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Journal of Chromatography B, 744 (2000) 423–431

JOURNAL OF CHROMATOGRAPHY B

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Liquid chromatography–electrospray tandem mass spectrometry of acidic monoamine metabolites

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Received 2 December 1999; received in revised form 7 April 2000; accepted 11 May 2000

Abstract

A new method based on liquid chromatography–tandem mass spectrometry has been developed for the determination of monoamine metabolites, i.e., homovanillic acid (HVA), vanilmandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) in human urine. Analytes were separated on a C_{16} amide (5 cm, 5 μm) column and ionized by negative ion electrospray. Operating in the selected-reaction monitoring mode, linearity was established over three-orders of magnitude and limits of detection were in the range 30–70 μg/l. Precision calculated as RSD was within 0.8–5.2% for all intra- and inter-day determinations. The method was applied to the quantitative analysis of monoamine metabolites in 700 urine samples from occupationally (adults) and environmentally (both children and adults) exposed people living in areas with different soil contamination from lead. The urinary excretion of monoamine metabolites was significantly higher ($P<0.001$) in the subgroup of children living in polluted areas as compared to the control group (HVA, 6.03 vs. 4.57 mg/g creatinine; VMA, 5.33 vs. 4.37 mg/g creatinine; 5-HIAA 3.24 vs. 2.45 mg/g creatinine). In adults belonging to both groups of subjects occupationally and environmentally exposed, no differences were detected in the urinary concentration of monoamine metabolites. However, adults showed lower values of HVA (2.57 mg/g creatinine), VMA (2.17 mg/g creatinine) and 5-HIAA (2.09 mg/g creatinine) as compared to children groups.

Keywords: Catecholamines; Homovanillic acid; Vanilmandelic acid; 3,4-Dihydroxyphenylacetic acid; 5-Hydroxyindoleacetic acid

1. Introduction

Vanilmandelic (4-hydroxy-3-methoxymandelic-)acid (VMA), homovanillic (4-hydroxy-3-methoxyphenylacetic) acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) are the main acidic catabolites of norepinephrine, dopamine and serotonin, respectively, and are excreted in urine. The concentrations of catecholamines, serotonin, and their precursor and metabolites are essential for diagnosis of neurological disorders. HVA and VMA are biological markers of neuroblastoma, the most solid tumor in children

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00285-1

[1–3]; the determination of the urinary concentrations of 5-HIAA is fundamental for the diagnosis of the carcinoid syndrome, a serotonin secreting tumor [4]. The lower limits of pathological concentrations are set at 35 and 20 mg/g creatinine for HVA and VMA, respectively [5]. Reference values in the range 0.50–6.55 mg/g creatinine have been reported for all the metabolites, VMA, HVA and 5-HIAA, in the case of healthy adult volunteers [6]. The HVA concentration in children is strongly age-related [7].

Recent studies on rat have shown that exposure to low levels of lead (Pb) result in significant depletion of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA [8]. The neurochemical effects of this metal seem to involve almost all the neurotransmitters [9], and alterations were observed also in the serotonin metabolism in rats following Pb treatment [10]. The mechanism leading to the Pb-induced impairment of dopaminergic activity in rat is still unknown and under investigation. In vitro studies indicated that Pb inhibits tyrosine hydroxylase (TH), the rate limiting enzyme in the dopamine biosynthesis [11]. Alterations in TH activity were also observed in the brains of rats exposed to low levels of Pb, suggesting that Pb-induced inhibition of TH activity may indeed contribute to the reductions in dopaminergic activity observed in Pb-exposed animals. Recently, Tang et al. [12] found a positive association between blood Pb and the urinary excretion of HVA in a group of workers heavily exposed to Pb (blood Pb, mean value 70.55 μg/l), from lead smeltery and storage-battery manufacturing factory. The urinary HVA was correlated with the impairment in neurobehavioral function, i.e., attention/response speed, manual dexterity, perceptual motor-speed, visual perception/memory, and motor speed/steadiness, as assessed by the World Health Organization (WHO) Neurobehavioral Core Test battery. Learning defects were also observed in children exposed to low-levels of Pb [13]. These findings suggest the possibility to detect alteration of dopamine metabolism by measuring its acidic metabolite HVA in urine. As such, HVA in urine is a useful biomarker of Pb-induced neurotoxicity to dopaminergic systems.

