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DETERMINATION OF SULFINPYRAZONE AND TWO OF ITS METABOLITES IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY AND SELECTIVE DETECTION

P. JAKOBSEN and A. KIRSTEIN PEDERSEN

Department of Pharmacology, University of Aarhus, DK-8000 Aarhus C (Denmark)

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SUMMARY

A selective and sensitive gas chromatographic method for simultaneous determination of sulfinpyrazone and two of its metabolites (the *para*-hydroxylated metabolite and the sulfone metabolite) in biological fluids using alkali flame ionization detection (AFID), electron capture detection (ECD) and mass fragmentographic detection is described. The compounds are extracted from the samples, methylated and separated on 2% OV-17 or 3% OV-225 columns. Phenylbutazone is used as internal standard. Standard curves are linear. The coefficient of variation at 10 μg/ml of sulfinpyrazone in plasma was shown to be 1.8% (AFID), and the detection limits were 0.1 μg/ml (AFID) and 10 ng/ml (ECD). Mass spectra of the methylated compounds are shown and serum concentration curves after oral administration of 100 mg sulfinpyrazone to two persons are determined together with the excreted amounts of drug and metabolites.

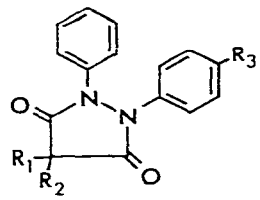
INTRODUCTION

Sulfinpyrazone, 1,2-diphenyl-4-[2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione, Anturan® (I, Table I), which was originally introduced as a uricosuric agent in the treatment of gout, has been shown to suppress platelet function presumably by competitive inhibition of platelet prostaglandin synthetase (fatty acid cyclooxygenase) [1]. Studies [2] have demonstrated that sulfinpyrazone is an effective drug in the management of certain thromboembolic conditions. Recently, a controlled clinical trial has shown that sulfinpyrazone reduces the annual death-rate after myocardial infarction with 46% compared to placebo [3].

Sulfinpyrazone is mainly excreted unchanged and as a monoglucuronide (VI) in urine. In addition two minor metabolites have been identified, namely the *p*-hydroxymetabolite (III) and the sulfone metabolite (IV). These two

TABLE I

COMPOUNDS INVESTIGATED



No.	Compound	R_1	R_2	R_3
I	Sulfinpyrazone	Ph—S(=O)—CH_2CH_2—	H	H
II	Phenylbutazone	$CH_3CH_2CH_2CH_2$—	H	H
III	G 32642	Ph—S(=O)—CH_2CH_2—	H	OH
IV	G 31442	Ph—SO_2—CH_2CH_2—	H	H
V	GP 52097	Ph—S(=O)—CH_2CH_2—	OH	H
VI	I-glucuronide	Ph—S(=O)—CH_2CH_2—	Glu	H

metabolites together with a third uncertain metabolite (V) represent only about 12% of the urinary excretion products from a single oral dose of 200 mg [^{14}C] sulfinpyrazone [4]. For the determination of sulfinpyrazone in biological fluids, Burns et al. [5] described a spectrophotometric assay. The limited sensitivity and poor selectivity of this method have resulted in development of several high-performance liquid chromatography (HPLC) methods [6—8]. Until recently [9] no gas chromatographic (GC) method has been published. The present report presents a selective and sensitive GC method for simultaneous determination of sulfinpyrazone and two of its metabolites in human plasma and urine.

MATERIALS AND METHODS

Apparatus

A Varian Model 2100 gas chromatograph equipped with a specific nitrogen-sensitive detector (AFID) and a ^{63}Ni electron capture detector (ECD) was used. The GC column for alkali flame ionization detection was a 100 cm × 2 mm I.D. U-shaped glass column filled with 2% OV-17 on Gas-Chrom Q (Pierce, Rockford, Ill., U.S.A.). The column was conditioned for 24 h at 300° with a nitrogen flow-rate of 30 ml/min and silylated at 200° by injecting 5 μl of Silyl-8 (Pierce) ten times. Operating conditions: injector temperature 280°, detector temperature 300°, column temperature 200° and carrier gas (nitrogen) flow-rate 30 ml/min. Air and hydrogen flow-rates for the alkali flame ionization detector were adjusted according to the manual. A number of chromato-

grams were recorded with different injector temperatures (230—300°). In the analysis of the sulfone metabolite the column temperature was raised to 280°.

