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Biological Monitoring of Exposure to Low Dose Aniline, *p*-Aminophenol, and Acetaminophen

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Aniline, p-aminophenol (p-AP), and acetaminophen are three structurely-related chemicals. Both aniline and p-AP are widely used industrial chemicals. p-AP is used, for example, as a photographic developer, as an intermediate in the manufacture of sulfur and azo dyes, and in the dyeing of furs and feathers. Aniline is used to manufacture rubber, dyes, medicinals, resins, varnishes, and perfumes. Acetaminophen, N-acetyl p-aminophenol (APAP), is used not only as an analgesic and antipyretic, but also to manufacture azo dyes and photographic chemicals (Merck Index 1983).

Humans overdosed with APAP may develop hepatic centrilobular necrosis and acute renal failure (Boyer and Rouff 1971). The same effects were also observed in experimental rats (McMurtry et al. 1978). p-AP has also been shown to induce nephrosis in rats and formation of methemoglobin (met-Hb) in rats and dogs (Lloyd et al. 1977). In addition to the induction of met-Hb (Kiesa 1966), aniline has also been shown to induce spleenic siderosis (Kao et al. 1978) and hemagiosarcomas in rats (Bus and Popp 1987).

The major urinary metabolites of aniline include p-AP, sulfate and glucuronide conjugated p-AP, and APAP (Kao et al. 1978; McCarthy et al. 1985). p-AP is also a metabolite of APAP (Newton et al. 1982). A sensitive and specific colorimetric method was developed to measure urinary p-AP at the μg/mL level (Chang et al. 1992). It was used to monitor the exposure to aniline ranging from 36.5 to 210 mg/kg, ip, in rats. In this report, we have modified the method to include a dilute acid hydrolysis that hydrolyzes the N-acetyl group as well as the sulfate and glucuronide groups from their respective conjugates.

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This modified colorimetric method was used to monitor low-dose exposure to APAP, p-AP, and aniline hydrochloride in rats ranging from 3.5 to 14.0 mg/kg as well as low-dose exposure to APAP in human volunteers ranging from 240 to 1000 mg/person.

MATERIALS AND METHODS

Female Wistar rats, 4 per dose group, were used. As reported previously, animals were trained in metabolic cages (Nalge, Rochester, NY) and provided with Lab Diet #5010 Rodent diet (PMI feeds, Inc., St Louis, MO) and tap water *ad libitum* before a gavage of chemicals. Three chemicals, aniline hydrochloride (AH, Sigma, St Louis, MO), p-AP (Merck, Darmstadt, FRG), and APAP (Fluka Chemie, Switzerland) were studied. The light cycle of the animal room was kept from 6 am to 6 pm. Each rat was given freshly dissolved aqueous AH, p-AP, or APAP at 5 mL/kg, once orally. Three dose groups per chemical of 3.5, 7.0, and 14.0 mg/kg were evaluated. Twenty-four hour urine samples were collected in polypropylene tubes at room temperature and protected from light. The urine samples were collected for the day before and a day after the exposure and stored at -75°C. The volume of each urine sample was recorded.

Urine samples were collected from human volunteers at pre-designated hours after an ingestion of either 500 or 1000 mg of APAP (1 and 2 caplets, respectively, of Tylenol, McNeil Consumer Products, PA). Several days later, 2 of the volunteers again ingested three 80-mg tablets each of a children's Tylenol.

Urinary creatinine was determined by the Jaffe reaction. Urinary p-AP was determined as reported previously after an initial acid (1N HCl) hydrolysis at 85°C for 2 hr. Briefly, frozen urine samples were quickly thawed and centrifuged (10,000 x g for 20 min at 4°C) to remove particulates. To 50 µL of cleared urine sample, 100 µL of deionized water, and 50 µL of 4N HCl were added. The mixture was incubated in a 85°C water bath for 2 hr. After 2-hr incubation, samples were cooled to room temperature before the addition of a 1.6 mL of a reagent mix that consisted of 11 volumes of 4M ammonium hydroxide and 5 volumes of aqueous o-cresol (Merck, Darmstadt, FRG) at 20 mg/mL. The color reaction was allowed to develop at room temperature for 30 min. At the completion of the reaction, a brief centrifugation at 10,000 x g for 5 min was used to eliminate the

denatured proteins. The blue color that developed was measured for its optical density at 620 nm. A working standard curve of 0.12 to 0.60 μmol free p-AP per mL water was included in every batch of a determination. No correction for recovery was made.