The present study is part of a European project aimed at the investigation of the possible effects of low doses of Pb in human, which occur as a consequence of environmental pollution near non-ferrous smelting factories. Environmental concentrations of Pb in soil are normally around 2–3 ppm, whereas around some smelters in Eastern Europe and in the north of France levels as high as 5000 ppm in soil were reported.

The analytical determination of monoamine acidic metabolites is mostly based on high-performance liquid chromatography (HPLC) with electrochemical detection [6,14–16], while gas chromatography–mass spectrometry (GC–MS) after derivatization of VMA and HVA to trimethylsilyl derivatives is also described [5]. A competitive enzyme immunoassay (EIA) with monoclonal antibodies has been proposed for HVA measurement in human urine samples [17].

The goal of the present work was to develop a new method for the rapid analysis of VMA, HVA, DOPAC and 5-HIAA in untreated urine samples using liquid chromatography–tandem mass spectrometry (LC–MS–MS). The high selectivity and sensitivity of tandem MS makes this technique suitable for the determination of low concentrations of metabolites in complex matrices, such as urine without any sample manipulation.

2. Experimental

2.1. Chemicals

VMA, HVA, DOPAC and 5-HIAA were obtained from Sigma (Milan, Italy). All chemicals were of analytical-reagent grade and were used without further purification. Standard stock solutions (approximately 1 g/l) were prepared in water. These solutions are stable for at least 1 month when stored in the dark at −20°C. Working solutions (10 mg/l) were obtained by diluting the stock solution to the appropriate concentration with the mobile phase. HPLC-grade water and methanol were from LabScan (Dublin, Ireland). Analytical grade formic acid, ammonium acetate, sodium dihydrogenphosphate, disodium ethylenediaminetetraacetate (disodium EDTA), sodium octyl sulfate (OSS) were supplied by Fluka (Buchs, Switzerland).

2.2. Calibration standards and control samples

Calibration samples were prepared by spiking urine with the standard solutions in order to obtain concentrations of the analytes in the 0.1–250 mg/l range. Control samples were prepared from a certified urine standard containing VMA (14.0 mg/l), HVA (15.0 mg/l) and 5-HIAA (24.0 mg/l) (Bio-Rad, Munich, Germany) by reconstituting and subsequent dilution (1:10) with water.

2.3. Liquid chromatography–mass spectrometry

LC–MS–MS analyses were carried out on a Perkin-Elmer (PE) Sciex API 365 triple-quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an ionspray interface for pneumatically-assisted electrospray and with a Power Macintosh 7200/120 computer for instrumental control, data acquisition and processing. The liquid chromatograph was a Perkin-Elmer series 200 binary system (Norwalk, CT, USA) equipped with an ASPEC XL autosampler (Gilson, Villiers-le-Bel, France). The analytes were separated on a Discovery C_{16} amide column, 50 mm×4.6 mm I.D., 5 μm (Supelco, Bellefonte, PA, USA). Separation was achieved by running a fast linear gradient starting from 18% (hold 1 min) to 80% methanol (in 2.5 min) in aqueous formic acid (20 m*M*). The chromatographic flow-rate was 0.75 ml/min and, after the column, the eluate was subjected to a 1:20 split. Ionization of the analytes was obtained by electrospray in the negative ion mode. All the interface parameters were optimized by infusing standard solutions (5 mg/l) of the analytes. Electrospray conditions: nitrogen curtain gas, 1.04 l/min; nebulizing gas (air), 1.23 l/min; ionspray voltage, −3600 V; orifice voltage, −40 V; ring voltage, −200 V. Full-scan single-quadrupole mass spectra were acquired in the 100–210 u mass range, scan rate, 0.63 s. Product ion mass spectra of the $[M-H]^-$ ions were obtained in the range *m/z* 50–200. The collision energy was 16 eV. MS acquisition was performed in the selected-reaction monitoring (SRM) mode, by monitoring the reactions *m/z* 197.0⇒137.0, characteristic of VMA; 181.0⇒137.0, characteristic of HVA; *m/z* 167.0⇒123.1, characteristic of DOPAC; 190.1⇒146.1, characteristic of 5-HIAA (200 ms for each transition). To elucidate the fragmentation pathways of the compounds studied and particularly that of VMA, high-resolution mass spectra were obtained using an ESI-oa time-of-flight (TOF) mass spectrometer PE Biosystem Mariner.

