Urinary 5-aminolevulinic acid in lead-exposed children

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Received 3 August 1998, revised form accepted 10 December 1998

Lead intoxication can interfere with haem synthesis and alter the concentration of haem precursors, such as the neurotoxin 5-aminolevulinic acid, in plasma and urine. The relationship between blood lead concentration (PbB), a biomarker of lead exposure, and 5-aminolevulinic acid concentration in urine (ALAU), a biomarker of the early biological effect of lead, was examined in lead-exposed children. ALAU was assayed by chemical derivatization and high performance liquid chromatography with fluorescence detection. The study subjects were 79 children with moderate to high lead exposure recruited from a lead-poisoning prevention clinic. Their urine had been previously analysed for creatinine (CR) concentration and the benzene metabolite *trans,trans*-muconic acid, and their blood had been analysed for lead. We found that ALAU was not correlated with PbB (Spearman $r = 0.088$, $p = 0.44$), but the ratio ALAU/CR was correlated with PbB (Spearman $r = 0.22$, $p = 0.054$). Creatinine and ALAU concentrations were higher in urine samples collected in the afternoon than those collected in the morning, a finding that is consistent with known diurnal variation. However the ratio ALAU/CR was not different in morning and afternoon urines, supporting the use of creatinine adjustment of ALAU analysis of spot urine samples. In view of the neurotoxic properties of ALA, future validation studies of biomarkers of lead exposure and effect in children should include ALAU or ALAU/CR as potential markers of lead effect.

Keywords: 5-aminolevulinic acid, lead, exposure, children.

Abbreviations: ALA, 5-aminolevulinic acid; ALAD, 5-aminolevulinic acid dehydratase; ALAU, 5-aminolevulinic acid in urine; CR, creatinine; HPLC, high performance liquid chromatography; MA, *trans,trans*-muconic acid; PbB, blood lead concentration.

Introduction

Lead is one of the major environmental hazards in urban areas. Children are at higher risk for exposure to lead, and more vulnerable to its toxicity, than adults (CDC 1991). A major source of indoor lead is lead paint; whereas, soil and dust near lead industrial sites, roadways, and lead-painted homes are major environmental sources of lead exposure outdoors (ATSDR 1992, Prpic-Majic *et al.* 1992). In addition, tobacco smoke is also a potential source since cigarettes contain small amounts of lead (World Health Organization 1977, Watanbe *et al.* 1987).

Lead adversely affects several organ systems in the body causing neurological, haematological or renal symptoms. Lead is known to interfere with haem synthesis

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 1999 Taylor & Francis Ltd
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by inhibiting several enzymes in the haem synthetic pathway, especially aminolevulinic acid dehydratase (ALAD), resulting in increased 5-aminolevulinic acid (ALA). ALA is the first intermediate substrate in the haem synthetic pathway generated from glycine and succinyl CoA by the action of ALA synthase. Two molecules of ALA are condensed to form one molecule of porphobilinogen by the action of ALAD (Kappas *et al.* 1995). Lead inhibits ALAD activity resulting in increased ALA which can be quantified in urine and plasma. Increased ALA in urine (ALAU) has been used as a biomarker for lead exposure or effect. Furthermore, a number of studies indicate that ALA is neurotoxic in animals (Brennan and Cantrill 1979, 1981, Audesirk 1985, Cutler *et al.* 1985, Muller and Snyder 1997) and may be responsible for some of the adverse neurological outcomes in lead poisoning and porphyria (McGillion *et al.* 1974, Doss *et al.* 1979, 1982, 1984, Silbergeld and Lamon 1980, 1982, Bonkowsky and Schady 1982, Yeung Laiwah *et al.* 1987, Thunell *et al.* 1987, Bloomer and Bonkovsky 1989, Hassoun *et al.* 1989).

