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Analysis of the metabolites of the sodium salt of 6-hydroxy-5-(phenylazo)-2-naphthalenesulfonic acid in Sprague–Dawley rat urine

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Abstract

The sodium salt of 6-hydroxy-5-(phenylazo)-2-naphthalenesulfonic acid (SS-AN), which is a subsidiary color present in Food Yellow No. 5 [Sunset Yellow FCF, disodium salt of 6-hydroxy-5-(4-sulfophenylazo)-2-naphthalenesulfonic acid], was orally administered to Sprague–Dawley rats. Metabolite A, metabolite B, and unaltered SS-AN were detected as colored metabolites in the rat urine. Analysis of the chemical structures showed that metabolite A (major peak) was 6-hydroxy-5-(4-sulfooxyphenylazo)-2-naphthalenesulfonic acid, the sulfuric acid conjugate of SS-AN, and metabolite B (minor peak) was 6-hydroxy-5-(4-hydroxyphenylazo)-2-naphthalenesulfonic acid (SS-PAP), which is a derivative of metabolite A without the sulfuric acid. The colorless metabolites *p*-aminophenol, *o*-aminophenol, and aniline present in the urine were analyzed by liquid chromatography–mass spectrometry. The orally administered SS-AN had been metabolized to the colorless metabolites (*p*-aminophenol 45.3%, *o*-aminophenol 9.4%, aniline 0.4%) in the 24-h urine samples. Analysis of the colored metabolites by high-performance liquid chromatography with detection at 482 nm indicated the presence of metabolite A (0.29%), SS-PAP (0.01%), and SS-AN (0.02%) were detected in the 24-h urine samples. Approximately 56% of SS-AN was excreted into the urine and the rest is probably excreted into feces.

Keywords: Azo dye; Metabolites; Orange RN

1. Introduction

The sodium salt of 6-hydroxy-5-(phenylazo)-2-naphthalenesulfonic acid (SS-AN, C.I. 15970, Orange RN) is a subsidiary color in Food Yellow No. 5 [Y-5, C.I. 15985, Sunset Yellow FCF, disodium salt of 6-hydroxy-5-(4-sulfophenylazo)-2-naphthalenesulfonic acid]. Y-5 is an azo dye synthesized by diazotizing a sulfanilic acid (4-aminobenzenesulfonic acid) and coupling it with Schaeffer's salt (sodium salt of 6-hydroxy-2-naphthalenesulfonic acid). The raw materials for manufacturing the sulfanilic acid include aniline, which can be a contaminant. SS-AN is produced when aniline is diazotized and coupled with Schaeffer's salt.

A survey of the subsidiary colors present in commercial Y-5 [1,2] showed that the SS-AN content in Y-5 is between N.D. (not detected) and 0.52%, which is within the limit stated in the Official Standard of Food Additives Ver. 7 [3]. However,

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since SS-AN is speculated to be reduced and to produce aniline, its metabolites should be studied. The metabolites of SS-AN present in urine are reportedly *p*-aminophenol (PAP), *o*-aminophenol (OAP), aniline, and 6-hydroxy-5-(4-hydroxyphenylazo)-2-naphthalenesulfonic acid (SS-PAP) [4,5]. In this study, we administered SS-AN to rats and analyzed the urinary metabolites by classifying them into colored and colorless metabolite groups.

The colored metabolites were analyzed by high-performance liquid chromatography (HPLC) with detection at 482 nm, which is within the visible range. Two unknown peaks (metabolites A and B) were detected. According to Larsen and Tarding [5], the colored metabolites are considered to contain SS-PAP. We diazotized *p*-aminophenol and coupled it with Schaeffer's salt to synthesize SS-PAP. The minor peak (metabolite B) matched a chromatogram of the SS-PAP and the HPLC chromatogram recorded at 482 nm. However, since there are no reports that describe the major peak, we isolated and identified the peak. Analysis of the colorless metabolites was attempted using UV–HPLC, but due to the number of peaks, PAP, OAP, and aniline were unable to be quantified. Therefore, we used liquid chromatography–mass spectrometry (LC–MS) to quantify the colored and colorless metabolites.

