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Short communication

Determination of FGN-1 (an active metabolite of sulindac) in human plasma, urine, and feces by HPLC ☆

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1. Introduction

Sulindac is presently being evaluated as a treatment for familial adenomatous polyposis (FAP) which is a hereditary condition in which multiple adenomatous polyps form in the colon and rectum [1–3]. FAP is typically manifested in adolescents with standard treatment normally involving colectomy, since these polyps usually become malignant [1,3,4]. Several investigators have found that sulindac (a non-steroidal anti-inflammatory drug, NSAID) has favorable effects in reducing both the size and number of rectal adenomatous polyps in patients with FAP [3–6]. However, current studies suggest a metabolite of sulindac (FGN-1) may also prevent or reduce the severity of rectal adenomatous polyp formation as indicated by growth inhibitory activity in cultured colon cancer cells and in the formation of colonic tumors of rats exposed to the tumor inducer azoxymethane [7].

Sulindac (Fig. 1) undergoes reversible sulfoxyl reduction to a sulfide metabolite and irreversible oxidation to a sulfone metabolite (FGN-1). Both of these compounds are active metabolites; however, FGN-1 is not active as a prostaglandin synthesis inhibitor (NSAID). Appreciable concentrations of sulindac and these two metabolites are present in plasma after oral administration of sulindac [8–11]. These compounds undergo extensive enterohepatic recycling with FGN-1 and the sulfide metabolite as the predominant forms excreted in the feces [8,12,13]. Sulindac and FGN-1 are also metabolized to their acyl glucuronide conjugates and excreted in the urine.

Published methods for sulindac and its metabolites have typically involved reverse phase high performance liquid chromatography (HPLC) with ultraviolet absorbance [14–20]. However, these reported methods were not optimized for FGN-1 analysis and therefore are less sensitive than the

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approach taken here. Electrochemical detection was not evaluated since the reported sensitivity (15 ng ml^{-1}, [21]) was not dramatically lower than the limit of quantitation observed with UV (20–25 ng ml^{-1}).

FGN-1 is being developed to determine if it can provide prophylaxis of adenomatous polyp formation in FAP patients without the typical side effects associated with NSAIDs such as sulindac. The purpose of this initial study is to characterize the safety, tolerability, and pharmacokinetics of single doses of FGN-1 in healthy male volunteers.

2. Experimental

2.1. Materials

FGN-1 (*Z*-5-Fluoro-2-methyl-1-(*p*-methylsulfonylbenzylidine)-idene-3-acetic acid) was synthesized by Cell Pathways, Denver, CO. Sulindac, indomethacin (internal standard), and β-glucuronidase (Type H-1, *Helix pomatia*) were purchased from Sigma Chemical Company (St. Louis, MO). HPLC grade methanol, acetonitrile, and methylene chloride were obtained from Burdick & Jackson (Muskegon, MI). All other reagents were analytical grade.

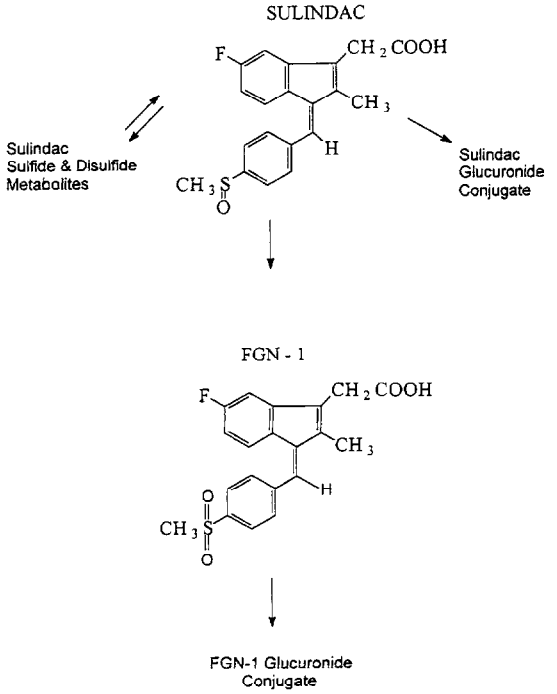

Fig. 1. Structure of sulindac and its oxidation and reduction reactions.