RESULTS AND DISCUSSION

In the very beginning, three series of p-AP standards were prepared in 1N HCl. Their final concentrations ranged from 0.12 to 0.60 μmol/mL. Two of them were prepared with a pooled unexposed rat urine (50 μL each) for the comparison of with and without incubation at 85°C for 2 hr. A stability of 96 ± 1% recovery (by slope ratio, n=3) was obtained. The third series were prepared without urine and also hydrolyzed at 85°C for 2 hr. A 99 ± 4% recovery (slope ratio of "with urine" over "without urine", n=3) suggested a negligible matrix effect. Thereafter, all the urinary analyses were performed with an aqueous p-AP standard curve with no correction for recovery.

To find out the efficiency of the acid hydrolysis for APAP, a series of equimolar spiked rat urine samples were prepared and hydrolyzed as the p-AP spiked urine standards. By slope ratio, a 93 ± 3% recovery was determined for the concentration range of 0.12 to 0.60 μmol/mL. Although no standard sulfate or glucuronide conjugated p-AP was available to test the completeness of acid hydrolysis, a continuous increase in p-AP color reaction of aniline HCl-exposed rat urine (both 14.0 and 7.0 mg/kg) was observed after 1 to 2 hours hydrolysis. An additional 1 to 2 hr incubation at 85°C did not significantly increase the color formation. Therefore, the 2-hr acid hydrolysis was used for this report.

Summarized in Table 1 are the rat data of three different exposures. The dose-response relationship of all 3 chemicals was found to be linear with a r^2 of ≥ 0.94. The LD50 of these 3 chemicals are 420 mg/kg for aniline in rats, ip (Registry of Toxic Effects of Chemical Substances, 1991), 671 mg/kg for p-AP in rats, orally (Lloyd et al. 1977), and 338 mg/kg for APAP in mice, orally (Merck Index 1983). The lowest dose studied in this report, 3.5 mg/kg, is about ≤ 1% LD50. At this dose level, all of the measured (12 rats) acid-released urinary p-AP were greater than 3 times the mean background level of pre-exposed rat urines which was determined to be 15 ± 7 μg p-AP/mg creatinine (± SD, n=36).

Table 1. Dose-response of acid-released urinary p-aminophenol obtained from rats gavaged with either aniline HCl, p-aminophenol or acetaminophen

	mean (μg p-AP/mg creatinine) ± SD, n=4		
Dose (mg/kg)	aniline HCl	p-AP	APAP
3.5	58 ± 12	74 ± 7	51 ± 4
7.0	135 ± 28	100 ± 21	94 ± 10
14.0	197 ± 23	185 ± 49	162 ± 16

24-Hour urine samples after the gavage of a chemical were analyzed. Data were not corrected for the background.

The results of human ingestion studies are summarized in Table 2. The human data for the APAP study were obtained on grab samples obtained at the pre-designated time points. No consideration was given to any possible confounding factors potentially present in a human study, such as smoking and drinking.