2. Experimental

2.1. Materials and reagents

2.1.1. Test chemical

SS-AN synthesized and refined as described by Bailey Jr. [1] was tested. When the substance was titrated with titanium trichloride, the purity was 99.0%. Also, by HPLC the purity of SS-AN was not less than 99.0% and one peak. In other words, the organic impurities of SS-AN were less than 1.0% by HPLC.

2.1.2. Standards and reagents

PAP, OAP and aniline were obtained from Wako (Osaka, Japan). SS-PAP was synthesized by the method of Danek et al. [6]. A solution of PAP (10.9 g) dissolved in 42% tetrafluoroboric acid (31 g) was diazotized with a solution of sodium nitrite (7.0 g) dissolved in 12 ml of water at −5 to 0°C for 3 h. After cooling to −10°C, the product was filtered, washed with 15 ml of cool ethanol and 20 ml of ether, and then air-dried. Schaeffer's salt (24 g) was coupled with the diazotized PAP (16.4 g). The electrospray ionization (ESI) MS of SS-PAP showed m/z 342, and the maximum absorption was 485 nm. Other reagents used were of HPLC grade and the highest grade available from commercial sources.

2.1.3. Animals and housing conditions

Five male and female 6-week-old specific pathogen-free (SPF) Sprague–Dawley (SD) rats were purchased from Charles River Japan (Atsugi, Japan) and were used at the age of 7 weeks, after 1 week of quarantine. The rats were kept in a chamber maintained temperature at 23±3°C and relative humidity at 50±20%, ventilated 10 to 15 times an hour, and illuminated for 12 h a day from 7:00 to 19:00. Each rat was kept in a placket-type metal mesh cage (254 W×350 D×170 H mm) within this chamber. The rats were free to take in solid feed (CFR-1: Oriental Yeast, Tokyo, Japan) and tap water.

2.1.4. Dosage and administration method

Oral administration was selected for the study of the metabolism of impurities contained in the food additives. SS-AN was dissolved in distilled water to make a concentration 0.1% and the dosage was set at 40 ml/kg according to body weight. The rats were starved for 1 night (about 16 h) before administration. The SS-AN solution was administered orally once to each rat forcibly using a metal gastric sound. Urine samples were collected from the rats 12 and 24 h after administration.

2.1.5. Instruments

HPLC was performed using a JASCO 900 series HPLC system (Tokyo, Japan) with a Borwin integrator. MS was obtained using a VG Biotech Platform spectrometer (Micro Mass, Manchester, UK). Nuclear magnetic resonance (NMR) spectra were determined with a JNM-LA400 (JEOL, Tokyo, Japan).

2.2. Analysis of colored metabolites

Rat urine samples were collected from −6 h to immediately before SS-AN administration, from 0 to 12 h after administration, and from 12 to 24 h after

administration. The urine samples were filtered individually through 0.45 μm filters to produce specimens for HPLC analysis. Analytical HPLC was performed on an L-column ODS (250×4.6 mm I.D.; Chemical Substance Evaluation Lab., Tokyo, Japan) at 30°C with the detection wavelength at 482 nm. A solvent flow rate of 1.0 ml/min was used with a 50-min linear gradient progressing from 100% 0.02 mol/l aqueous ammonium acetate to 40% acetonitrile in 0.02 mol/l ammonium acetate. The injection volume was 20 μl.

2.3. Isolation and identification of colored metabolites (metabolites A and B)

Ten male SPF SD rats at the age of 6 weeks were purchased from Charles River Japan and were used at 7 weeks, after 1 week of quarantine. SS-AN was dissolved in a physiological salt solution to make a concentration 2.0% and the dosage was set at 10 ml/kg according to the body weight. The solution was injected into the tail vein of each rat at a rate of about 2 ml/min using a winged syringe with a Millipore MILLEX-HA 0.45 μm filter unit (Japan Millipore, Tokyo, Japan). The urine sample was obtained 24 h later. The urine (74 ml in total) was adjusted to pH 4 with 10% acetic acid, and then adsorbed onto a Polyamido C-100 column (300×20 mm I.D., particle size 150~425 μm; Wako), and washed with water, water–methanol (1:1), and methanol. After eluted with methanol–2% ammonia solution (8:2), the specimen was solidified by evaporation, and dissolved in a small amount of water. To separate the fraction of metabolites A and B, the colored metabolites were charged on a Sephadex LH-20 column (300×20 mm I.D., particle size 25~100 μm; Pharmacia LKB, Japan) and eluted with water. After the column purification, the fractions of metabolites A and B were evaporated to dryness and dried in a vacuum oven 40°C for 48 h to give metabolites A (7.0 mg) and B (1.0 mg).