Table 1
FGN calibration standards

	LOQ	LOW	MID	HIGH
Plasma				
Conc. (ng ml^{-1})	25	42	438	9000
Recovery ($\bar{x}$) (%)	90.6	102.6	102.2	89.8
RSD (%)	9.5	10.4	5.7	5.4
n	41	41	41	40
Urine				
Conc. (ng ml^{-1})	20	46	200	1640
Recovery ($\bar{x}$) (%)	104.1	96.8	95.3	99.8
RSD (%)	5.2	2.8	2.6	1.6
n	30	29	30	30
Feces				
Conc. (μg ml^{-1})	0.25	0.38	10	75
Recovery ($\bar{x}$) (%)	108.0	97.4	91.3	89.7
RSD (%)	7.4	10.8	9.5	8.1
n	36	35	36	35

2.2. Chromatographic conditions

A Shimadzu HPLC 6A series chromatograph (Columbia, MD) equipped with a variable wavelength detector, limited volume autosampler, and integrator was used for the analyses. The HPLC column (octyl, 5μ, 150 mm × 4.6 mm i.d.) was obtained from Burdick & Jackson. The mobile phase consisted of acetonitrile/acetic acid/water (37.5:1:61.5, v/v/v); flow rate, 2.0 ml min^{-1} (1.0 ml min^{-1} for fecal samples); temperature, ambient. The analytes were detected by ultraviolet absorbance (329 nm, 0.005 AUFS). Injection volume was 200 μl (plasma and fecal extracts) and 100 μl (urine extracts).

2.3. Sample handling and collection

Blood samples (7 ml) were collected using sodium heparin as anticoagulant and the plasma was frozen (−20 °C) in polypropylene tubes within 60 min of collection. Urine samples were

refrigerated during 24 h collection intervals and the total volume and pH recorded; aliquots were frozen in polypropylene containers. Stool samples were refrigerated during collection intervals into tared plastic containers (2 l). The fecal sample weights were recorded and the samples combined with an appropriate volume of 1% acetic acid in water (2 ml per gram feces). The mixtures were shaken for 10 min (Eberbach, 120 osc min^{-1}), methanol (6 ml per gram feces) was added, and the samples were shaken again. The fecal contents were transferred to Stomacher bags (14″ × 20″) and homogenized using a Stomacher (Seward Medical, London) homogenizer on low speed until a homogenous slurry was achieved (10–20 min). Portions of each slurry were centrifuged (10 min at 2000 rev min^{-1}) and the supernatant stored at −20 °C in polypropylene tubes.

2.4. Extraction procedures

Plasma samples (1 ml) were combined with phosphoric acid (50%, 100 μl), internal standard (indomethacin, 50 μg ml^{-1}, 200 μl) and methylene chloride (10 ml). The mixtures were shaken horizontally (10 min, ≈60 osc min^{-1}) and centrifuged (10 min, 3000 rev min^{-1}). The aqueous layer was aspirated to waste and the organic layer evaporated to dryness (N_2, 37 °C). The samples were reconstituted in 500 μl mobile phase.

Urine samples (1 ml) were combined with β-glucuronidase (8000 unit ml^{-1}, 250 μl; 0.1 M acetate buffer, pH 5.0) and incubated at 37 °C for 2 h. After incubation, phosphoric acid (50%, 100 μl) internal standard (indomethacin, 50 μg ml^{-1}; 200 μl) and methylene chloride (10 ml) were added and the mixture extracted as described above for plasma.

