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Two metabolites of sulphinpyrazone and their identification and determination by mass spectrometry

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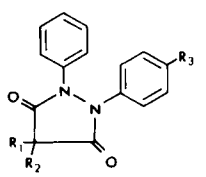
Sulphinpyrazone is an antiplatelet agent in vivo and in vitro. Two active metabolites, a sulphide (S) and a hydroxylated sulphide (S-OH) have been identified in rabbit and human plasma and a selective and sensitive g.c.-m.s.-method for quantitative determination of the sulphide and hydroxylated sulphide in plasma and urine has been evolved which allows concentrations down to 5 ng ml^{-1} for the sulphide and 30 ng ml^{-1} for the hydroxylated sulphide to be detected. The time course of the metabolite concentrations in plasma corresponds to the biological findings, suggesting that the metabolites contribute significantly to the in vivo effects of the drug.

Sulphinpyrazone (I) is reported to suppress platelet function in patients suffering from thromboembolic disorders (Weily & Genton 1970; Steele et al 1975) and a significant reduction of the incidence of sudden death after acute myocardial infarction has been attributed to the drug (The Anturane Reinfarction Trial Research Group 1980).



		R^1	R_2	R_3
I	Sulphinpyrazone (SO)	C_6H_5-SO-CH_2CH_2-	H	H
	Phenylbutazone	$CH_3CH_2CH_2CH_2$-	H	H
II	G 25671 (S)	C_6H_5-S-CH_2CH_2-	H	H
III	G 33378 (S-OH)	C_6H_5-S-CH_2-CH_2-	H	OH
IV	G 31442 (SO_2)	C_6H_5-SO_2-CH_2-CH_2-	H	H
V	G 32642 (SO-OH)	C_6H_5-SO-CH_2CH_2-	H	OH
	Internal std. (BuS)	C_6H_5-S-CH_2-CH_2-	CH_3CH_2-CH_2-CH_2-	H

Structure of sulphinpyrazone and related compounds.

A biphasic effect of the drug has been reported (Buchanan et al 1978) in the rabbit. This was described as an immediate concentration-related platelet suppressive action followed by a late and more pronounced depression of platelet function after all the drug had disappeared from plasma. It also occurred in guinea-pigs (Butler et al 1979) and in man (Maguire et al 1979), and was assumed to be caused by a so far unknown, but slowly eliminated and very potent, metabolite.

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A sulphide (II) and a hydroxylated sulphide (III) metabolite were identified in the rabbit (Kirstein Pedersen & Jakobsen 1979); we now report a sensitive and selective method for their determination in plasma or urine by g.c.-m.s.

METHODS AND MATERIALS

Experiments in rabbits

To four rabbits (3–4 kg) were given intraveneously 100 mg kg^{-1} sulfinpyrazone. Heparinized blood samples were drawn at 0, 0·5, 3, 12, 24 and 48 h after administration. Plasma samples were kept at −20 °C (until analysis; see below).

Experiments in man

From two patients treated with sulphinpyrazone 800 mg day^{-1} for 2 years, plasma samples collected before the first morning dose were analysed for drug and metabolites II–V. Samples of 24 h urine from the same two patients were collected and analysed as described below for the same compounds before and after treatment with β-glucuronidase (Sigma G-0251, 2000 uml^{-1} at pH 5 for 24 h at 37 °C).

Procedure

Plasma or urine samples (0·1–0·5 ml) to which were added 1 ml of 0·1 M hydrochloric acid were extracted with 8·0 ml of dichloromethane for 30 min. After centrifugation 7·0 ml was transferred to a tapered centrifuge tube and evaporated to dryness at 45 °C under nitrogen. The residue was dissolved in 50 μl of the TMAOH-solution and 25 μl methyliodide was added. The tube was vortex mixed and left at room

temperature (20 °C) for 10 min. Then 0·5 ml of 0·05 M sulphuric acid, 50 μl of the internal standard solution (1 μg BuS) and 1 ml of ethylacetate were added. The tube was vortex-mixed for about 30 s. and after centrifugation the organic phase was transferred to a centrifuge tube and evaporated to dryness at 45 °C under nitrogen. The residue was dissolved in 100 μl ethylacetate of which 1–5 μl was injected onto the g.c.-m.s. system, which was adjusted to measure the intensity of the fragment ions $m/z = 266$, (methylated sulphide (S-Me)), $m/z = 296$ (methylated OH-sulphide (S-OH-Me)) and $m/z = 308$ (BuS).

Preparation of standard curves

Known amounts of sulphide and OH-sulphide were added to plasma and the samples were treated as described under procedure. Standard curves were constructed by plotting the ratio of the peak heights of the methylated compounds to that of the internal standard against the concentration of sulphide and OH-sulphide.