Mauzerall and Granick quantified ALAU by a colorimetric method in 1956. Although variations of this assay were used to measure ALA in plasma and urine in several studies, it was later discovered that the method overestimated ALA concentration in urine because other α-amino ketones also yield the chromogen (Chisolm 1968, MacGee *et al.* 1977, Witting *et al.* 1987, Bloomer and Bonkovsky 1989, Morita *et al.* 1994). In recent years, a high performance liquid chromatography (HPLC) assay for ALA was developed and improved (Meisch *et al.* 1985, Ho *et al.* 1986, Meisch and Wannemacher 1986, Minder 1986, Okayama *et al.* 1986, Tomokuni *et al.* 1987, 1992). This method is more specific, has a lower limit of detection, and requires smaller volumes of urine (50 μl) or plasma than the colorimetric method.

Although ALA measured in 24-h urine collections from children is correlated with blood lead concentration (PbB), no such correlation is found between PbB and ALA measured in random ‘spot’ urines from children (Chisolm *et al.* 1976, Hudák *et al.* 1994). The purpose of the present study is to examine the relationship between ALAU in randomly collected urines and PbB in lead-exposed children using the more specific HPLC assay.

Materials and methods

Population

All children who were seen at the Kennedy Krieger Institute’s Lead Poisoning Prevention Clinic during a 4 week study period in September and October of 1994 were eligible for enrolment (Weaver *et al.* 1995). These children were originally referred to the clinic for evaluation of elevated blood lead levels; some were receiving ongoing follow-up for persistently elevated blood leads or other lead-related concerns such as learning disorders.

The study design and consent procedures were approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Health Institutions. Parents/guardians were invited to participate while waiting for the subjects’ physician visit (Weaver *et al.* 1995). Explanations to the parents and children were provided and informed consent was obtained from all participants. The parents/guardians of 117 children were approached regarding the study. Thirty-one of the children were excluded for one of the following reasons: not toilet trained, could not produce a specimen during the clinic visit, or the guardian was not present. The parents of 79 of the remaining 86 children (91.9 %) agreed to participate. Seventy-six subjects (96 %) were African-American, two were Caucasian and one was Asian.

A questionnaire, administered to the parents, elicited basic information on the child, consisting of medical and environmental histories with data on sources of benzene exposure including environmental tobacco smoke. Urine samples were coded and kept at –80 °C until analysis. Venous blood lead levels were obtained as part of routine clinical care. Urinary *trans,trans*-muconic acid (MA) concentration, creatinine (CR), and other questionnaire data were also available from the benzene exposure assessment study (Weaver *et al.* 1995).

Chemicals and equipment for ALAU assay

Acetylacetone (2,4-pentanedione) was obtained from Aldrich Chemical Company; ethanol, formaldehyde, methanol and glacial acetic acid from J.T. Baker; water from an in-house MilliQ purification system; 5-aminolevulinic acid hydrochloride from Sigma Chemicals. All chemicals were of HPLC grade or the highest grade available. The HPLC system included a PM-30A dual piston pump (Bioanalytical Systems, Inc.), an LO-Pulse pulse dampener (Rainin), a Rheodyne 7125 injector, a Guard-Pak precolumn guard (Waters), a YMC-Pack ODS-A 150 × 4.6 mm i.d., S-5 mm, 120A column (YMC, Inc.), a fluorescence detector (Waters 420-AC), and an integrator (Hewlett Packard 3390A).

Derivatization and analysis for ALA

ALAU levels were determined by a modification of Tomokuni's method (Tomokuni *et al.* 1993a,b). Acetylacetone solution was prepared as acetylacetone/ethanol/water 3:2:15 v/v/v. A 10 % formaldehyde solution was prepared by mixing 37 % formaldehyde/water 27:73 v/v. Fifty µl of standard ALA solution or urine were mixed with 1.75 ml of acetylacetone solution and 225 µl of formaldehyde solution. The mixture was vortexed, heated at 100 °C in a multi-block heater for 10 min, cooled in an ice-cold water bath to stop the derivatization reaction, and filtered through a disposable filter (25 mm × 0.45 µm, nylon (Whatman)). The filtrate was kept shielded from light until injected, within 6 h after derivatization, into the HPLC system. The isocratic mobile phase contained methanol/water/glacial acetic acid 50:50:1 v/v/v, and ran at a flow rate of 0.7 ml min^{-1}. The injection volume was 20 µl. The system was operated at room temperature and the retention time of derivatized ALA was 8.6 min. The limit of detection of the ALAU assay was 45 ng ml^{-1}. The coefficients of variation of the assay were 2.8 % for intraday replicates ($n = 5$ at 911 ng ml^{-1}), and 5.9 % for interday replicates (five sets at concentration ranging from 911 ng ml^{-1} to 3348 ng ml^{-1} measured on 3 days).