For electron capture detection a 150 cm × 2 mm I.D. U-shaped glass column filled with 3% OV-225 on Gas-Chrom Q (Pierce) was used. The column was conditioned at 290° for 2 h with nitrogen flow. Operating conditions: injector temperature 270°, column temperature 210°, detector temperature 290° and carrier gas (oxygen-free nitrogen) flow-rate 40 ml/min.

Mass spectra were recorded with a Jeol D 100 mass spectrometer in connection with a gas chromatograph (Jeol, JGC-20K), a three-channel multiple ion detector unit and the Jeol mass data system. The GC column was a 100-cm glass column (3% OV-17) and the carrier gas was helium.

Standards and reagents

Sulfinpyrazone, phenylbutazone and sulfinpyrazone metabolites (G 31442 and G 32642) were obtained as pure crystalline compounds from Ciba-Geigy (Copenhagen, Denmark). Standard solutions of 5 and 100 μg/ml of sulfinpyrazone and metabolites in methanol and internal standard solutions of 1 mg/ml and 20 μg/ml of phenylbutazone in methanol were prepared and kept at 4°. Dichloromethane, methanol, ethyl acetate, toluene and methyl iodide were of analytical grade obtained from Merck (Darmstadt, G.F.R.). The methyl iodide was distilled before use. Dimethylacetamide and tetramethylammonium hydroxide (TMAOH), 20% in methanol, were obtained from EGA-Chemie (Steinheim, G.F.R.). The derivatization reagent (TMAOH solution) was prepared immediately before use by mixing 1 ml of 2% tetramethylammonium hydroxide in methanol with 8 ml of dimethylacetamide.

Procedure

One ml of sample (urine, serum or plasma), to which were added 25 μl of the internal standard solution (1 mg/ml for alkali flame ionization detection and 20 μg/ml for electron capture detection) and 1 ml of 2 *N* hydrochloric acid, was extracted with 6 ml of dichloromethane. An amount of 5.5 ml of the organic phase was transferred into a new tube and back extracted into 4 ml of 1 *N* sodium hydroxide; 3.5 ml of the aqueous phase were then transferred to a third centrifuge tube, acidified with 0.5 ml of 10 *N* hydrochloric acid and extracted with 6 ml of dichloromethane.

After centrifugation 5.5 ml of the organic phase was transferred to a tapered centrifuge tube and evaporated to dryness at 45° under a gentle stream of nitrogen. The remanence was dissolved in 100 μl of the TMAOH solution by vortex mixing for 10 sec. Methyl iodide 50 μl was added, the tube was shaken for 10 sec and left at room temperature for 10 min. Then 0.5 ml water and 1 ml dichloromethane were added, the tube was vortex mixed for about 30 sec and after centrifugation the organic phase was transferred to a centrifuge tube and evaporated to dryness at 45° under nitrogen flow. For alkali flame ionization detection the remanence was dissolved in 100 μl of ethyl acetate of which 1 μl was injected onto the gas chromatograph. For electron capture detection 200 μl of toluene and 1 ml of a hot saturated silver sulfate solution was added. The tube was shaken and after centrifugation 1—2 μl of the organic phase was injected.

Preparation of standard curves

Known amounts of sulfinpyrazone, the *p*-hydroxymetabolite or sulfone metabolite were added to plasma or urine and the samples were treated as described under *Procedure*. Standard curves were constructed by plotting the ratio of the peak heights of the derivatized sulfinpyrazone or *p*-hydroxymetabolite to that of the derivatized phenylbutazone against the concentration of sulfinpyrazone or the *p*-hydroxymetabolite. In constructing the standard curve for the sulfone metabolite, the peak height was plotted against the concentration.

