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Short-chain aliphatic amines in human urine: a mathematical examination of metabolic interrelationships

Stephen C. Mitchell*, Mary E. Bollard, AiQin Zhang

SORA Division, Biomolecular Medicine, Faculty of Medicine, Imperial College London, South Kensington, London SW7 2AZ, UK

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Abstract

The relationships between several small molecular weight aliphatic amines (methylamine, dimethylamine, trimethylamine, and ethylamine) and an associated *N*-oxide (trimethylamine *N*-oxide) quantified in human urine collected from 203 healthy volunteers have been assessed mathematically. Principal component analysis highlighted a female subgroup with raised trimethylamine levels and the possibility of hormonal influence on the N-oxidation of trimethylamine has been proposed. A second subgroup of men, who ate a large meal of fish before the study, displayed raised levels of all compounds except ethylamine. In all cases, ethylamine was least significantly correlated with the other urinary components and appeared metabolically unrelated.

1. Introduction

Several small molecular weight aliphatic amines are known to be present invariably in human urine and all appear to have both endogenous and exogenous origins. Despite their detection many decades ago, their basic physiological significance and possible pathological associations are still under question, although some have been shown to influence cell growth [1,2]. A significant role in the central nervous system disturbances that are observed during renal and hepatic dysfunction has been proposed for these amines, especially when the blood-brain barrier also is compromised [3,4]. This is a reasonable assumption, because owing to their low molecular weight, ease of solubility in both aqueous and lipid environments, and their electron-rich amino groupings, these molecules are able to readily access the brain and spinal tissues and interfere with neurologic function.

Nevertheless, the relationships between these amines are not known and the relative importance of metabolic suggestions based on knowledge of chemical structures is uncertain, especially in humans. In the present study, mathematical analysis of data concerning the daily urinary excretion of 4 of these aliphatic amines (methylamine, dimethylamine, trimethylamine, and ethylamine) and 1 related *N*-oxide (trimethylamine *N*-oxide) from a large healthy population has provided insights into the interrelationships of these materials. The advantage of analyzing parallel data sets is also evident, and the use of such mathematical approaches may help to render transparent relationships that are otherwise unclear.

2. Subjects and methods

2.1. Volunteers and urine collection

A total of 203 randomly selected healthy volunteers (102 men, age, 22.2 ± 4.5 years [mean ± SD]; range, 19-47 years; 101 women, age, 21.6 ± 5 years; range, 19-48 years) were recruited from the staff and students of Imperial College Medical School (St Mary's) London. All participants gave full informed consent before entering the study and appropriate ethical approval was obtained from the local ethics committee. Subjects were maintained on their normal diet, which they recorded on an individual diet sheet. A complete 0- to 24-hour urine sample was collected into a plastic container to which hydrochloric acid (6 mol/L, 15 mL) had been added to prevent microbial growth and to maintain the amines as their water-soluble hydrochloride salts. The total urine volume was recorded and multiple

* Corresponding author. Tel.: +44 0207 594 3180.
E-mail address: s.c.mitchell@ic.ac.uk (S.C. Mitchell).

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Table 1
Statistical values and correlation coefficients (product moment r, Spearman rank R) obtained from urinary (0-24 hours) data measured in 203 healthy volunteers

	MMA	DMA	TMA	EA	TMAO
	(μmol/24 hours)				
Statistical values					
Mean	354.1	389.8	30.6	181.3	926.2
SD	262.9	261.3	42.9	167.6	2052.3
Minimum	54.2	15.1	0.4	4.9	22.3
Maximum	2007.4	2427.5	235.8	1029.1	16368.4
Median	268.1	347.2	15.3	118.9	370.7
Kurtosis	8.8	23.6	7.3	3.8	35.0
Skewness	2.4	3.9	2.7	1.8	5.6
Correlation coefficients	Product moment correlation coefficient (r)				
MMA	–	0.396[a]	0.327[a]	0.112	0.180
DMA	0.488[a]	–	0.319[a]	0.147	0.589[a]
TMA	0.422[a]	0.329[a]	–	0.003	0.524[a]
EA	0.227	0.130	0.115	–	0.097
TMAO	0.383[a]	0.568[a]	0.631[a]	0.197	–
	Spearman rank correlation coefficient (R)				

MMA indicates methylamine; DMA, dimethylamine; TMA, trimethylamine; EA, ethylamine; TMAO, trimethylamine N-oxide.

[a] With a sample size of n = 203, a value for r of 0.14 is the lower limit where the coefficient of correlation is only about twice (2.03) that of its standard error and the level of significance (from zero) is doubtful ($P \approx 5\%$). A value for r of 0.23 is where the coefficient of correlation is 3.5 times its standard error and is regarded as being significantly different from zero ($P \approx .1\%$). Values in between (0.14-0.23) have a possible significance.

aliquots (25 mL) were stored in the dark at −20°C until analysis for their methylamine, dimethylamine, trimethylamine, trimethylamine N-oxide, and ethylamine content, which was carried out as soon as possible [5,6].