Statistics

SAS for Windows v6.08 was used. Univariate analysis for each continuous variable, Spearman rank correlation between variables, and Wilcoxon rank sum test for group data were performed. Multiple linear regression was used to examine predictors of ALAU/CR and to evaluate potential interaction.

Results

All urine samples contained detectable (> 45 ng ml^{-1}) levels of ALA. Table 1 shows summary data of ALAU, ALAU/CR, ALAU divided by log(CR) [ALAU/lnCR], MA, MA/CR, PbB, age, and creatinine. Blood lead levels ranged

Table 1. Descriptive data of urine and blood assays of 79 children with lead exposure.

Variable	N	Mean	SD[a]	Median	Min	Max
Age (year)	79	4.33	1.65	3.84	1.65	10.48
PbB (µg/dl)[b]	77	23.64	8.51	22	5	45
MA (ng/ml)[c]	79	144.49	296.11	59.5	8	2001
MA/CR (ng/mg)[d]	79	176.57	341.73	78.84	7.12	2579
CR (mg/dl)[e]	79	77.88	43.28	70.43	15.59	236
ALAU (ng/ml)[f]	79	1240	720.87	1139	253.66	3563
ALAU/CR[g] (µg/mg)	79	1.80	1.00	1.66	0.26	6.87
ALAU/lnCR[h]	79	286.6	143.8	269.4	67.1	842.5

[a] standard deviation
[b] blood lead concentration
[c] trans,trans-muconic acid in urine
[d] MA divided by creatinine
[e] creatinine in urine
[f] 5-aminolevulinic acid in urine
[g] ALAU divided by creatinine
[h] ALAU divided by natural log of creatinine

from 5 to 45 md dl^{-1}, indicating that this group of patients had considerable lead exposure, as expected in a referral clinic for lead poisoning. Figure 1 shows the frequency distribution of ALAU in this population. Most of the subjects had ALAU in the range of 250 to 2500 ng ml^{-1}, and only three subjects had ALAU higher than 3000 ng ml^{-1}. MA concentrations for these urine samples are included for the purpose of comparing the ALAU data to an unrelated urine metabolite in subsequent analyses to assess non-specific variation in ALAU due to urine dilution.

By Wilcoxon rank sum test, we found no significant differences between boys and girls for age ($p = 0.15$), ALAU ($p = 0.63$), ALAU/CR ($p = 0.23$), ALAU/lnCR ($p = 0.79$), PbB ($p = 0.14$), and CR ($p = 0.30$) (data not shown). Scatterplots of ALAU vs PbB and ALAU/CR vs PbB for all subjects are shown in figure 2. Spearman rank correlations between ALAU, ALAU/CR, ALAU/lnCR, MA, MA/CR, PbB, age, and creatinine are summarized in table 2. ALAU was highly correlated with CR, ALAU/CR, ALAU/lnCR, and MA, but not with PbB; whereas ALAU/CR was correlated with ALAU, ALAU/lnCR, MA, age, and PbB, but not CR. Creatinine adjustment was performed only for CR concentrations ≥ 30 mg dl^{-1}. CR was highly correlated with ALAU, ALAU/lnCR, and MA. Thus, adjustment of ALAU by CR (ALAU/CR) improved the correlation with PbB (Spearman $r = 0.22$, $p = 0.054$) compared to unadjusted ALAU ($r = 0.088$, $p = 0.44$) or ALAU/lnCR ($r = 0.14$, $p = 0.21$).

