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Simple Colorimetric Method for the Assessment of Aniline Exposure—Analysis of Urinary p-Aminophenol

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Aniline is widely used to manufacture rubber, dyes, medicinals, resins, varnishes, and perfumes (Merck Index 1983). Aniline toxicity is characterized by methemoglobin (met-Hb) formation (Kiese 1966), induction of siderosis of the spleen in various species (Kao et al. 1978), and spleen hemangiosarcomas in rats (Bus and Popp 1987).

The major urinary metabolites of aniline included free and sulfate and glucuronide conjugated para-aminophenol (p-AP) and N-acetyl p-AP. Free and conjugated ortho-AP (o-AP) and N-acetyl o-AP were minor metabolites (Kao et al. 1978; McCarthy et al. 1985). Met-Hb and p-AP are the two biological exposure determinants for aniline listed by the American Conference of Governmental Industrial Hygienists (ACGIH 1990). Both determinants are not specific to aniline. More than a few analytical methods were described in literature for determining urinary p-AP. There were semiquantitative analyses by direct color reaction with o-cresol in ammonia solution (Simpson and Stewart 1973) or 1-naphthol in the presence of sodium nitrite (Svirbely 1989). Quantitative analyses included (1) a reaction with 1,2-naphthoquinone-4-sulfonic acid after an organic solvent extraction (Chrastil 1976), (2) thin layer chromatography (TLC) with a detection by reacting with dimethyl aminobenzaldehyde (Bieniek et al. 1984), and (3) high performance liquid chromatography (HPLC) coupled with an UV detection (Brega et al. 1990). Both of the TLC and HPLC analyses also involved an organic solvent extraction in sample preparation. This can be cumbersome when multiple samples are to be analyzed.

The detection limits reported in the literature were 5 μg/mL by the TLC method, 100 nmol/mL (about 11 μg/mL) by color reaction with

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1,2-naphthoquinone-4-sulfonic acid, and 1 µg/mL by HPLC at a signal to noise ratio of 2. Although the HPLC method had the highest sensitivity, it was utilized to monitor the urinary phenol, not p-AP per se, for an occupational exposure to benzene (Brega et al. 1990). In that report, p-AP eluted at an early retention time and overlapped with several unknown peaks derived from a control urine. The direct colorimetric reaction with o-cresol in ammonia solution was used to screen paracetamol poisoning. A detection limit of 1 mg/dL for urinary p-AP was reported when a spectrophotometer was used. The method was therefore used with some modifications in an attempt to monitor the excretion of p-AP in rat urine after an acute exposure to aniline hydrochloride.

MATERIALS AND METHODS

Female Wistar rats, three to four per dose group, were used. Prior to the intraperitoneal injection of aniline hydrochloride (AH, obtained from Sigma, St Louis, MO), animals were trained one per cage in the metabolic cages (Nalge, Rochester, NY) and provided with Purina rat chow and tap water *ad libitum*. The light cycle of the animal room was kept from 6 am to 6 pm. Each rat was injected once with freshly prepared aqueous AH at 1 mL/kg b. wt. Four dose groups equivalent to aniline proper of 26, 53, 105, and 210 mg/kg were studied. Twenty-four hour urine samples were collected at room temperature for the day before and two days after the exposure in polypropylene tubes and stored at -30°C. The volume of each urine sample was recorded.

Urinary creatinine was determined by the Jaffe reaction. p-AP was determined in the quickly thawed and centrifuged (10,000 x g for 20 min at 4°C) urine supernatant by a colorimetric method described below. To a 0.2 mL cleared urine sample, 1.6 mL of a special reagent mix was added. The special reagent mix consisted of 10 volume of 4 M ammonium hydroxide, 5 volume of aqueous o-cresol (Merck, Darmstadt, FRG) at 20 mg/mL, and 1 volume of concentrated HCl. The reaction was carried out at room temperature for 100 min. At the completion of the reaction, a brief centrifugation at 10,000 x g for 5 min was used to eliminated any denatured proteins. The blue color that developed was measured for its optical density at 620 nm. A working standard curve of 25 to 200 nmol/mL was included in every batch of a determination. The percent relative error of each standard was kept at no greater than ± 5 percent. The final result was corrected for an 86% recovery.

A recovery study was carried out with a control urine. As presented in Table 1, in addition to the aqueous standards, a series of urine samples, spiked with various aqueous p-AP stocks were prepared in triplicate and analyzed simultaneously. A linear regression equation was computed separately for the aqueous standards and spiked urine samples. The recovery was calculated from the ratio of the slope.

To determine the specificity of the method, phenol, m-AP, o-AP, acetanilide, and N-acetyl p-AP (all from Fluka Chemie, Switzerland) were prepared and analyzed as for aqueous p-AP standards.