2.2. Urine analysis

Thawed urine (5 mL) spiked with 0.2% (vol/vol) isopropylamine (30 μL, 20.8 μg; Sigma-Aldrich, Dorset, UK) as internal standard was placed into a screw-capped glass vial (15 mL) and pelleted potassium hydroxide (2 g) was added before sealing with an airtight polytetrafluoroethylene-lined septum cap and leaving on ice to cool. The vial was then vortex mixed and heated at 90°C for 20 minutes in an aluminum heating block, after which an aliquot (2 mL) of the generated headspace gas was injected directly onto the analytical column of a gas chromatograph. Trimethylamine N-oxide was quantified as an increase in trimethylamine after reduction of the sample (2 mL) with aqueous titanous chloride (0.2 mL, 30% [wt/vol] aqueous HCl) in a vial at 30°C for 30 minutes. Reduced samples were then diluted with water and analyzed as above. The use of authentic amine hydrochlorides and trimethylamine N-oxide dihydrate (Sigma-Aldrich) added to distilled water and to urine enabled the construction of calibration curves (0.1-150 μg/mL), which enabled the quantification of endogenous materials. All analyses were undertaken in duplicate, urine samples with a high amine concentration being diluted before analysis as appropriate [6].

Gas chromatography was performed on a Pye Unicam 4500 series gas chromatograph (Pye Unicam, Cambridge, UK) with a flame ionization detector. The silanized glass column (170 cm × 4 mm ID) was packed with 4% (wt/wt) Carbowax 20M and 0.8% (wt/wt) potassium hydroxide on Carbopack B (60-80 mesh) graphitized support (Supelco, Philadelphia, PA). The operating temperatures of the column, injection port, and detector unit were 70°C isothermal, 150°C, and 200°C, respectively, with a nitrogen carrier gas flow rate of 60 mL/min [6]. Under these conditions, the observed retention times (and relative retention times [rrt] compared with the internal standard, isopropylamine) for the various amines were as follows: methylamine, 0.9 minutes (rrt 0.23); dimethylamine, 1.6 minutes (rrt 0.42); ethylamine, 1.8 minutes (rrt 0.47); trimethylamine, 2.59 (rrt 0.65); isopropylamine, 3.9 minutes (rrt 1.0).

3. Results

Each measured component was individually examined for linear association with each of the other 4 urinary compounds by scatterplotting and mathematical calculation of the correlation coefficients (product-moment correlation coefficient, Spearman rank correlation coefficient) (Table 1). The results from the population data revealed that ethylamine was the outlier, displaying least significant association with any of the other substances. The remaining 4 compounds all exhibited a significant tendency to be grouped in a direct or positive linear association (the lack of significance between methylamine and trimethylamine N-oxide disappeared when the data were ranked). However, although statistically significant, the actual numerical values (greater than zero) of the correlation coefficients were small,

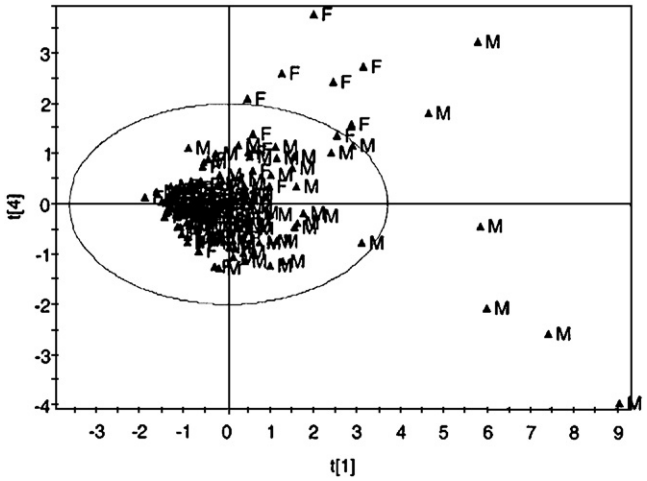

Fig. 1. PC1 (t[1]) vs PC4 (t[4]) scores plot of mean-centered urinary data ± SD ellipse for the 203 human volunteers, illustrating partial separation with an outlying group (outside centroid) of men (▲M; n = 6) toward higher PC1 values and an outlying group of women (▲F; n = 6) toward higher PC4 values. PCA is an unsupervised pattern recognition method for multivariate data, independent of any knowledge of class membership. PCA reduces the urinary data X to a set of orthogonal principal components describing the main directions of variation within X. Each principal component is attached to an "eigenvalue" λ, summarizing the amount of variance explained, and a dual set of values: the "scores" t, describing the coordinates of the individuals in the orthogonal basis, and the "loadings" p or "eigenvectors" highlighting the influence of input features (amine data variables) used to build the orthogonal basis.

indicating that although the parameters tended to drift in the same direction there were still considerable independent elements present.