Experiments in humans

To each of two healthy male volunteers, body weight 79 kg (AK) and 68 kg (PJ) a 100-mg Anturan® tablet was given orally. Blood samples were drawn at certain intervals (0—16 h) and the 24-h urine was collected. Serum samples and urine samples were analysed according to *Procedure*. The urine samples were tentatively treated with β-glucuronidase at pH 5 for 24 h at 37° and reanalysed.

RESULTS AND DISCUSSION

Gas chromatography

Sulfinpyrazone and its metabolites could not be chromatographed directly. Sulfinpyrazone showed more than one peak and the metabolites no peaks.

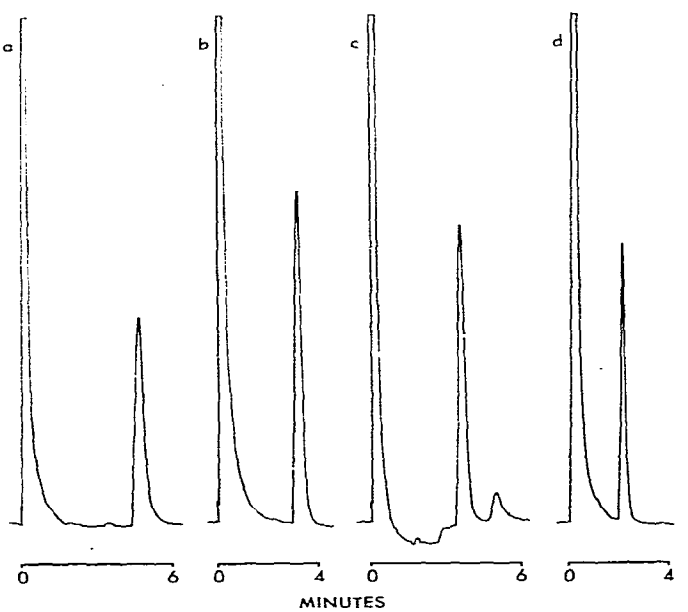


Fig. 1. Gas chromatograms with alkali flame ionization detection of (a) phenylbutazone (100 ng), (b) methylphenylbutazone (100 ng), (c) sulfinpyrazone (500 ng) and (d) methylsulfinpyrazone (100 ng). Column, 100 cm 2% OV-17, column temperature 200°, injector temperature 270°. Attenuation 64 × 10^{-13} A/mV.

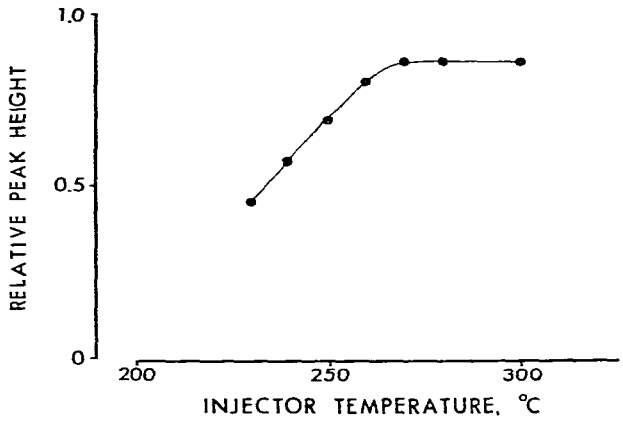


Fig. 2. Influence of injector temperature on the peak height of methylsulfinpyrazone. The ratios of the peak heights of methylsulfinpyrazone to methylphenylbutazone (50 ng of each) are plotted against the injector temperature.

Underivatized phenylbutazone gave a single peak. Methylation of the compounds gave derivatives with excellent GC properties when the injector port temperature was kept above 270° (Fig. 1). In contrast to methylated phenylbutazone, methylated sulfinpyrazone seems to degrade in the injector port as the peak shape and peak height improve with increasing injector temperature. At temperatures above 270° the degradation appears to be complete (Fig. 2). In order to analyse the methylated sulfone metabolite it was necessary to elevate the column temperature to 280° to reduce the retention time. Retention times for the methylated compounds in question are listed in Table II.