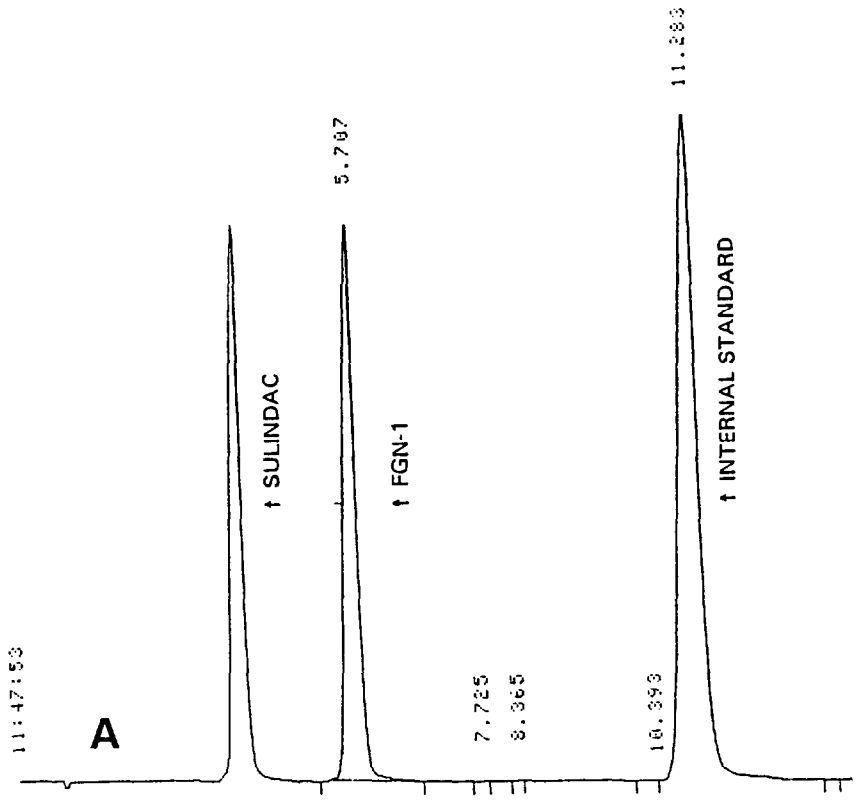

Fig. 2(A)

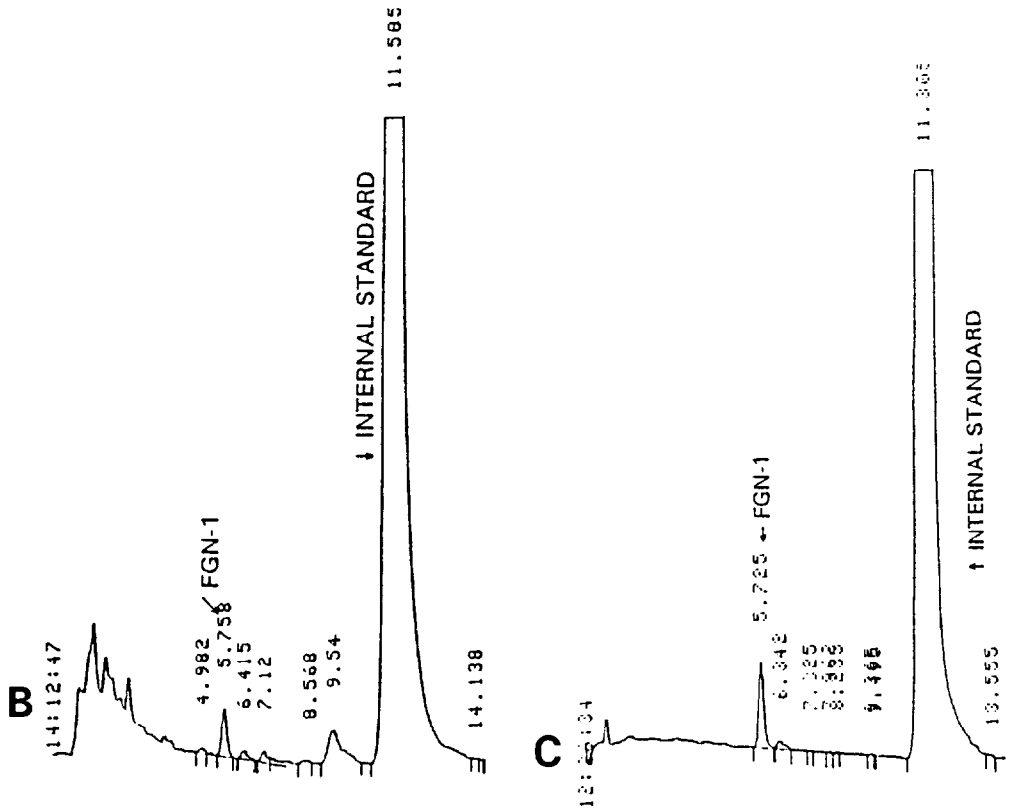

Fig. 2. Chromatograms for: (A) neat standard solution; (B) plasma calibration standard (25 ng ml^{-1}); (C) fecal calibration standard (250 ng ml^{-1}).