Apparatus

The g.c.-m.s. analysis was carried out using a Jeol D-100 mass spectrometer equipped with data reduction system and a three channel multi ion detection unit. The gas chromatograph was a Jeol K-25 connected to the m.s. via a heated double stage jet separator. The g.c. column was 1 m glass (i.d.: 2·5 mm) packed with 3% OV-1 on Gas-Chrom Q 80/100 mesh. Operating conditions were: injector temp. 270 °C, column temp. 255 °C, separator temp. 270 °C and carrier gas (helium) inlet pressure 1 kg cm^{-2}. Adjustment of the mass spectrometer was done by injection of known sample mixtures into the g.c. Extractions were made using 15 ml glass stoppered tubes placed horizontally in a mechanical shaker (200 strokes min^{-1}, ampl. 5 cm) (Edmond Bühler, Tübingen GFR).

Standards and reagents

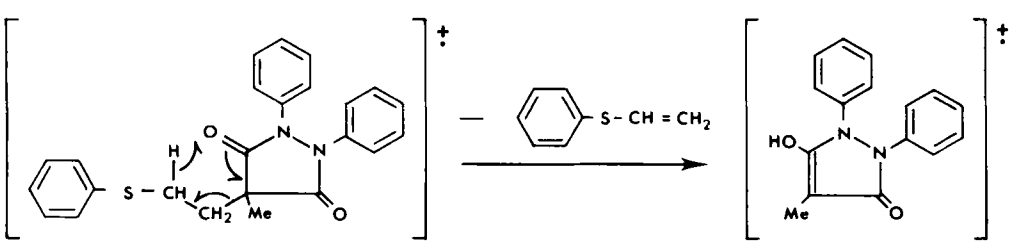
Sulphinpyrazone (I) and its synthetic reference compounds (G 25671 II, G 33378 III, G 31442 IV and G 32642 V) were obtained as pure crystalline compounds from Ciba-Geigy (Copenhagen, Denmark). Standard solutions were made up in ethanol. Dichloromethane, methanol, ethylacetate, methyliodide and butyliodide were of analytical grade from Merck (Darmstadt, GFR). Dimethylacetamide and tetramethyl ammonium hydroxide 20% in methanol were from EGA-Chemie (Steinheim, GFR) and tetrahexyl ammonium hydrogen sulphate from Fluka. The derivatization reagent (TMAOH) was prepared immediately before use by mixing 1 ml of 2% tetramethyl ammonium hydroxide in methanol with 8 ml of dimethylacetamide as previously described (Jakobsen & Kirstein Pedersen 1979). The internal standard, butylated sulphide (BuS), was prepared by extractive butylation of the sulphide II.

RESULTS AND DISCUSSION

During g.c.-analysis of methylated blood samples from rabbits receiving sulphinpyrazone, two unknown peaks appeared in the gas chromatograms. M.s. analysis of the g.c.-peaks showed molecular ions corresponding to the molecular weight of methylated sulphinpyrazone with one oxygen atom missing (m/z 402). Base peaks at m/z 266 and 296 respectively, corresponded to loss of C_6H_5-S-CH = CH_2 by a McLafferty rearrangement (Scheme 1), suggesting that the sulphoxide in sulphinpyrazone had been reduced to a sulphide.

Mass spectra of the methylated two authentic compounds II, III were identical to those obtained from the plasma samples and their retention times were 2·4 and 4·2 min respectively while the internal standard had Rt 3·5 min on a 100 cm 3% OV-1 column at 255 °C. Attempts to analyse the compounds without alkylation were not successful.

Dichloromethane, which was used in the quantitative extraction of the drug and the known metabolites IV and V_2 (Jakobsen & Kirstein Pedersen 1979;



SCHEME 1. McLafferty rearrangement of the methyl derivative of the sulphide metabolite.

Dieterle et al 1975) was able to extract both II and III quantitatively from buffer solutions at acidic pH. Extraction from acidified plasma samples, however, was not quantitative (50–60% II after 10 min extraction), but efficiency improved with extraction time, implying that the metabolites are strongly bound to plasma constituents. An extraction time of 30 min was chosen since about 80% II and about 90% III were then recovered. Recovery through the alkylation step was more than 95%.

Fig. 1 shows mass fragmentograms of a human plasma blank and a plasma sample containing II and III carried through the analytical procedure. The

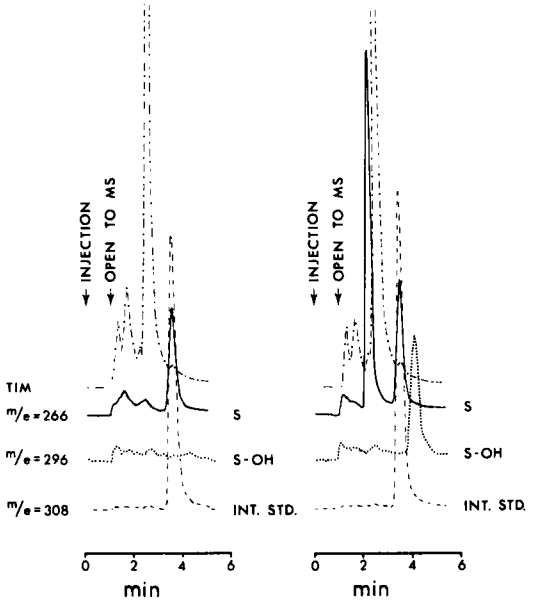


Fig. 1. Mass fragmentograms of a plasma blank sample (left) and a plasma sample (right) containing 0·5 μg ml^{-1} of the sulphide II and 0·5 μg ml^{-1} of the *p*-hydroxy-sulphide metabolite III.

large peak in the total ion current chromatogram with about the same retention time as that of II did not interfere with the measurements at *m/z* 266. The presence of this peak, however, prevented quantitation of II using other g.c. detectors.