TABLE II

RETENTION TIMES FOR THE METHYL DERIVATIVES (min)

Compound	100 cm 2% OV-17, 200°	150 cm 3% OV-225, 210°
Sulfinpyrazone	2.1	3.1
Phenylbutazone	3.2 (4.7)*	4.1 (5.9)*
p-Hydroxy metabolite	5.6	7.5
sulfone metabolite	7.0**	35

*Underivatized phenylbutazone.
**Column temperature 280°.

Extraction and derivatization

Single extraction of acidified plasma blanks followed by derivatization gave a peak of variable size with the same retention time as methylated phenylbutazone together with a peak with a retention time of about 30 min. Back extraction into alkali with subsequent acidification, re-extraction and deriva-

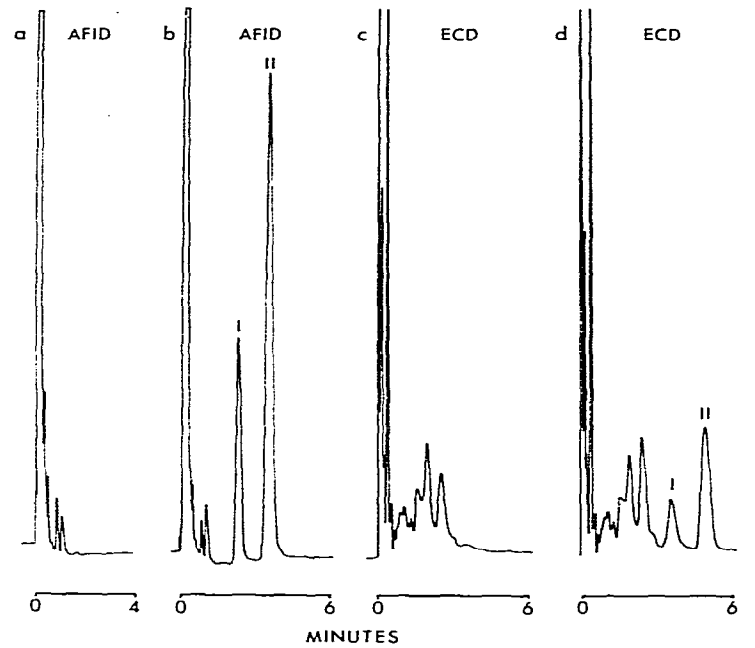


Fig. 3. Gas chromatograms with electron capture (ECD) and alkali flame ionization detection (AFID) of derivatized plasma extracts. a and c, plasma blanks; b, plasma containing sulfinpyrazone (I) (10 μg/ml) and phenylbutazone (II) (25 μg/mg); d, plasma containing sulfinpyrazone (I) (100 ng/ml) and phenylbutazone (II) (500 ng/ml). Conditions as described under Materials and methods.

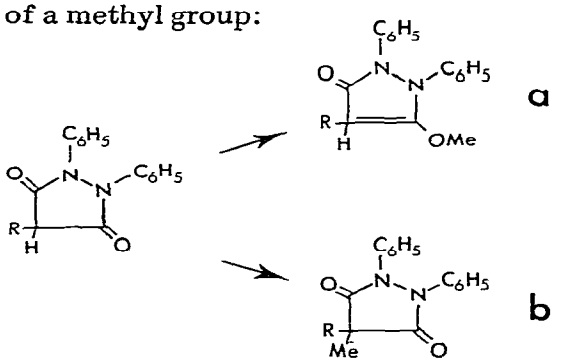
tization resulted in disappearance of the disturbing peaks. Fig. 3 shows gas chromatograms of plasma blanks together with plasma samples spiked with both sulfinpyrazone and phenylbutazone.

The method chosen for alkylation of the compounds has been applied in butylation of theophylline [10]. The reaction scheme for compounds with active hydrogen atoms is shown below:

$$HA + (CH_3)_4N^+ OH^- \rightarrow (CH_3)_4N^+ A^- \xrightarrow{RI} R{-}A$$

The reaction is based on formation of an ion pair between the quaternary ammonium ion and the appropriate anion which in turn reacts with an alkyl iodide to form an alkylated compound.