Fecal supernatants (100 μl) were pipetted into tared culture tubes and the sample weights recorded. Internal standard (indomethacin, 50 μg ml^{-1}; 200 μl) was added and the mixtures evaporated to dryness (N_2, 37 °C). Samples were reconstituted with 500 μl mobile phase.

2.5. Validation procedures

The precision (% RSD) and accuracy (% recovery) of the methods were determined on at least five separate occasions. The lower limit of quantitation was defined as the concentration where the observed precision and deviation from the theoretical values were ≤20%. Quantitation of FGN-1 was determined using a weighted ($1/x$) regression of the peak height ratio (FGN-1/internal standard) versus concentration.

2.6. Efficiency of glucuronide hydrolysis

The extent of hydrolysis of FGN-1 glucuronide was evaluated after 0, 1, 2, and 16 h incubation of a post-dose urine sample with β-glucuronidase at 37 °C (2000 units, pH 5.0).

2.7. Pharmacokinetic data analysis

Plasma pharmacokinetic parameters were calculated using standard non-compartmental methods [22]. The peak plasma concentration, C(max), was the highest observed concentration. The peak time [t(max)] was the time that C(max) occurred. The lag time, t(lag), was the time immediately preceding the first time that the plasma concentration exceeded the limit of quantitation (LOQ). Area under the concentration–time curve (AUC) and area under the first moment curve (AUMC)

Table 2
Plasma pharmacokinetic parameters[a] of FGN-1 following single oral doses of FGN-1

Parameter	Units	Dose of FGN-1 (mg) ($N = 6$)						
		50	100	150	200	250	300	400
Time to peak plasma concentration	h	1.5 (1.0–1.5)	1.5 (1.0–12.0)	1.5 (1.0–2.0)	1.5 (1.5–3.0)	2.0 (1.5–3.0)	1.8 (1.5–3.0)	2.5 (2.0–3.0)
Peak plasma concentration	ng ml^{-1}	1994 (577)	3146 (1451)	5649 (1512)	6667 (778)	7832 (1461)	7852 (3707)	13145 (2885)
Plasma area under the curve ($0 \rightarrow \infty$)	ng h ml^{-1}	9250 (2546)	17330 (3684)	26962 (9227)	32254 (8290)	39533 (7129)	40488 (6277)	85038 (18202)
Apparent oral clearance	ml h^{-1} kg^{-1}	77.1 (30.9)	73.8 (14.6)	82.2 (28.7)	80.0 (15.9)	83.6 (15.8)	98.8 (13.8)	62.5 (18.3)
Mean residence time in plasma	h	9.06 (1.76)	11.25 (3.22)	9.42 (2.33)	9.24 (2.08)	9.35 (0.88)	9.30 (1.22)	10.63 (2.23)

[a] Values are mean (standard deviation) for peak concentration, area under the curve, apparent oral clearance, mean residence time, urinary excretion, and renal clearance. Values are median (range) for time to peak plasma concentration.
[b] $N = 5$.

were calculated by the linear trapezoidal method. C(max) and AUC ($0 \rightarrow \infty$) were normalized to a 50 mg dose. Mean residence time (MRT) was determined as AUMC/AUC. Apparent oral clearance (CL/F) was calculated as Dose/AUC and was normalized for body weight. All concentrations reported below the lower limit of quantitation (LLOQ) for the assay were set to zero.

Urine excretion of total FGN-1 (free + conjugated) was determined for each collection interval as the product of urine volume and concentration in the hydrolyzed urine sample. Since the rate of urinary FGN-1 excretion was very low by day 4, total urinary excretion was summed over days 0–4 and not corrected to infinite time. Renal clearance of total FGN-1 was calculated as urinary excretion divided by plasma AUC from t_0 to the first time point that plasma FGN-1 was not detected.

Fecal excretion of unchanged FGN-1 (in milligrams) was calculated by multiplying the total homogenate volume (where volume = total weight/specific gravity of homogenate) by the observed concentration (μg ml^{-1}) × 1000.