2.4. Spectrometric analyses

ESI-MS analysis was performed under the following conditions: negative ion mode, scan of 50 to 600 *m/z* over 2 s, needle temperature at 70°C, and cone voltage of 30 V. A small amount of the sample was dissolved in water and directly injected.

^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR spectra were measured in dimethylsulfoxide (DMSO)-d_6 with tetramethylsilane as the internal standard. The signals for the ^{1}H- and ^{13}C-NMR spectra of metabolite A and SS-AN were assigned on the basis of chemical shifts and according to the results of ^{1}H-^{1}H correlation spectroscopy (COSY), ^{13}C-^{1}H COSY, a distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple bond connectivity (HMBC).

2.5. Analysis of colorless metabolites

A 1-ml volume of a specimen was mixed with 0.5 ml of 36.0% hydrochloric acid and 1 ml of distilled water and then the mixture was hydrolyzed in an autoclave at 121°C for 90 min. A 5-g amount of K_2HPO_4 was added to adjust the pH of the mixture to 7. The specimen was extracted twice with 25 ml of diethyl ether containing 1.5% isoamyl alcohol and then centrifuged (8000 *g*, 10 min). After adding 5 ml of 0.05% H_2SO_4, the specimen was concentrated and solidified by evaporation. Then, 0 h and 12–24 h rat urine samples were dissolved in 1 ml of 5% K_2HPO_4 solution, and 0–12 h rat urine samples were dissolved in 1 ml of 5% K_2HPO_4 solution and 7 ml of water. After the samples were prepared, they were immediately analyzed by LC–MS.

HPLC was carried out on an L-column ODS (250×4.6 mm I.D.) at 30°C under the following conditions: a solvent flow-rate of 1.0 ml/min with 5 mmol/l ammonium acetate–methanol (92:8). The injection volume was 20 μl. The MS conditions were as follows: ESI in positive ion mode was used. The split ratio was 98:2. The source temperature was set at 70°C. The monitor ions were *m/z* 110 (PAP protonated molecule), *m/z* 93 (without hydroxyl group), *m/z* 110 (OAP protonated molecule), *m/z* 92 (without amino group), and *m/z* 94 (aniline protonated molecule).

3. Results and discussion

3.1. Chemical structures of unknown colored metabolites

In order to identify colored metabolites A and B, it was necessary to obtain a large quantity of them. It

was confirmed that metabolites A and B were also detected in rat urine by HPLC, after SS-AN was administered via tail vein of rat. According to this intravenous (i.v.) injection method, large amounts of metabolites A and B were obtained compared to the oral administration method. Therefore, 2% SS-AN solution was injected into the tail vein of rat, and rat urine was collected for 0–24 h. After the column separation, 7.0 and 1.0 mg of metabolites A and B, were obtained, respectively.

Fig. 1 shows chromatograms of 0 h and 0–12 h rat urine after the SS-AN administration. Metabolite A was detected as a major peak at 30.5 min, metabolite B as a minor peak at 40.2 min, and unchanged SS-AN at 47.9 min.