Two locations in the 3,5-dioxopyrazolidine ring structure are available for introduction of a methyl group:



O-Alkylation of malonic ester type compounds has to our knowledge not been found, so compound b is probably more easily formed than compound a. The structure has not been confirmed.

The reaction proceeds quickly with approximately 100% yield and the only disadvantage is the use of dimethylacetamide as solvent. This solvent has to be completely removed before injection as its presence will cause large tailing of the solvent peak when using a nitrogen sensitive detector. Other solvents such as acetonitrile, methanol, dichloromethane and ethyl acetate were tried without success as yields were poor. However, evaporation to dryness at 45° under nitrogen could be achieved within 30 min without loss of methylated compounds. In case of electron capture detection it was necessary to treat the remanence with silver sulfate in order to minimize solvent tailing. Attempts to methylate sulfinpyrazone with etheral diazomethane was not successful as more than one GC peak appeared. In course of preparation of the present analytical method Rosenfeld et al. [9] have described a GC method for the determination of sulfinpyrazone based on extractive methylation and normal flame ionization or mass spectrometric detection. In our preliminary experiments this extractive methylation technique was tried but analytical yields were in our hands not satisfactory.

Recovery and standard curves

For sulfinpyrazone and phenylbutazone no trace of underivatized compounds was found in the gas chromatograms after methylation of pure substances. Presumably all compounds mentioned are methylated quantitatively. When peak heights of derivatized pure compounds were compared to the peak heights of the same amounts of compounds carried through the complete sample preparation procedure, the following recoveries were found: 94.4% ± 4.8 (sulfinpyrazone), 93.8% ± 3.2 (phenylbutazone), 89.7% ±6.8 (the *p*-hydroxymetabolite) and 93.8 ± 8.2 (the sulfone metabolite). The values are corrected for loss of solvents during the extraction procedure.

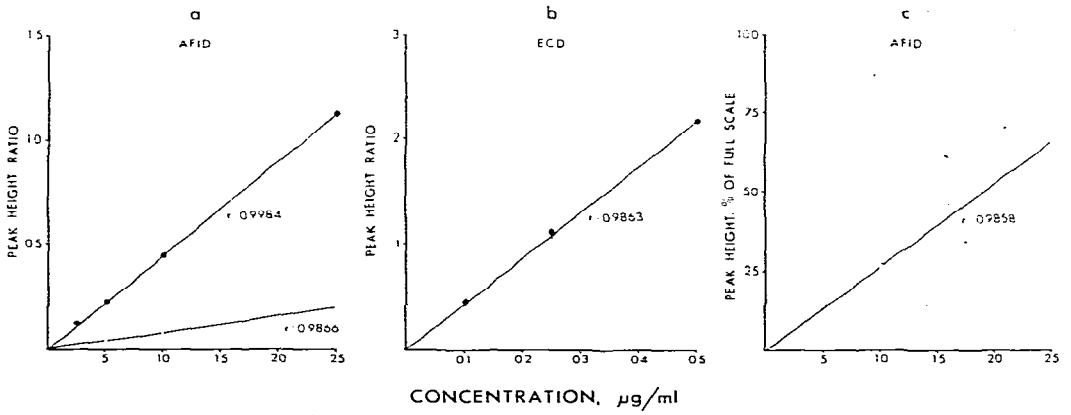


Fig. 4. Standard curves for determination of sulfinpyrazone (●———●), the *p*-hydroxy metabolites (G 32642) (△———△) and sulfone metabolite (G 31442) (○———○). Peak height ratio refers to the ratio between the peak heights of derivatized drug or metabolite and the derivatized internal standard (phenylbutazone).

A linear relationship between detector response and plasma concentration was found for sulfinpyrazone and its two metabolites (Fig. 4). As no suitable internal standard was available in the determination of the sulfone metabolite the peak height versus concentration was used as standard curve. For sulfinpyrazone the minimum detectable concentration was about 100 ng/ml sample using AFID and 10 ng/ml using ECD.

The analytical precision of the method depends on the concentration of drug. For sulfinpyrazone, the coefficient of variation was found to be 6.0% (n = 4) using electron capture detection of 100 ng/ml plasma samples and 1.8% (n = 6) using alkali flame ionization detection of 10 μg/ml plasma samples.