Further analysis by multiple linear regression indicated that age and PbB were significant independent predictors of ALAU/CR (table 3). The interaction term between age and PbB was not significant (not shown).

In order to examine possible variation in concentration of urinary metabolites due to biological diurnal variation, we compared measurements in urines collected in the morning and afternoon. By Wilcoxon rank sum test, we found that morning urines had marginally lower concentrations of ALAU ($p = 0.099$) and significantly lower concentrations of creatinine ($p = 0.03$) than afternoon urines; whereas morning and afternoon ALAU/CR were not different ($p = 0.91$), as shown in figure 3. Afternoon urines also had higher MA and MA/CR than morning urines;

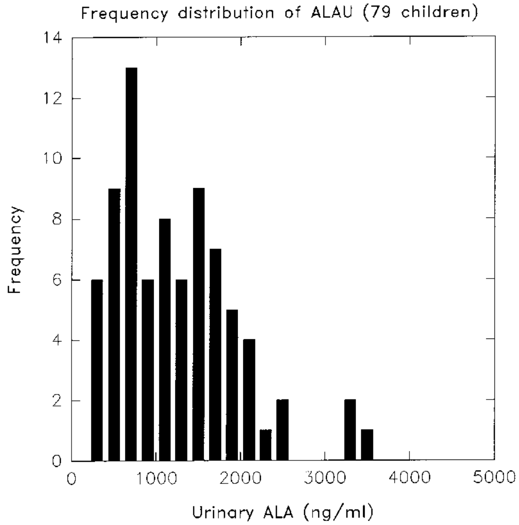

Figure 1. Frequency distribution of urinary ALA in 79 children.

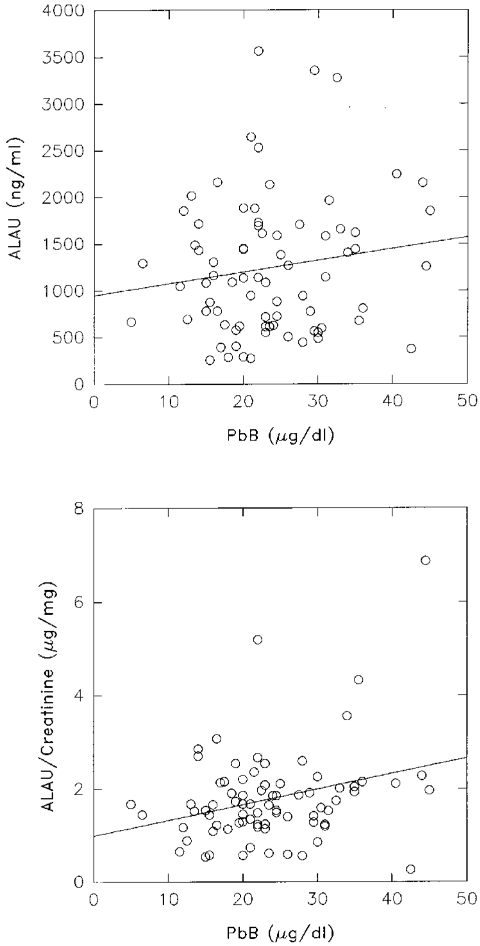

Figure 2. Scatterplots of ALAU vs PbB [top panel; Spearman $r = 0.096$, $p = 0.41$], and ALAU/CR vs PbB [bottom panel; Spearman $r = 0.21$, $p = 0.06$] for 77 children.

whereas, no difference in morning and afternoon PbB was found ($p = 0.16$) as expected.

Discussion

We examined the relationship between PbB and creatinine-adjusted ALAU in randomly collected 'spot' urines from lead-exposed children. Two methods of creatinine-adjustment were used: ALAU/CR and ALAU/lnCR. We found that ALAU/CR correlated more closely with PbB than did ALAU/lnCR, indicating that ALAU/CR may be superior to ALAU/lnCR for assessing lead exposure and lead-specific effects on haem synthesis. Age and PbB were also independent predictors of ALAU/CR by multiple linear regression, although no effect modification between age and PbB was observed.