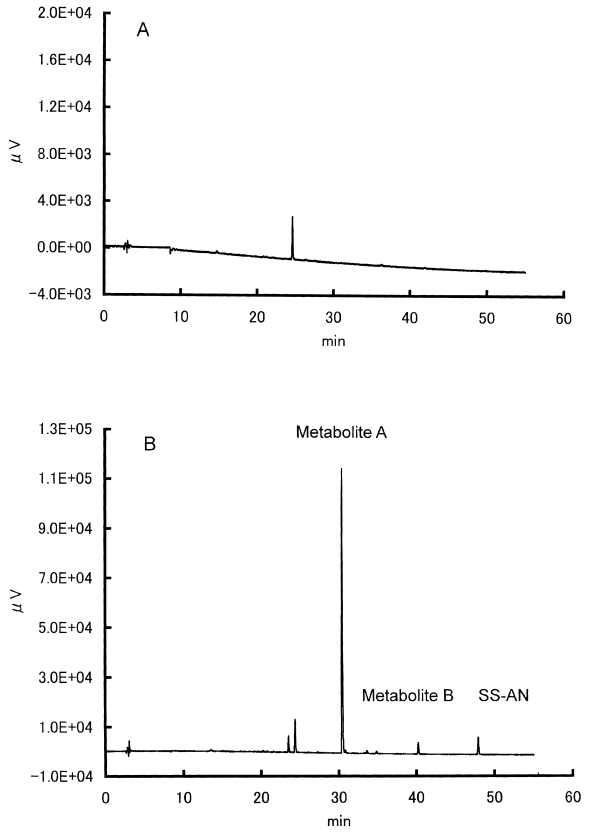

Fig. 1. Chromatograms of the 0 h rat urine (A) and the 0–12 h rat urine (B) after oral administration of SS-AN. HPLC conditions: Column: L-column ODS (250×4.6 mm I.D.), Mobile phase: 0–50 min linear gradient from 0 to 40% acetonitrile in 0.02 *M* ammonium acetate, flow-rate: 1.0 ml/min, column temperature: 30°C, injection volume: 20 μl, detection: 482 nm.

Since Larsen and Tarding [5] determined the main colored metabolite to be SS-PAP, HPLC analysis was conducted under the same conditions. According to analysis, the metabolite was detected at 40.2 min, which was identical to the retention time of metabolite B.

When the mass spectrum of the separated metabolite B was measured, the deprotonated molecule $[M-H]^-$ m/z 343 was detected and its mass spectrum matched that of SS-PAP. This proved that metabolite B was SS-PAP.

Since there are no reports describing the chemical structure of the main colored metabolite (metabolite A), which was detected as a peak at 30.5 min, this fraction was separated and its chemical structure was determined.

The ESI mass spectrum of metabolite A showed two deprotonated molecules, m/z 211 $[M-2H]^{2-/2}$ and m/z 423 $[M-H]^-$. Using a high-resolution fast atom bombardment (FAB) mass spectrum in the positive mode, $[M+H]^+$ with a molecular formula of $C_{16}H_{13}O_8N_2S_2$ (observed value: 425.0089, calculated value: 425.0113) was determined. Thus, the molecular formula of $C_{16}H_{12}O_8N_2NaS_2$ was determined for metabolite A. Comparison with the molecular formula for SS-AN ($C_{16}H_{11}O_4N_2NaS$), showed that a molecular increase equivalent to the addition of a sulfate group SO_4 was obtained. Based on these results, metabolite A is determined to be a sulfate of SS-AN. NMR analysis was carried out to determine the combination site of this sulfate group.

In Tables 1 and 2, the ^{1}H- and ^{13}C-MNR data of metabolite A and SS-AN are compared. According to the ^{1}H-NMR data, the SS-AN 4′-position proton

Table 1
^{1}H-NMR data (400 MHz) of SS-AN and metabolite A in DMSO-d_6

Position[a]	SS-AN	Metabolite A
1	8.12 (s)	7.94 (s)
3	7.92 (d, J=8.9 Hz)	7.84 (d, J=8.7 Hz)
4	8.56 (d, J=8.9 Hz)	8.64 (d, J=8.7 Hz)
7	8.07 (d, J=8.9 Hz)	8.05 (d, J=9.2 Hz)
8	6.98 (d, J=9.5 Hz)	7.10 (d, J=9.2 Hz)
2′, 6′	7.89 (d, J=8.5 Hz)	7.93 (d, J=9.0 Hz)
3′, 5′	7.57 (t, J=7.6 Hz)	7.38 (d, J=9.0 Hz)
4′	7.41 (t, J=7.6 Hz)	–

[a] Position on azobenzene moiety and benzene moiety is indicated by prime.

Table 2
^{13}C-NMR chemical shifts (100 MHz) of SS-AN and metabolite A in DMSO-d_6

Position[a]	SS-AN	Metabolite A	Position[a]	SS-AN	Metabolite A
1	125.7	125.2	1′	145.2	142.9
2	145.4	144.8	2′	119.1	121.4
3	126.6	126.2	3′	129.8	121.0
4	120.9	120.9	4′	128.3	155.0
4a	132.7	132.4	5′	129.8	121.0
5	129.1	128.8	6′	119.1	121.4
6	168.3	160.6			
7	140.0	137.7			
8	124.1	122.2			
8a	127.0	127.0			

[a] Position on azobenzene moiety and benzene moiety is indicated by prime.