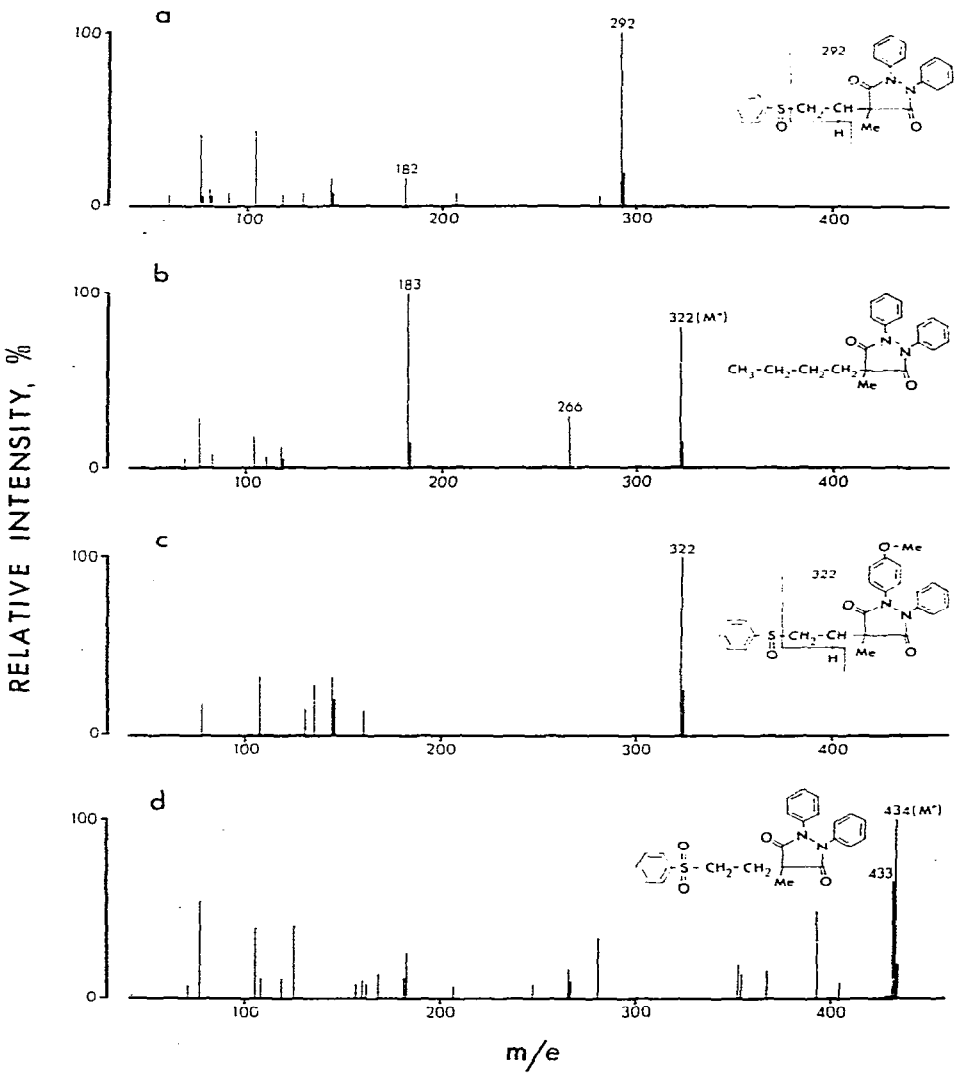


Fig. 5. GC—MS (26 eV) of the methyl derivatives of (a) sulfinpyrazone, (b) phenylbutazone, (c) p-hydroxy metabolite (G 32642) and (d) sulfone metabolite (G 31442).