A potential weakness of the current study design was the fact that many of the children recruited from the lead exposure prevention clinic came from homes that had undergone varying degrees of lead remediation. Such an intervention might be

Table 2. Spearman's correlation coefficients and p values for ALAU, PbB, and other variables in 79 children ($n = 77$ for PbB; $n = 72$ for CR).

	ALAU/CR	ALAU/lnCR	MA	MA/CR	PbB	Age	CR
ALAU	0.31	0.80	0.28	0.02	0.088	–0.08	0.50
	0.005	<0.0001	0.01	0.87	0.44	0.47	<0.0001
ALAU/CR		0.64	–0.24	–0.09	0.22	–0.42	–0.14
		<0.0001	0.03	0.43	0.054	0.0001	0.23
ALAU/lnCR			0.05	–0.02	0.14	–0.27	0.58
			0.63	0.84	0.21	0.02	<0.0001
MA				0.91	–0.16	–0.04	0.26
				<0.0001	0.17	0.74	0.02
MA/CR					–0.13	–0.17	0.06
					0.24	0.14	0.58
PbB						–0.16	0.02
						0.17	0.87
Age							0.04
							0.73

Table 3. Multiple linear regression analysis of ALAU/CR by age and PbB for 77 subjects (2 missing).

Variable	β	standard error β	p-value	model r
Age (year)	–0.15	0.07	0.026	0.37
PbB (μg/dl)	0.03	0.01	0.033	

expected to reduce or weaken the observed association between PbB and ALAU or ALAU/CR, suggesting that our results may underestimate the strength of such an association.

The correlations between urine variables are subject to confounding due to urine dilution. This is illustrated by the colinearity of both ALAU and MA with creatinine. We found no correlation between MA or MA/CR and PbB as expected since presumptive sources of benzene and lead exposure in these subjects are different. The major source of lead is most likely lead paint, whereas the major source of benzene may be gasoline or environmental tobacco smoke.

Since creatinine, ALAU, and MA are urinary metabolites, their concentrations in urine will depend, at least partly, on hydration status of the subjects. We found that urines collected in the afternoon had higher concentrations of creatinine and ALAU than those collected in the morning, which is consistent with previous knowledge of diurnal variation of excretion of creatinine and other compounds in the general population (Botta *et al.* 1987, Letourneau *et al.* 1988, Boeniger *et al.* 1993). This difference disappeared when we adjusted for creatinine (ALAU/CR), supporting the use of creatinine adjustment for ALAU measured in 'spot' urines. When morning and afternoon samples were analysed together, we found a positive correlation between ALAU and CR, consistent with a previous report (Nishima 1977), suggesting that common factors determine their renal excretion (e.g. renal blood flow, glomerular filtration rate, etc) and supporting the use of ALAU/CR for spot urine samples.

In contrast to the study by Hudák *et al.* (1994) which used the colorimetric method to assay ALAU, we used the more specific method of HPLC with fluorescence detection. The colorimetric method overestimates ALA because other α-aminoketones also become chromogenic (MacGee *et al.* 1977, Witting *et al.*