(σ 7.41 ppm) was not found in metabolite A. According to the ^{13}C-MNR data, the SS-AN 4′-position carbon (σ 128.3 ppm) was shifted to the position of σ 155.0 ppm with a lower magnetic field. Consequently, metabolite A can be regarded as 6-hydroxy-5-(4-sulfooxyphenylazo)-2-naphthalenesulfonic acid (Fig. 2), i.e., SS-AN with a sulfate at the 4′-position.

3.2. Analysis of colored metabolites

Metabolite A, metabolite B and SS-AN analysis was carried out by HPLC. Calibration curves for metabolite A, metabolite B and SS-AN were linear over the range 1–20 ppm and their detection limits were 0.1 ppm. The recovery added to a concentration of 10 ppm metabolite A in rat urine was 88.0–96.0%±SD (standard deviation) 4.3% (n=3).

3.3. Analysis of colorless metabolites

Analysis of the colorless metabolites was carried out. We attempted to analyze the colorless metabolites at 254 nm under the same HPLC conditions as used for the colored metabolites. However, this attempt ended in failure because numerous peaks were detected. Using the preprocessing method of Larsen and Tarding [5], the specimen was hydrolyzed with hydrochloric acid and extracted with diethyl ether. LC–MS analysis was selected to enable simultaneous substance identification. To monitor the ions, PAP was quantified with m/z 110 (proton-added $[M+H]^+$) and m/z 93 (without the amino group), OAP was quantified with m/z 110 (proton-added $[M+H]^+$) and m/z 92 (without hydroxyl group), and aniline with m/z 94 (proton-added $[M+H]^+$).

Calibration curves for PAP, OAP and aniline were linear over the range 1–100 ppm. Table 3 shows the recovery rates of PAP, OAP, and aniline when 10 ppm of each substance was added to the rat urine collected before SS-AN administration and their detection limits. With a standard recovery rate of 76.7% and a SD of 8.5%, PAP showed great fluctuations. However, the overall results were good.

Fig. 3 shows SIM chromatograms of 0 h rat urine

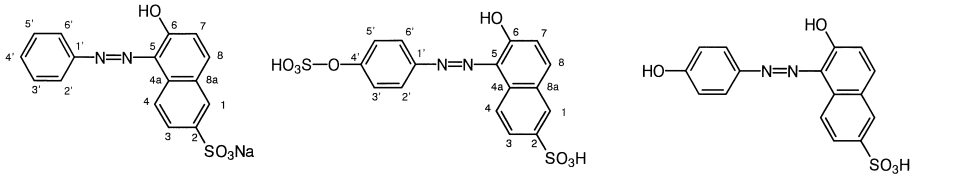

Fig. 2. Structures of SS-AN, metabolite A and metabolite B.

Table 3
Recoveries of PAP, OAP and aniline in the urine of rat[a]

	Recovery (%)			
	1	2	3	Mean±SD
PAP	71.9	71.7	86.5	76.7±8.5
OAP	83.0	82.0	82.2	82.4±0.5
Aniline	79.7	77.6	81.9	79.7±2.2

[a] PAP, 0.1 μg/g; OAP, 0.1 μg/g; Aniline, 0.2 μg/g.

and the rat urine collected from 0 to 12 h after SS-AN administration. The peaks were detected at *m*/*z* 110, *m*/*z* 93, *m*/*z* 92, and *m*/*z* 94.

3.4. Colored and colorless metabolite quantification results

Table 4 lists the metabolites detected in rat urine collected from the rats 12 and 24 h after the administration, when the administered volume of SS-AN is 100%.

Table 4
Excretion of metabolites in rat urine after oral administration of SS-AN

		Excretion (%)		
		0–12 h	12–24 h	Total (0–24 h)
Colorless metabolites	PAP	42.3	3.0	45.3
	OAP	6.4	3.0	9.4
	Aniline	0.4	N.D.*	0.4
Color metabolites	Metabolite A	0.29	N.D.**	0.29
	SS-PAP	0.01	N.D.**	0.01
	SS-AN	0.02	N.D.**	0.02

N.D., Not detected. *N.D.<0.1%; **N.D.<0.01%. Average of 10 rats.