Mass spectrometry

Mass spectrometry (MS), ionization energy 10 eV or 26 eV, using direct inlet of the methylated sulfinpyrazone gave a molecular ion (*m/e* = 418) of 14 mass units above the molecular weight of sulfinpyrazone showing substitution of a single methyl group in the sulfinpyrazone molecule. Combined gas chromatography—mass spectrometry (GC—MS) at 26 eV ionization energy did not show this ion but a loss of 126 mass units from the methylated sulfinpyrazone indicating elimination of the fragment [C_6H_5SOH] (Fig. 5a). GC—MS of methylated phenylbutazone (Fig. 5b) showed no degradation. The same cleavage as that of methylsulfinpyrazone with splitting off of the fragment [C_6H_5SOH] also takes place during GC-MS of the methylated-hydroxy metabolite. Fig. 5c shows substitution of the phenolic hydrogen atom as well as in the pyrazolidine ring structure. GC—MS of the sulfone metabolite (Fig. 5d) showed introduction of one methyl group. The high-intensity molecular ion together with the long retention time indicate thermal stability of this compound.

The loss of [C_6H_5SOH] seen in the mass spectra of methylated sulfinpyrazone and the methylated *p*-hydroxy metabolite is due to a thermal cleavage, which probably takes place in the gas chromatograph. Sulfoxides are usually not thermally stable compounds. They undergo 1,2-elimination [11] reactions at elevated temperature resulting in formation of alkenes:

$$R-\underset{\underset{O}{\|}}{S}-CH_2-CH_2-R' \xrightarrow{\Delta} [RSOH] + CH_2 = CH-R'$$

When sulfinpyrazone undergoes this elimination reaction a compound with a structure very similar to that of phenylbutazone is formed. The two compounds are however easily separated on 2% OV-17 or 3% OV-225 columns.

GC—MS of methylsulfinpyrazone shows a base peak of *m/e* 292, an ion which is not present in the mass spectrum of the methylphenylbutazone. The high-intensity molecular ion of this compound (*m/e* 322) is present as base peak in the mass spectrum of the methyl derivative of the *p*-hydroxy metabolite. The presence of these two ions makes it possible simultaneously to determine sulfinpyrazone and its hydroxylated metabolite using multiple ion detection with only two channels. Injection of 0.1—50 ng of the methyl derivatives of sulfinpyrazone and the *p*-hydroxy metabolite together with a fixed amount of methylated phenylbutazone (50 ng) gave linear standard curves for both methylated sulfinpyrazone (r = 0.996) and the methylated *p*-hydroxy metabolite (r = 0.987). Single extraction from acidified plasma samples was found to be satisfactory as ions of *m/e* 292 and *m/e* 322 from the impurities in plasma did not interfere (Fig. 6). This improves the capacity and speed of the method. With a proper internal standard, determination of the sulfone metabolite is possible with probably just as good a sensitivity using the molecular ion of the methylated sulfone metabolite.

Since both the selectivity and the sensitivity of the mass fragmentographic detection method is superior to that of electron capture detection, the mass fragmentographic method is suitable for the determination of trace amounts of the substances.

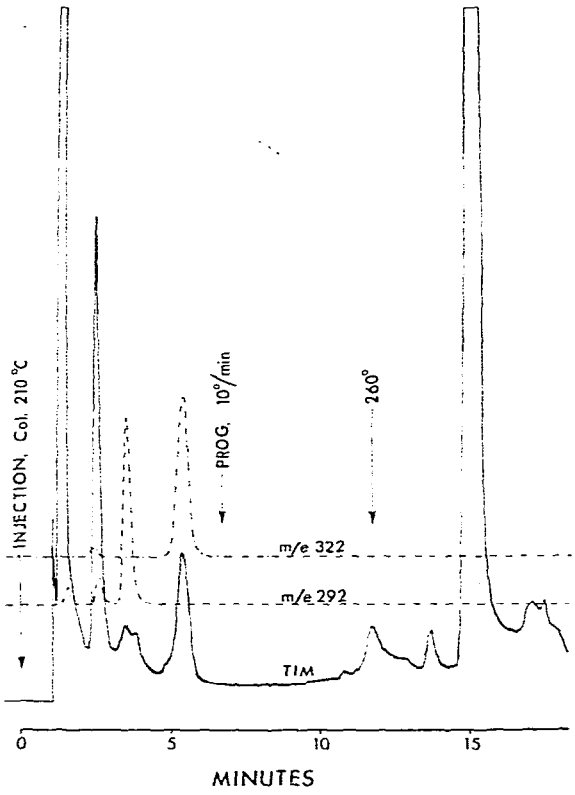


Fig. 6. Total ion monitoring (TIM) and selected ion monitoring of a derivatized extract of plasma containing sulfinpyrazone and phenylbutazone (single extraction). The mass spectrometer was set to detect the base peak in the mass spectra of methylsulfinpyrazone (*m/e* 292) and the molecular ion of methylphenylbutazone (*m/e* 322).