2.4. Liquid chromatography with electrochemical detection

For comparative purposes, the HVA concentration was also determined in 30 randomly chosen samples by LC with electrochemical detection (ED). Analyses were carried out on a Gilson 305 LC pump, equipped with ESA Coulochem II coulometric detector (Bedford, MA, USA). Separations were obtained using a Supelcosil LC-18-DB (750 mm×4.6 mm I.D., 3 μm) column and a mobile phase consisting of 100 m*M* $NaH_2PO_4 \cdot H_2O$–methanol (84:16, v/v), containing also $2.6 \cdot 10^{-3}$ *M* OSS and 10^{-4} *M* EDTA (final pH 3.5). The flow-rate was 0.8 ml/min. The potentials applied to the first (E_1) and second cell (E_2) were +50 mV and +400 mV, respectively.

2.5. Urine collection and analysis

Four groups of people were recruited, two groups of adults, occupationally exposed (investigated) and environmentally exposed (controls), and two groups of children living in areas with different soil contamination from Pb, i.e., near non-ferrous smelters (investigated) and in rural areas (controls). Seven hundred urine samples (about 10 ml) were collected in dark vials containing 1% HCl (pH<4.0) and stored at −20°C until analysis, which was carried out within 1 month to avoid analyte degradation. LC–MS analyses were done on 0.25 ml of urine samples diluted with 0.75 ml of 0.1 *M* ammonium acetate buffer. A volume of 20 μl was injected onto the LC column. All the analyses were performed within 1 working week, also running the instrument overnight. A control sample was analyzed after each 10 samples. Samples for LC–ED analysis were centrifuged and diluted 1:20 with water.

2.6. Statistics

Statistical analysis was carried out using SPSS for Windows (9.0) statistical package (SPSS, Chicago, IL, USA). Parametric statistic analysis was carried out. Log-transformed values were used when necessary to obtain a normal distribution, which was verified using the Kolmogorov–Smirnov test. Differences between groups were primarily assessed using the Student's *t*-test for independent samples. Linear regression analysis was carried out using the least-square method (Pearson's correlation).

3. Results and discussion

3.1. Mass spectrometry

The structures and the molecular masses of VMA, HVA, DOPAC and 5-HIAA are shown in Fig. 1. Mass spectra of the catecholamine metabolites show intense protonated and deprotonated molecules in the positive ion and negative ion modes, respectively. $[M+H]^+$ and $[M-H]^-$ ions showed comparable abundances when methanol–water (1:1) solutions of the analytes were infused, except for VMA. VMA was not detectable as an intact protonated molecule in the positive ion mode, since it loses water during ionization. In order to obtain the maximum sensitivity, orifice fragmentation was avoided. In fact, an increase in the orifice voltage not only leads to fragmentation of the analytes, which can be useful for structural characterization and confirmation, but it also results in a loss in sensitivity.

The fragmentation of $[M+H]^+$ and $[M-H]^-$ ions in the collision cell led to few specific ions useful for SRM analysis. The positive and negative product ion mass spectra of all the compounds studied except VMA are characterized by a base peak at the same *m/z*, at *m/z* 137.0 for HVA, *m/z* 123.1 for DOPAC, and *m/z* 146.1 for 5-HIAA. These very stable fragment ions are attributable to the cleavage of the C–C bond next to C=O and the release of HCOOH from the protonated molecule (PI) or CO_2 from the deprotonated molecule (NI). Fig. 2 shows the product ion mass spectra of monoamine metabolites as obtained in the negative ion mode. Although VMA and HVA are molecules with different structures, their NI product ion spectra are apparently similar, with an intense signal at *m/z* 137.0. However, when acquired with an electrospray ionization (ESI)-TOF-MS system, differences show up as a consequence of