Based on the recovery average, 45.3% of SS-AN was metabolized into PAP, 9.4% into OAP, and 0.4% into aniline within 24 h. On average, 0.29% of metabolite A, 0.01% of SS-PAP, and 0.02% of SS-AN were detected in the rat urine. Fig. 4 shows the

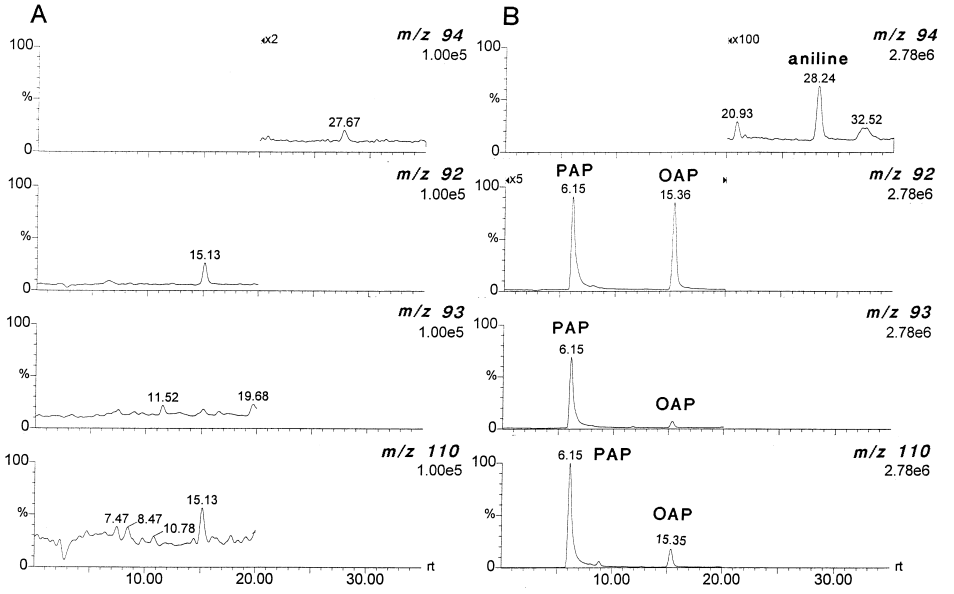

Fig. 3. SIM chromatograms of the 0 h rat urine (A) and the 0–12 h rat urine (B) after oral administration of SS-AN. LC–MS conditions: column: L-column ODS (250×4.6 mm I.D.), mobile phase: 5 m*M* ammonium acetate–methanol (92:8), flow-rate: 1.0 ml/min, column temperature: 30°C, injection volume: 20 μl, sprit ration: 98:2, ionization: ESI positive, ion source temperature: 70°C, monitor ion: PAP *m*/*z* 110 30 V, *m*/*z* 93 50 V, OAP *m*/*z* 110 30 V, *m*/*z* 92 50 V, aniline *m*/*z* 94 30 V.

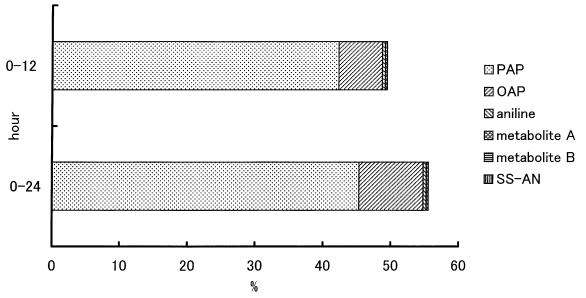

Fig. 4. Excretion of metabolites in rat urine after oral administration of SS-AN.

average excretion of the metabolites. Within 24 h, almost 56% of SS-AN was metabolized and excreted into urine.

4. Conclusion

We administered SS-AN orally to rats and analyzed the colored and colorless metabolites present in rat urine.

The main colored metabolite of SS-AN was 6-hydroxy-5-(4-sulfooxyphenylazo)-2-naphthalenesulfonic acid, but SS-PAP and SS-AN were also detected. To analyze the colorless metabolites, PAP, OAP, and aniline were quantified by LC–MS.

The orally administered SS-AN was metabolized into colorless metabolites (PAP, OAP, and aniline) and into colored metabolites (metabolite A, SS-PAP, and SS-AN) and excreted in 24-h urines. Approximately 56% of SS-AN is excreted into urine and the rest is probably excreted in the feces.

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