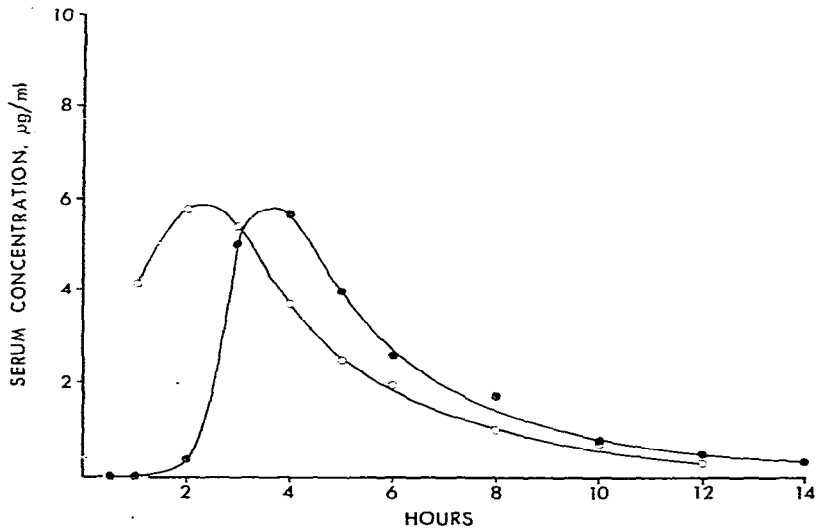


Fig. 7. Sulfinpyrazone in serum (alkali flame ionization detection) after oral administration of 100 mg of the drug to two volunteers. o——o: AK; ●——●: PJ.

TABLE III

AMOUNTS (mg) OF UNCHANGED DRUG AND METABOLITES EXCRETED IN URINE IN 24 H AFTER ORAL ADMINISTRATION OF 100 mg OF SULFINPYRAZONE

Compound	PJ	AK
Sulfinpyrazone	36.1	57.3
p-Hydroxy metabolite	7.3	9.0
Sulfone metabolite	2.5	2.4

Fig. 7 shows the serum concentration curves obtained from the human experiments. No measureable amounts of metabolites were found in serum using alkali flame ionization detection. The excreted amounts of sulfinpyrazone and two of its metabolites in the 24-h urine are listed in Table III. Treatment of the urine with β-glucuronidase did not increase the measured amounts.

A number of commonly used acidic drugs including barbiturates, weak analgesics and diuretics did not interfere with the determination.

The finding that β-glucuronidase is not able to increase the measured amounts of sulfinpyrazone in urine is consistent with the earlier work of Dieterle et al. [4], who found that glucuronic acid is conjugated to sulfinpyrazone via a C—C bond, which cannot be cleaved by β-glucuronidase. This metabolite is not measured by our method.

At present only limited information is available about plasma levels of sulfinpyrazone during therapy. A convincing correlation between the dose of sulfinpyrazone and inhibition of platelet function, measured by [^{14}C]serotonine release, has been reported [1]. The plasma levels in patients with transient ischemic attacks receiving 800 mg per day reported by Rosenfeld et al. [9] showed a considerable scatter within the range of 4—29 μg/ml. Furthermore, the presence of active metabolites, which seem to be equipotent with the parent molecule in inhibiting the platelet release reaction [12], has been suggested to cause the biphasic effect of sulfinpyrazone in rabbits in vivo reported by Buchanan et al. [13]. These data call for more detailed investigations of the metabolism and pharmacokinetics of sulfinpyrazone in man.

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