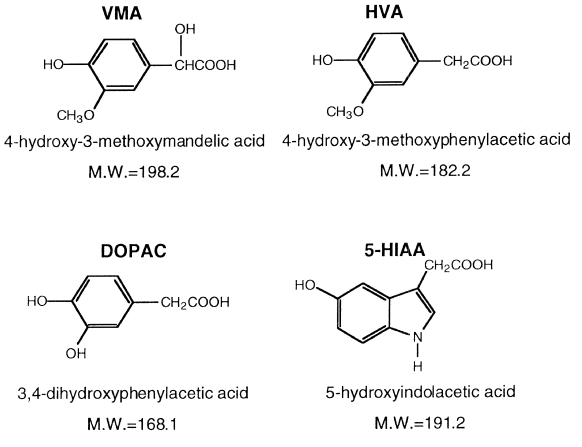

Fig. 1. Structures and molecular masses of the compounds studied.

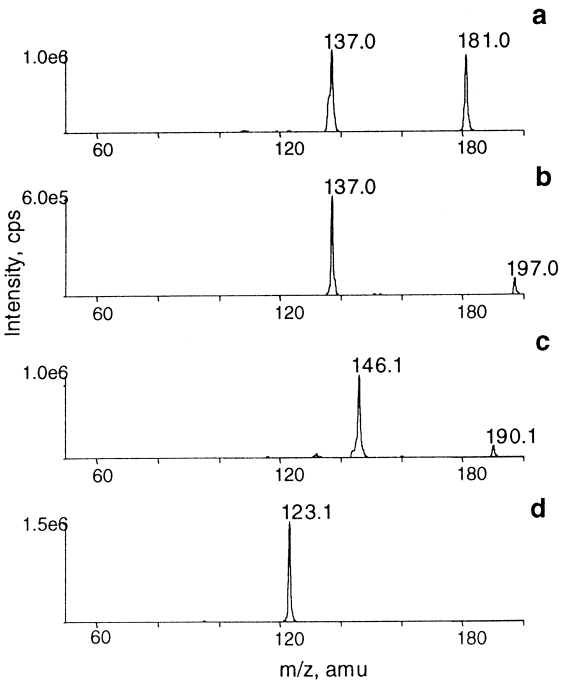

Fig. 2. Product ion mass spectra of HVA (a), VMA (b), 5-HIAA (c) and DOPAC (d) obtained in the negative ion mode. Scan range 50–200 u. For other conditions: see Experimental.

two different fragmentation pathways. The high-resolution mass spectrum of VMA showed the fragment ion at m/z 137.0265, corresponding to the elemental composition $C_7H_5O_3$. This fragment of the VMA spectrum could be undoubtedly attributed to the neutral loss of acetic acid, whereas in the case of HVA, the loss of CO_2 (m/z 137.0618, $C_8H_9O_2$) was confirmed also by ESI-TOF-MS. The loss of CO_2 was also observed for the other metabolites, 5-HIAA (m/z 146.0637, C_9H_8NO) and DOPAC (m/z 123.0452, $C_7H_7O_2$).

Since urine samples were diluted with ammonium acetate prior to analysis, the effect of ammonium acetate addition on the ESI-MS spectrum was investigated. As expected, the formation of the $[M+NH_4]^+$ adduct was observed in the positive ion mode for all the compounds. Negative ionization was preferred over positive ionization because the ammonium adduct could not be efficiently fragmented in tandem mass spectrometry.

3.2. Chromatography

Fast chromatography of catecholamine and serotonine metabolites was performed using a short column packed with a new kind of stationary phase, C_{16} amide under ion-suppressed reversed-phase chromatographic conditions. Formic acid was chosen as modifier because of its compatibility with electrospray ionization. Gradient elution was preferred to isocratic elution since it allowed faster analyses and reduced the risk of column degradation due to lipophilic compounds present in untreated urine. A LC–MS chromatogram of a urine sample containing VMA, DOPAC, 5-HIAA and HVA (in order of elution), and obtained under selected ion monitoring (SIM) conditions is shown in Fig. 3. It should be noted that the retention time of the first eluting compound, VMA, is strongly influenced by the concentration of ammonium acetate buffer added for dilution. A shift from 1.9 to 3.5 min was observed when the certified urine standard was used undiluted or diluted 1:100 with 0.1 *M* ammonium acetate. This behavior could be explained considering that VMA could give an intramolecular hydrogen bond, whose extent is influenced by the total amount of salt of the sample. Since the different salt content of individual urine samples could influence the retention time of