Only two urine samples were collected at 24 hours after a single ingestion of 500 mg APAP. One had an acid-released μg p-AP/mg creatinine value of greater than 4 x mean background level of 4 ± 3 μg

Table 2. Amount of acid-released human urinary p-aminophenol after an ingestion of acetaminophen[a]

Hour after ingestion	1000 mg n=5	500 mg n=3	240 mg n=2
	mean (μg p-AP/mg creatinine) ± SD		
2	-[b]	397 ± 173	253
4	783 ± 185	529[c]	216
6	-	375 ± 102	-
8	-	226 ± 85	82
24	-	13[c]	-

a. Grab urine samples were obtained from human volunteers.
b. No collection of urine.
c. Each value represents the average of 2 urine samples collected.

p-AP/mg creatinine (n=9), and the other of slightly greater than 2 x mean background level. Based on these results, it is recommended that in order to monitor a low dose exposure of APAP biologically , urine samples should be collected before or at the point of 8 hours after exposure. A linear dose response relationship of $r^2 > 0.94$ was also found for human study at 4 hours past ingestion of APAP.

Without acid hydrolysis, no free p-AP was detected in human urines. An additional analysis of urinary p-AP, without acid hydrolysis but with a sulfatase/glucuronidase digestion at 37°C for 3 hours, was performed on the human urines collected at 4 hours after ingestion of 1000 mg APAP. Approximately 28 ± 7 µg p-AP/mg creatinine (n=5) was measured. The mean background level of enzyme-released p-AP was determined to be 2 ± 3 µg p-AP/mg creatinine (n=5). This result suggested that an endogenous deacetylation of APAP occurred in humans.

To compare with the previous study, 2 series of p-AP and APAP spiked urine standards were prepared in 1N HCl at concentration ranging from 25 to 200 nmol/mL. The hydrolysis was performed at 85°C for 2 hours. The results were summarized in Table 3. When the slope of the p-AP spiked standards of this study (2.280×10^{-3}) was compared with the slope of the aqueous standards of the previous study, 2.266×10^{-3}, a 101% recovery was obtained. This again suggests the stability of p-AP under the acid hydrolysis conditions as well as negligible matrix effect when 50 µL of an unexposed rat urine was present. The completeness of the APAP hydrolysis was calculated to be $97 \pm 3\%$. This is slightly better than the higher APAP concentration range of 0.12 to 0.60 µmol/mL. The percent relative error of all standards was no greater than 5% and the CV values less than 10%.

In conclusion, by adding a dilute acid hydrolysis, the previously reported simple colorimetric method for the analysis of urinary p-AP has been further improved for its capability to monitor a low dose exposure to aniline, p-AP, and APAP. Only 50 µL urine, about 1 drop, was needed. The previously recommended (Simpson and Stewart 1973) screening colorimetric method for acetaminophen poisoning takes 5 times the urine volume used in this report. Although the method is non-specific to the chemicals studied, we have shown in this report that it is capable of monitoring human exposure to APAP

Table 3. Hydrolysis of a low concentration range of N-acetyl p-AP (APAP) with 1N HCl at 85°C for 2 hours[a]

Concn.	p-AP spiked		APAP spiked		
nmol/mL	A620[b]	% rel. E	A620 ± SD	% rel. E	CV, %
25	0.054	-1.1	0.063 ± 0.006	+0.7	9.5
40	0.091	+2.4	0.097 ± 0.006	+1.5	6.2
50	0.113	+1.2	0.119 ± 0.009	+1.1	7.6
100	0.223	-1.2	0.226 ± 0.007	-0.9	3.1
150	0.336	-1.1	0.333 ± 0.008	-1.6	2.4
200	0.457	+0.7	0.453 ± 0.006	+1.0	1.3
r^2 (n=6)		0.999		0.999	
slope $x10^3$,		2.280		2.206	

a. The volume of unexposed rat urine used was 50 μL per standard.
b. The A620 of both p-AP and APAP spiked standards was the mean of three determinations.

at a dose level not only much lower than a poisoning dose but also a recommended analgesic dose, which is 1000 mg/dose/adult for a maximum of 4 doses/day.

Without the acid hydrolysis, the colorimetric method was proposed to be used in monitoring an acute exposure to aniline (Chang et al. 1992). With the acid hydrolysis, we now propose this method to be used in biological monitoring of an occupational exposure to aniline, of which a BEI of 50 mg p-AP/g creatinine has been proposed by the American Conference of Governmental Industrial Hygienists (ACGIH 1990).

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