RESULTS AND DISCUSSION

p-AP is not only itself an important industrial chemical, but also one of the major metabolites for aniline and various fungicides, pesticides and drugs (Simpson and Stewart 1973; Svirbely 1989; Eiche et al. 1990). One of the colorimetric methods used to screen paracetamol poisoning was rather specific to p-AP. The method involved the addition of 1 mL aqueous o-cresol (10 mg/mL) and 2 mL ammonium hydroxide (4M) to a solution of 2 drops concentrated HCl and 5 drops urine. A blue color would develop in the presence of p-AP and N-acetyl p-AP (paracetamol). By coupling with a spectrophotometer, the method was reported to be able to detect p-AP at 1 mg/100 mL and paracetamol 12 mg/100 mL (Simpson and Stewart 1973).

The method was modified for sensitivity, linearity, and reproducibility. A final version of the assay was detailed in the Materials and Methods. By our assay procedure, a p-AP standard at 25 nmol/mL (2.7 μg/mL) resulted in an absorbance at 620 nm of slightly greater than 2.5 times the reagent blank (Table 1). It was used as the lowest standard for the working standard curve. As shown in Table 1, the linearity was very good over a concentration range of 25 to 150 nmol/mL for both aqueous standards and spiked urine samples. All percent relative errors were less than 5%. Later, in order to bracket the highest dose group, the standard curve was extented to 200 nmol/mL. This did not change the linearity nor the percent relative error. The percent relative errors were consistently less than 5%. The coefficients of variance were $\leq$ 6.2% within analysis (n=3) and $\leq$ 11% between analysis (n=6). A recovery of 86% was calculated from the slopes.

The specificity of the assay is demonstrated in Figure 1. A slope of

zero was observed for phenol, m-AP, aniline, acetanilide and N-acetyl p-AP. The slope of o-AP was about one-tenth of that determined for p-AP. No color was developed for N-acetyl p-AP even at a concentration twice the reported detection limit (Simpson and Stewart 1973), i.e. 24 mg/100 mL.

Table 1. Recovery and linearity studies of the p-AP spiked control urine[a]

p-AP nmol/mL	aq. std. A620	% rel. E	spiked urine A620 ± SD	% rel. E	CV, %
0	0.036 (reagent blank)				
25	0.094	+ 1.7	0.081 ± 0.005	+ 4.4	6.2
50	0.150	+ 0.3	0.127 ± 0.004	- 0.8	3.1
100	0.260	- 1.3	0.222 ± 0.002	- 1.9	0.9
150	0.378	+ 0.5	0.326 ± 0.004	+ 0.8	1.2
r^2 (n = 4)	0.999		0.999		
slope x10^3	2.266		1.958		
y-int'cept	0.036		0.030		

a. Sample size was 0.2 mL for the aqueous standards and 0.19 mL of a control urine which was then spiked with 10 µL of various aqueous p-AP stocks, n=3.

Table 2. Individual excretion of non-conjugated (Free) p-AP in urine determined after a single dose of aniline hydrochloride

Dose (mg/kg)[a]	Total free p-AP, nmol in 24 hr[b]				mean ± SD
210	5623	7022	4784	4811	5560 ± 1050
105	4658	2031	3419	-	3369 ± 1314
53	461	394	1040	-	632 ± 355
26	430	94	297	163	246 ± 149

a. Dose was expressed as per aniline.
b. A total of 14 rats were used in this study.

Shown in Table 2 is the individual excretion of non-conjugated p-AP in urine collected in the first 24 hours after an exposure to AH. No