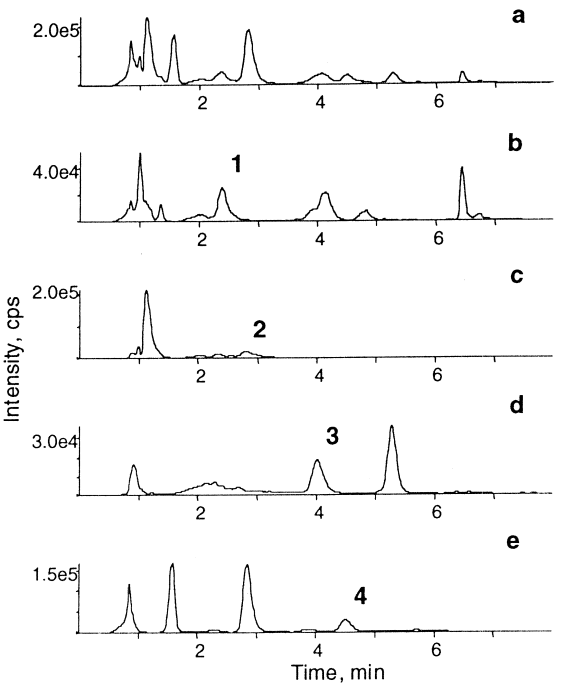

Fig. 3. (a) LC–MS SIM chromatogram of a urine sample from a occupationally exposed subject and mass chromatograms of m/z (b) 197.0, (c) 167.0, (d) 190.1 and (e) 181.0. Peak identification: 1=VMA, 2=DOPAC, 3=5-HIAA, 4=HVA. Column, Discovery C_{16} amide 5 cm×4.6 mm I.D., 5 μm. For other conditions: see Experimental.

VMA, samples were buffered with ammonium acetate. When the dilution factor is 1:4 (as for sample analysis), the retention time is ''locked'' at 2.3 min.

3.3. Validation of the LC–MS–MS method

Although ESI-MS offers adequate sensitivity for the analysis of these metabolites, low-mass background ions were found to cause significant interference resulting in a lack of specificity when authentic urine samples were analyzed. As a result, LC–ESI-MS overestimated (up to two-fold) the analyte concentrations in comparison to those obtained by LC–ED, which is considered as the reference technique for catecholamine metabolites. Thus, the reliability of the method in terms of selectivity and consequently quantification power was greatly improved using tandem mass spectrometry, by operating in the SRM mode.

Table 1
Calibration graph results for LC–ESI-MS–MS of monoamine metabolites[a]

Compound	Range (mg/l)	$b \cdot 10^{-4}$[b]	r^2	LOD (μg/l)[c]
VMA	0.5–50.0	2.23±0.01	0.999	70
HVA	0.1–25.0	1.18±0.01	1.000	30
DOPAC	0.1–10.0	5.27±0.02	1.000	40
5-HIAA	0.1–25.0	2.21±0.01	1.000	30

[a] Calibration fitting: $y=bx$ ($n=18$).
[b] ±Values are confidence intervals for 95% probability level.
[c] Limit of detection ($S/N=3$) calculated under SRM conditions.

The experimentally determined linear ranges, the equations, the linear correlation coefficients (r^2) and the limits of detection (LODs) for VMA, HVA, DOPAC and 5-HIAA are shown in Table 1. Experimental data fitted a linear model, $y=bx$, where x is the concentration and y the chromatographic peak areas, the intercept values a being negligible and not significantly different from the origin ($P>0.03$ in all cases). Calibrations in urine showed a three-orders of magnitude linear behavior with linear correlation coefficients (r^2) of 0.999 for VMA and 1.000 for HVA, DOPAC and 5-HIAA. Saturation of the signal was observed at concentrations higher than 50 mg/l for all the compounds except VMA, for which the response still linearly increases at higher concentrations.