3. Results and discussion

Calibration curves for plasma (25 ng ml^{-1} to 10 μg ml^{-1}), urine (20 ng ml^{-1} to 2 μg ml^{-1}) and feces (250 μg ml^{-1} to 100 μg ml^{-1}) were linear ($r \geqslant 0.999$) over the specified ranges. The results, presented in Table 1, indicate the precision and accuracy of the methods at the LLOQ and at three higher concentrations. Typical chromatograms are illustrated in Fig. 2.

The extraction efficiency of FGN-1 was approximately 75% from plasma, 90% from urine, and

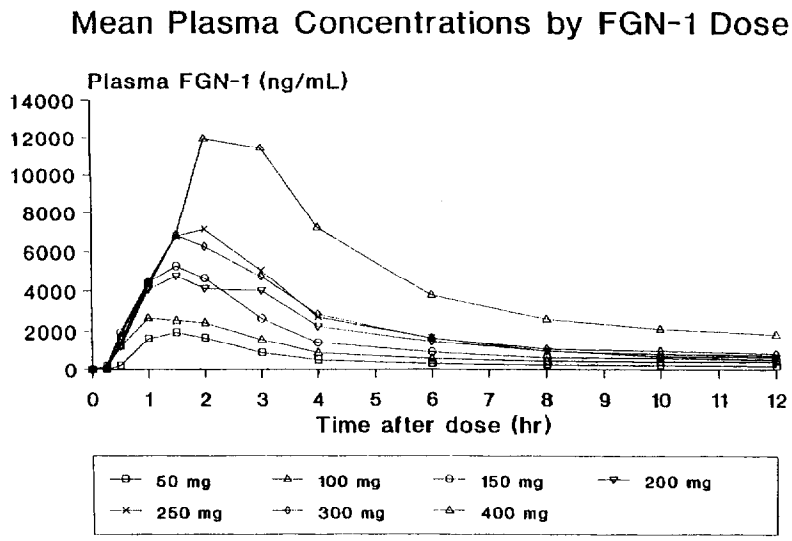

Fig. 3. Mean plasma concentrations vs. time following single FGN-1 oral doses ranging from 50 to 400 mg.

97% from feces. The hydrolysis of FGN-1 glucuronide with β-glucuronidase (2000 units) was determined as complete within 2 h at 37 °C, pH 5.0. Stability data indicate that FGN-1 is stable during the extraction procedures and during long term storage in plasma and fecal supernatant at −20 °C for at least 1 year. The internal standard selected (indomethacin), however, was determined as being unstable under alkaline conditions. Therefore, the samples were acidified prior to the addition of internal standard.

Concentrations of FGN-1 increased in a dose-related fashion. The plasma pharmacokinetic parameters following single FGN-1 oral doses ranging from 50 to 400 mg are presented in Table 2, with mean plasma concentrations illustrated in Fig. 3. Oral capsule doses of FGN-1 were rapidly absorbed after a short lag time. In most individual profiles, peak plasma concentrations were reached after 1.5–3 h with the majority of the dose eliminated after 12 h, as suggested by AUC (0–12) values approximating 70% of AUC ($0 \rightarrow \infty$). The declining phase of the log plasma concentration–time profiles often showed irregularities suggestive of enterohepatic recycling (Fig. 4). Mean residence times of FGN-1 in plasma averaged 9–11 h. Despite intersubject variability, peak concentrations and areas under the curve of FGN-1 normalized to a 50 mg dose were similar among the dose levels. Values of apparent oral clearance (normalized for subject weight) and mean residence time (MRT) did not show any obvious dose-related trends.

The pharmacokinetic parameters of FGN-1 in urine and feces following single oral FGN-1 administration are summarized in Table 3. The ma-

Table 3
Urinary and fecal excretion[a] of FGN-1 following a single 200 mg oral dose of sulindac

Parameter	Units	200 mg dose of sulindac ($N = 9$)
Urinary excretion[b]	mg	34.44 ± 10.68
	% of dose[c]	17.0 ± 5.1
Renal clearance[b]	$l\,h^{-1}$	2.135 ± 0.487
Fecal excretion	mg	0.71 (0.00–5.82)
	% of dose[b]	0.3 (0.0–2.8)

[a] Values are mean ± standard deviation for urinary excretion and renal clearance. Values are median (range) for fecal excretion.