Standard curves were linear at least up to 2 μg ml^{-1} (r = 0·9986, n = 16 for II and r = 0·9940, n = 16 for III). Larger concentrations than 2 μg ml^{-1} were determined after dilution of the samples. Minimum detectable concentrations using 0·5 ml samples were 5 ng ml^{-1} for II and 30 ng ml^{-1} for III.

Within-run precision was determined from 8 separate determinations of plasma samples containing 500 ng II and 500 ng III ml^{-1} of plasma. The within-run coefficient of variation was 2·9% for II and 3·0% for III.

Use of deuterated II and III added to the plasma samples would improve the accuracy of the method. The BuS is added after the extraction and methylation steps, but serves as a good internal standard in the g.c.-m.s. measurement.

By changing one channel of the MID-unit to measure *m/z* = 434 (M^+, base peak), determination of the metabolite IV can be performed from the same plasma extract; it has Rt 4·8 min.

The plasma concentration curves of drug, II and III from the rabbit experiments (Fig. 2) clearly show,

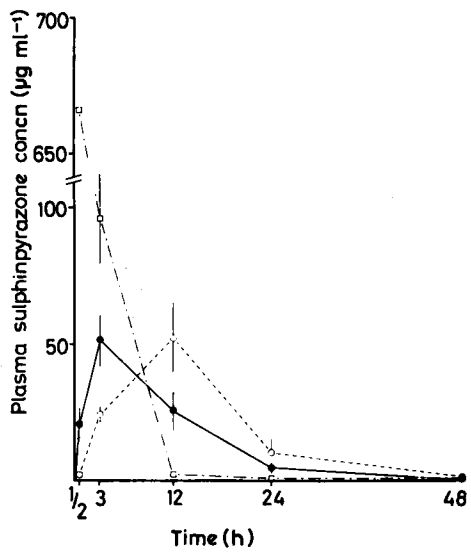


FIG. 2. Plasma concentrations of sulphinpyrazone (□ — · — □), the sulphide II (●—●) and the *p*-OH-sulphide III (○ - - - ○) after i.v. administration of drug 100 mg kg^{-1} to four rabbits. Each point represents mean ± s.e.m.

that both metabolites are formed in vivo after intravenous administration of drug, although II was found as a minor impurity in the injection preparation used (<2%). The time courses of the metabolite concentrations in plasma indicate, that II is partly oxidized to III before elimination. The maximum of the metabolite curves occur earlier than the maximum of biological effect in rabbits (18 h) stated by Buchanan et al (1978). This might be due to biological variation in formation and excretion of the metabolites or in platelet sensitivity to the inhibitory actions of the compounds.

Analysis of human plasma and urine concentrations for patient 1 are shown in Fig. 3 and are similar

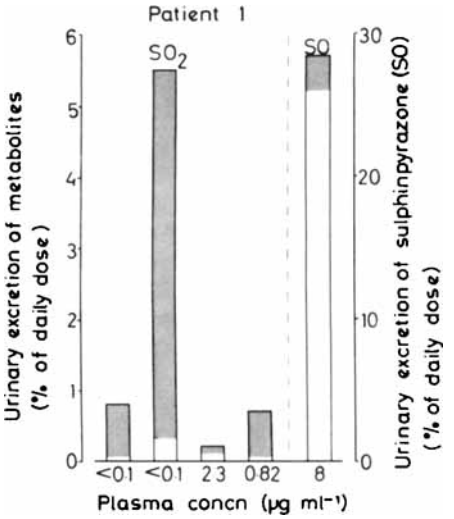


FIG. 3. Plasma concentrations and excreted amounts in urine of sulphinpyrazone and metabolites in one patient. The amounts indicated by the dark columns represent the additional amounts found after treatment with β-glucuronidase.

in patient 2. These results imply that the reductive metabolic pathway is also important in man, and metabolites II and III may well account for the major part of "platelet suppressive activity" in plasma if a three times greater potency of II than of drug is assumed (Kirstein Pedersen & Jakobsen 1979). The excreted amounts of the compounds are low; this is probably due to extensive formation of *C*-conjugated metabolites, which cannot be cleaved by β-glucuronidase (Richter et al 1975). However, formation of *O*-glucuronides of some of the metabolites (II, III, IV) is likely, since the concentrations of these increase after glucuronidase treatment.

The drug was originally described as a urinary metabolite of II (Burns et al 1957), now the reverse transformation seems to be an important metabolic route.

In conclusion, this reductive metabolic pathway of sulphinpyrazone should be further investigated in the light of the biological activity of the new metabolites. In man especially, this might lead to possibilities of improving antithrombotic therapy and prophylaxis.

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