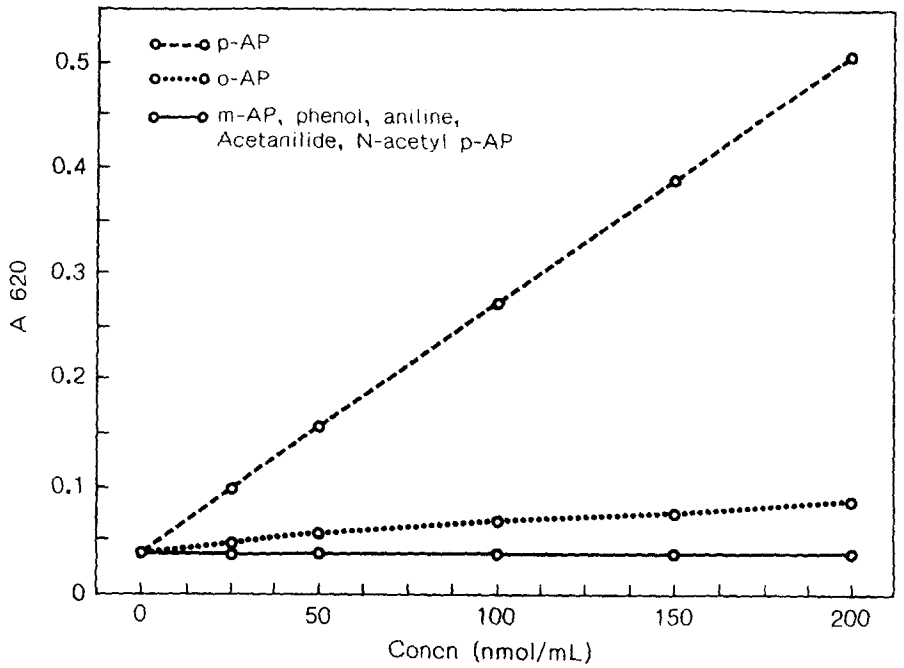

Figure 1. Differential sensitivity of various compounds analyzed by the method.

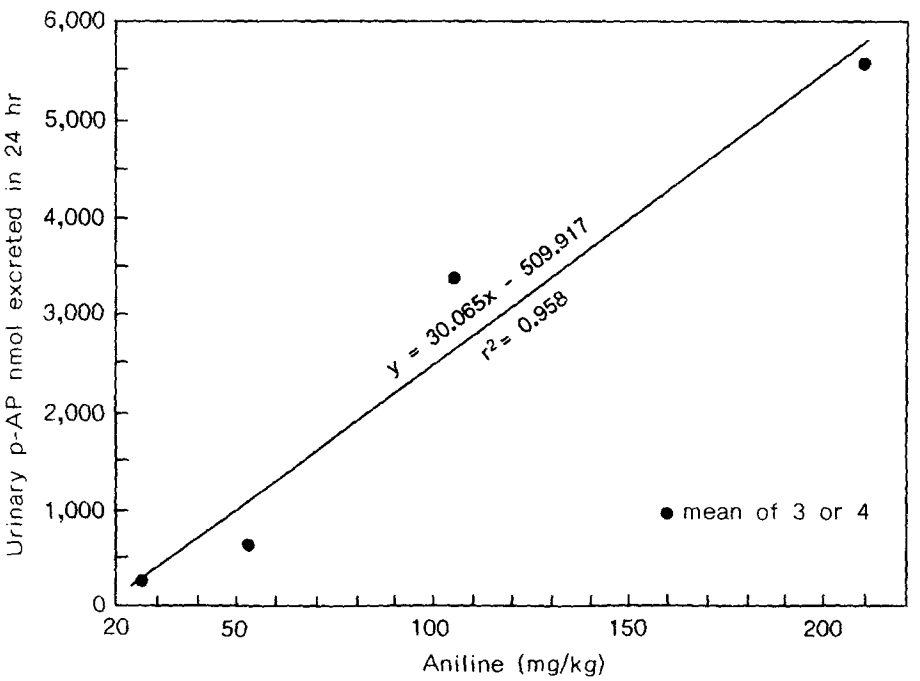

Figure 2. Linear regression of rat urinary p-aminophenol excreted in 24 hours vs exposure dose.

detectable free p-AP was demonstrated in urines collected before or the second day after exposure. More than 40 control urine samples were tested and no trace of free p-AP was found. Individual variation in the excretion of free p-AP was clearly seen. It was estimated that about 1 to 2% of the dose given to the two higher dose groups was eliminated as free p-AP and less than 1% for the two lower dose groups. No loss of free p-AP was observed in urine after thawing and storage at 0°C for 26 hours (data not shown). The measured total excretion of free p-AP correlated well (Figure 2, $r^2 = 0.96$) with the dose given. The linear relationship also held true for nmol p-AP per mg creatinine vs dose (data not shown). The dose levels studied in this report corresponded to 6 to 50% of the LD50 for aniline which was registered as 420 mg/kg, rat ip, in the Registry of Toxic Effects of Chemical Substances (1991).

As reported in literature, as much as 60% of the aniline administered was oxidized to give o- and p-AP. The ratio of p-AP to its ortho-isomer was about eight (Kao et al. 1978). A major part of these two isomers were further acetylated and conjugated with sulfate and glucuronide (Boot et al. 1989; McCarthy et al. 1985). A preliminary analysis on the sulfatase and glucuronidase released p-AP by the present colorimetric method suggested that approximately 95% and higher of the eliminated p-AP was in conjugated forms.

In conclusion, a sensitive colorimetric method for the direct quantitative determination of urinary p-AP was developed. The method, involving minimal sample preparation, was shown to be adequate even before a hydrolysis with sulfatase and glucuronidase in monitoring an acute exposure to aniline.

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