[b] Unchanged plus conjugated FGN-1 in urine.

[c] Converted to sulindac equivalents using multiplication by the factor 356.42/372.41.

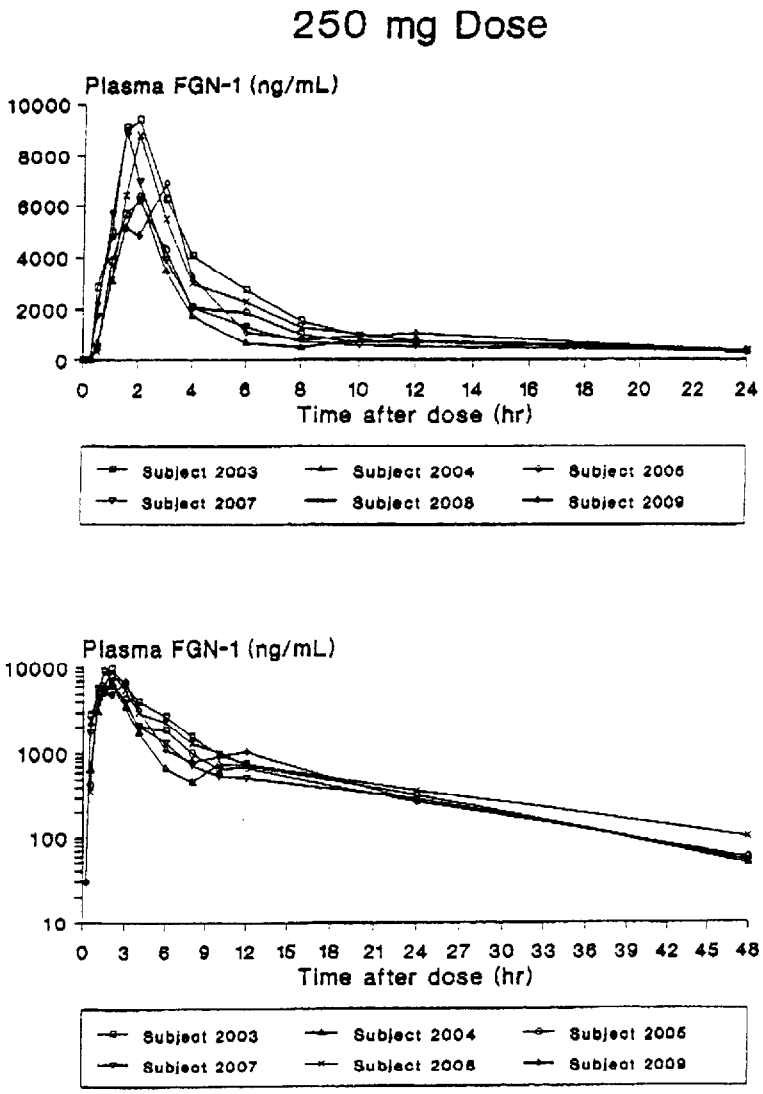

Fig. 4. Log plasma concentration vs. time following 250 mg dose of FGN-1.

jor portion of the total FGN-1 excreted in urine was found in the first 24 h of collection. Urinary concentrations were often undetectable after three or more days at lower FGN-1 doses. The average urinary recovery of total FGN-1 (free and conjugated) accounted for ≈30% of the oral dose and did not vary in any consistent manner over the dose range. Mean values for renal clearance of total FGN-1 ranged from 1.4–2.0 l h^{-1} and showed no apparent relationship to dose. The largest amounts of FGN-1 excreted in feces were found in the collections made during the first or second day after dosing. Fecal concentrations of FGN-1 were frequently, but not always, below the detection limit of the assay by the fourth day. The amount of FGN-1 in feces varied enormously within each dose group but was usually less than 10% of the dose. A few subjects exhibited fecal levels ranging up to 34.4% of the dose; however, plasma AUCs and urinary values were not markedly decreased in these subjects. The low mass balance recovery of FGN-1 (≈30% in

urine; < 10% in feces) appears to involve the reported difficulties noted at the clinical site in processing the fecal samples.

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