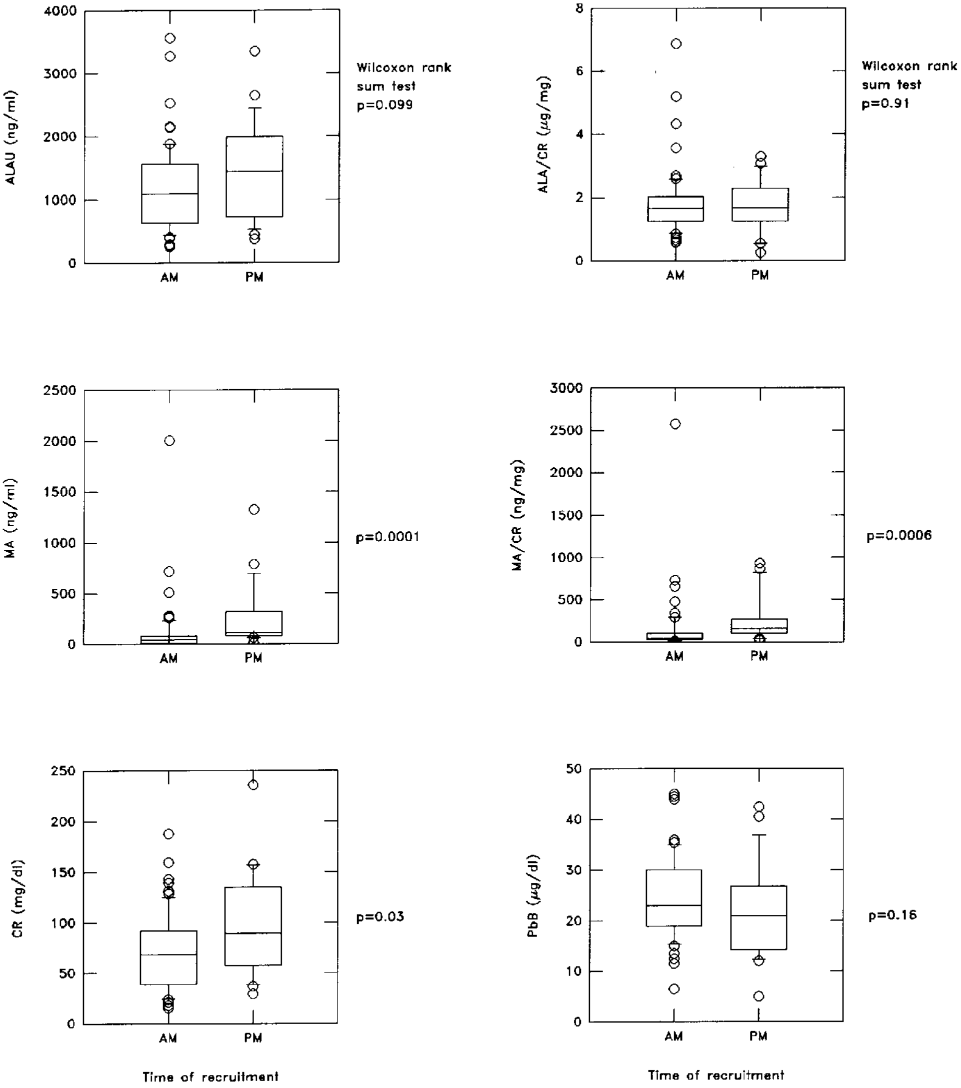

Figure 3. Boxplots comparing morning and afternoon urine measurements (ALAU, ALAU/CR, MA, MA/CR, CR) and PbB. All comparison tests are Wilcoxon rank sum.

1987). Also, the range of PbB (1.0–15.7 μg dl^{-1}) in their subjects was lower and narrower than the range in our subjects (5–45 μg dl^{-1}). Our finding that there were no differences between boys and girls for ALAU and ALAU/CR was consistent with Hudák's results.

Our study did not provide any evidence that would clarify whether or not a threshold for ALAU/CR with increasing PbB exists (figure 2). A previous study in adults (Bernard and Lauwerys 1987) reported a threshold for ALAU/CR at 30–35 μg dl^{-1} PbB. Another study in children (Chisolm *et al.* 1976) reported a threshold at 40–50 μg dl^{-1} PbB; however, such a threshold would have been undetectable in our study since the maximum PbB value was 45 μg dl^{-1}. These relationships may be clarified for both adults and children using the more sensitive HPLC assay in subjects with moderate to high lead exposure. Since children are at increased risk for environmental lead poisoning compared with adults, particular attention should be focused on the validation of biomarkers of exposure and effect in this population. The potential use of ALAU or ALAU/CR as markers of lead effect should be investigated further, particularly in view of the neurotoxic properties of ALA.

Acknowledgements

We thank Dr Marie Diener-West for statistical advice. The research was supported in part by DHHS grants ES03819, ES06052, and ES07780.

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