The limits of detection calculated on a signal-to-noise ratio of 3 were 70 μg/l for VMA, 40 μg/l for DOPAC, and 30 μg/l for both HVA and 5-HIAA, and were well below the reference values for healthy adult people reported in the literature [6]. LODs were comparable to those reported in the case of LC–ED [6]. Only stable isotope dilution GC–MS [5] offers superior sensitivity with detection limits of 4.0 pg (HVA) and 0.8 pg (VMA), but requires complex sample pretreatment before analysis.

The repeatability and the intra- and inter-day precision of LC–MS–MS method were determined at two concentration levels and the results expressed as relative standard deviation (RSD) are summarized in Table 2. The accuracy of the method was evaluated by assessing the agreement between the measured and the nominal concentrations of certified control samples. The measured concentrations of VMA, HVA and 5-HIAA are the means of the concentrations obtained. The bias does not exceed ±4%. For the randomly-chosen samples ($n=30$), the concentrations of HVA obtained using tandem MS were in good agreement with those measured by LC–ED (±11%, $r=0.88$).

3.4. Application

The method was applied to the quantitative determination of the concentrations of VMA, HVA and 5-HIAA in urine, as biomarkers of environmental and occupational exposures to Pb. Samples were

Table 2
Repeatability, intra- and inter-day precision of the LC–MS–MS method calculated as RSD (%) at two concentration levels for VMA, HVA, DOPAC and 5-HIAA

Compound	Concentration (mg/l)	Repeatability[a] (% RSD)	Precision		Accuracy (% bias)
			Intra-day[a]	Inter-day[b]	
VMA	1.4	0.9	1.2	1.8	3.3
	14.0	0.4	0.8	1.1	2.1
HVA	1.5	2.3	3.4	5.2	3.9
	15.0	1.5	1.4	2.6	2.8
DOPAC	1.0	1.9	3.5	4.7	3.6
	10.0	1.1	1.5	2.2	2.4
5-HIAA	2.4	2.4	2.1	4.1	4.0
	24.0	0.7	1.4	3.5	3.1

[a] $n=6$.
[b] $n=10$.

analyzed by LC–MS–MS after dilution (1:4) with ammonium acetate to prevent column degradation. Fig. 4 shows one out the 700 chromatograms of urine samples analyzed. A screening for conjugated metabolites (both glucuronides and sulfates) was performed using LC–MS–MS in the precursor ion mode (M−176, diagnostic for glucuronides) and neutral-loss (M−80, diagnostic for sulfates). No evidence of conjugates of these metabolites was found in urine samples using tandem MS.

The concentrations found for all subjects examined but one were within the physiological range for all the metabolites. Quantitative results of VMA, HVA and 5-HIAA are summarized in Fig. 5, where

