes of plasma. This estimate of accuracy covers the entire range of the assay procedure, from 50 ng to 50 μg of sulfinpyrazone per sample. Calibration curves for the sulfone metabolite (n=12) and the *p*-hydroxy metabolite (n=2) from plasma and urine had mean coefficients of variation of 5.9% and 7.1%, respectively. These estimates for the metabolites cover the concentration range of 0.05 or 0.1 to 5.0 μg/ml. Reproducibility studies on replicates of sulfinpyrazone and its sulfone metabolite provided similar estimates of accuracy of the method (Table II). It should be noted that both procedures of sample preparation yield similar estimates of accuracy. As it may be necessary to use variable volumes of plasma for sulfinpyrazone measurement, the effects of plasma volume on the method (see Experimental) were examined as judged by the

TABLE II

ESTIMATES OF ACCURACY OF THE METHOD FOR DETERMINING SULFINPYRAZONE AND TWO OF ITS METABOLITES IN BIOLOGICAL FLUIDS

Biological fluid (volume)	Concentration range, μg/ml	Average mean normalized peak height ratio (number of studies)	Average coefficient of variation, % (range)
Sulfinpyrazone			
Calibration curve data			
Plasma (1.0 ml)	0.1—50	0.270 (7)	4.3 (1.5—6.1)
Plasma (0.5 ml)	0.1—50	0.174 (2)	3.9 (3.8—4.0)
Plasma (0.1 ml)	0.5—50	0.092 (2)	3.7 (3.4—4.0)
Plasma* (1.0 ml)	0.1—50	0.250 (1)	1.9
Urine (1.0 ml)	0.1—50	0.221 (2)	3.3 (2.1—4.5)
Reproducibility at a given concentration			
Plasma (1.0 ml)	0.5—50	0.265 (5)	3.8 (1.8—5.9)
Sulfone metabolite			
Calibration curve data			
Plasma (1.0 ml)	0.05—5.0	0.235 (7)	6.6 (5.1—9.4)
Plasma (0.5 ml)	0.05—5.0	0.152 (2)	5.3 (4.8—5.8)
Plasma* (1.0 ml)	0.1 —5.0	0.160 (1)	4.8
Urine (1.0 ml)	0.05—5.0	0.181 (2)	4.8 (3.8—5.8)
Reproducibility at a given concentration			
Plasma (1.0 ml)	0.1—5.0	0.225 (5)	5.0 (1.2—7.2)
p-Hydroxy-sulfinpyrazone			
Calibration curve data			
Plasma* (1.0 ml)	0.1—5.0	0.273 (2)	7.1 (5.6—8.6)

*Using the triple extraction with ethylene dichloride and a buffer wash.

peak height ratio, which has to be corrected for the different amounts of internal standard added, and the coefficient of variation; these estimates are independent of the volume of plasma used between 0.1 and 1.0 ml.

Application of the method to the determination of sulfinpyrazone and its sulfone metabolite in plasma from a healthy male volunteer is demonstrated in Fig. 4. It can be seen that the concentrations of the sulfone metabolite are approximately one-tenth of those of sulfinpyrazone and that they appear to fall parallel to those of the parent drug. It should be noted that no detectable concentrations of *p*-hydroxy-sulfinpyrazone were observed (following the triple extraction with ethylene dichloride).

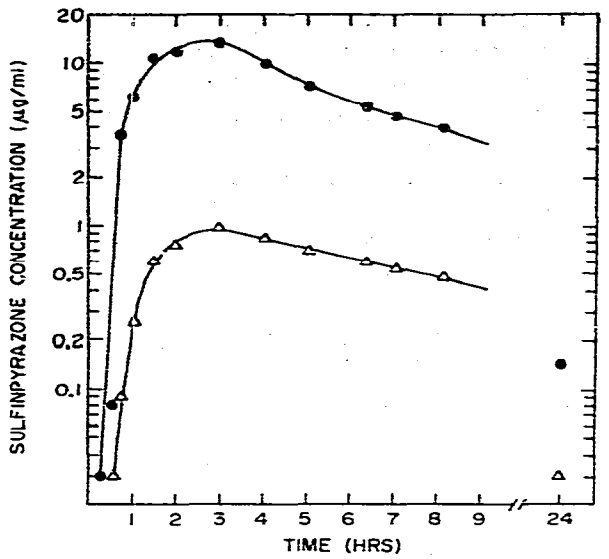

Fig. 4. Semi-logarithmic plot of plasma concentrations of sulfinpyrazone (●) and its sulfone metabolite (△) in a healthy male subject after a single oral dose of 200 mg of sulfinpyrazone.

The method described here for the quantitative determination of sulfinpyrazone and its sulfone metabolite, using the single extraction with 1-chlorobutane, is simple and rapid. By using the techniques described, 20—30 samples can easily be assayed in a day. When it is also desired to determine the concentrations of *p*-hydroxy-sulfinpyrazone, the method using the triple extraction with ethylene dichloride and the buffer wash is used. Such sample preparation, although more time consuming, allows the simultaneous determination of sulfinpyrazone, and its sulfone and *p*-hydroxy metabolites.

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