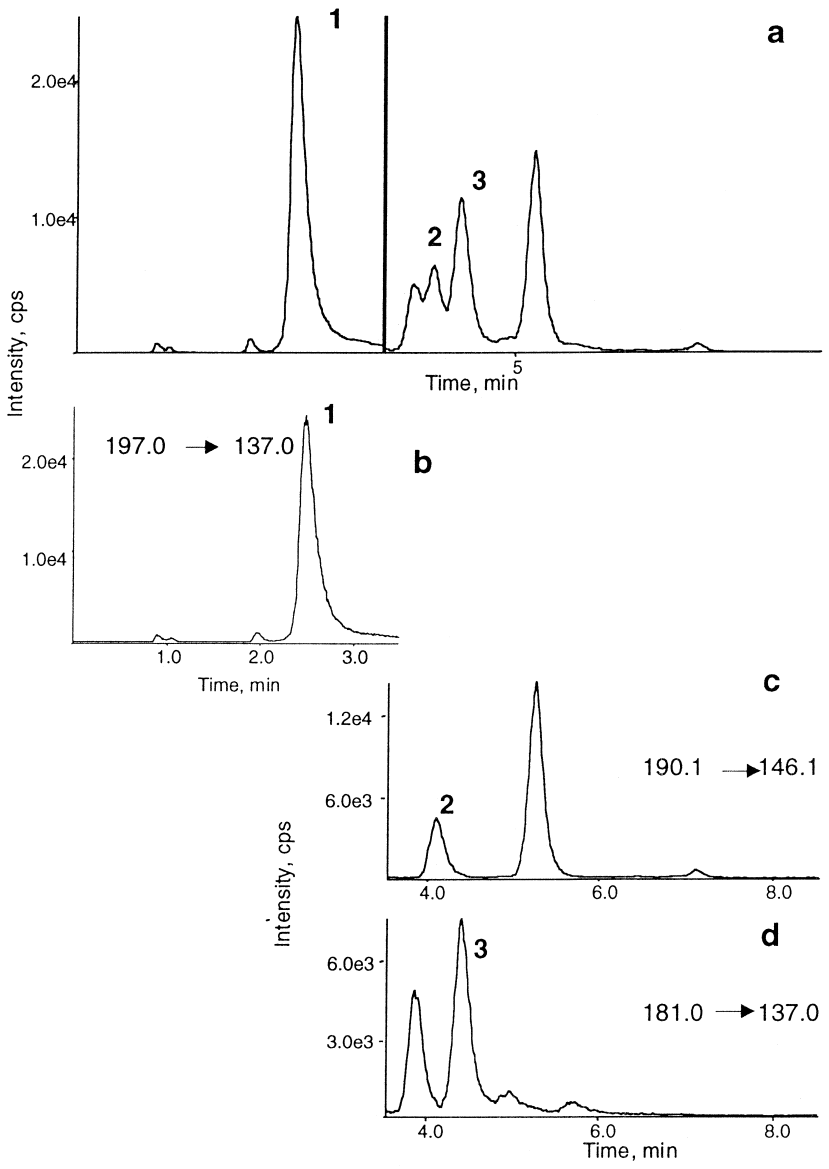

Fig. 4. (a) LC–MS–MS time-scheduled SRM chromatogram of a urine sample from a occupationally exposed subject and separate traces for the transitions (b) 197.0⇒137.0, (c) 190.1⇒146.1, (d) 181.0⇒137.0. Peak identification: 1=VMA, 2=5-HIAA, 3=HVA.

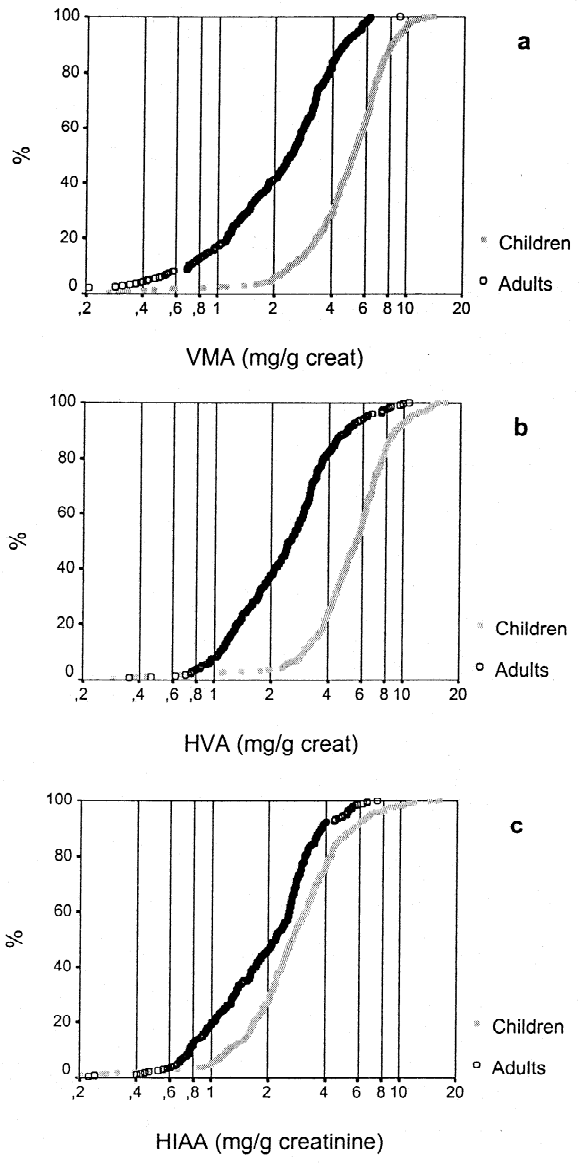

Fig. 5. Cumulated frequency (%) for VMA (a) HVA (b) and 5-HIAA (c).

the cumulated frequency for each analyte (a–c) and for children and adults are shown. As expected, urinary concentrations of all acidic monoamine metabolites are higher in children than in adults. The difference between children and adults is smaller in the case of 5-HIAA, the serotonin metabolite. The concentrations of HVA, VMA and 5-HIAA were inter-correlated ($P<0.01$). Table 3 shows the mean values of urinary metabolites in the groups under study. Whereas no differences were observed between adults belonging to either the control or the investigated groups, the subgroup of children living in the polluted areas near non-ferrous smelters showed significantly higher values compared to their controls. Fig. 6 shows the correlation between blood Pb and the urinary excretion of homovanillic acid, obtained in the case of occupationally exposed adult subjects. A weak but statistically significant relationship was found between blood Pb and the urinary excretion of HVA in adults. Although this finding is inconsistent with the positive association found by other authors [12], it is in line with the reported inhibitory effect of lead on TH [11] and with both experimental [18] and epidemiological studies [19,20] suggesting respectively that Pb exposure is associated with DA depletion and dysfunction resulting in neuroendocrine changes, namely increased serum prolactin. On the other hand, the effect of Pb on dopamine metabolism could be largely influenced by exposure levels and thus this controversial issue needs further investigations. Also 5-HIAA, but not VMA, showed a negative correlation with blood Pb ($r^2=0.29$), suggesting an effect of lead also on serotonin metabolism. Although this has been demonstrated in brain of lead-treated chicks [21], no evidence exists about possible changes in 5-HIAA excretion in urine of lead exposed people. Furthermore, the serotonin concentration would be strongly influenced by dietary habits, e.g., consumption of chocolate, bananas, coffee and foods containing vanilla, etc.

Acknowledgements

The authors would like to thanks Dr. Bruno Casetta (PE Biosystems, Italy) who performed the ESI-TOF-MS analyses. This work was financially supported by European Community, Inco Copernicus IC14-CT96-0311 and by the Azienda Ospedaliera di Parma (Italy).

Table 3
Geometric means (GMs) and geometric standard deviations (GSDs) of urinary metabolites VMA, HVA and 5-HIAA in subgroups of children and adults[a]

	Variable	Controls		Investigated		*t*-Test	
		GM[b]	GSD[b]	GM[b]	GSD[b]	*t*	*P*
Children	VMA	4.37	1.86	5.37	1.66	3.638	<0.001
	HVA	4.57	2.09	6.03	1.45	4.444	<0.001
	5-HIAA	2.45	2.09	3.24	1.86	3.829	<0.001
Adults	VMA	2.29	2.09	2.04	2.40	1.245	0.210
	HVA	2.57	1.91	2.57	2.00	0.100	0.921
	5-HIAA	2.00	2.14	2.24	2.24	1.190	0.235

[a] Differences between group means (controls and investigated) were assessed by Student's *t*-test for independent samples.
[b] mg/g creatinine.

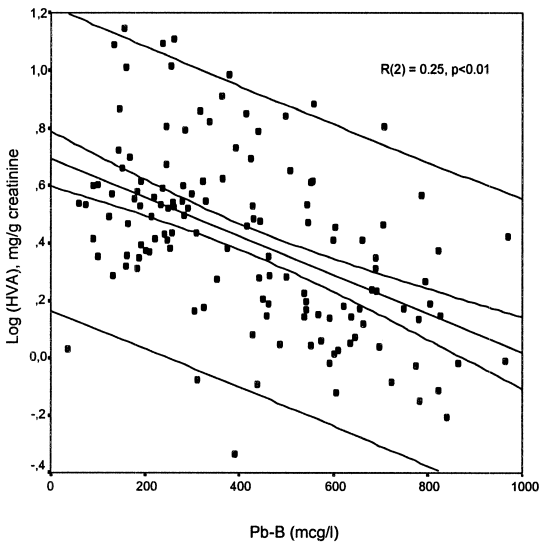

Fig. 6. Correlation between blood lead and urinary excretion of homovanillic acid for adult occupationally exposed subjects (investigated).

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