Some relationships only become apparent when several variables are examined at the same time, as the complete interactive picture is not always evident from the sum of its dissected constituent parts. One suitable mathematical visualization method that is useful for overviewing relationships or clusters within multivariate data is principle component analysis (PCA) [7].

Briefly, the data are represented in K-dimensional space (where K is equal to the number of variables) and subsequently reduced from a multidimensional array to a few principal components (descriptive dimensions) that can be readily understood and are able to describe the maximum variation present within the original data. Graphical representations use the first few principal components as axes and the resulting "score plots" display any patterns or clusters within the data; any separation from the central control region can be useful in discerning outliers [8,9].

Its application to the present data produced a score plot in which the majority (191/203, 94.1%) of the data points lay within the centroid (mean ± SD) and 2 outlying clusters were evident (Fig. 1). One cluster of 6 male subjects was displaced (to higher PC1 values) from the main group, and the factors that had teased them away were collectively higher numerical values for all of the compounds measured. Closer examination of the original data for these 6 selected male subjects showed that, as a subgroup, their excretion values for everything except ethylamine were significantly greater ($P < .5\%$, Student t test) than those observed for the entire population (methylamine, population, 354.1 ± 262.9 μmol/24 hours [n = 203], male outliers, 712.9 ± 358.1 μmol/24 hours [n = 6]; dimethylamine, 389.8 ± 261.3, 1360.0 ± 800.0; trimethylamine, 30.6 ± 42.9, 145.8 ± 64.4; trimethylamine N-oxide, 926.2 ± 2052.3, 7901.3 ± 5748.0). The second cluster (to higher PC4 values) comprised 6 female subjects with the suggestion that levels of trimethylamine were higher and those of trimethylamine N-oxide were lower than the majority. Inspection of the original data for these 6 female subjects could only confirm, as a subgroup, significantly raised trimethylamine levels; the values for trimethylamine N-oxide were not significantly different owing to the large variation observed (trimethylamine population, 30.6 ± 42.9 μmol/24 hours (n = 203); female outliers, 179.7 ± 30.5 μmol/24 hours (n = 6); $P < .5\%$, Student t test).

4. Discussion

Many previous metabolic studies in humans have been seriously hampered by the unknown and variable dilution of any administered amine dose with that co-ingested in food, produced by gut microflora or by intermediary metabolism. However, the few investigations in humans using radiolabeled materials have shown that dimethylamine is excreted mainly unchanged with a small proportion (~5%) undergoing demethylation to methylamine [10]. Trimethylamine is usually converted to its N-oxide (~90+%) with the remainder being voided unchanged [11], although some reports have intimated at demethylation to produce trace amounts of dimethylamine [12]. Trimethylamine N-oxide is excreted unchanged [11], although at higher dose levels (up to 2.25 g) a proportion (up to 15%) may be recovered as dimethylamine [13]. There are no investigations reported in the literature where either radiolabeled methylamine or ethylamine has been examined in humans. However, early studies in humans suggest that ethylamine may be converted to ethylurea and other small molecules [14].

In accord, the correlation coefficients calculated for the present population data suggest several interrelationships between the above amines and N-oxide. Ethylamine is the incongruous member within the group of compounds, and it is perhaps metabolically ill-founded to suggest that the ethyl moiety would be split to produce methylamine or that methylamine would be further methylated to yield ethylamine as opposed to dimethylamine. The correlation between trimethylamine N-oxide and dimethylamine ($r = 0.589$, $R = 0.568$) was greater than that between trimethylamine and dimethylamine ($r = 0.318$, $R = 0.329$) indicating an alternative pathway from the N-oxide to dimethylamine that was not through the intermediate, trimethylamine, such as the previously suggested direct removal of formaldehyde [15].

Such a reaction has been observed in the soil bacteria *Bacillus PM6* and *Pseudomonas aminovorans* [16,17] and in fish muscle where the enzymatic nature has been investigated tentatively [18-20]. Contrariwise, it has been suggested that this type of reaction may be catalyzed by nonspecific macromolecules such as hemoglobin, cysteine residues, or even reduced metal ions [21,22]. For tertiary amine oxides with no β-hydrogen, migration of an alkyl group from the nitrogen to the oxygen may occur on heating to yield *O*,*N*,*N*-trisubstituted hydroxylamines (Meisenheimer rearrangement) [23]. This rearrangement has been shown to be predominantly intramolecular [24,25], although free radical or ion pair mechanisms cannot be discounted [26,27]. During the production of aldehydes and secondary amines from aliphatic amine oxides (via acylation with acetic anhydride or acetyl chloride) transfer of oxygen from the initial nitrogen to the adjacent carbon occurs (Polonovski reaction) [28]. In addition, reaction with sulfur dioxide converts amine oxide into a zwitterionic sulfitoamine that then undergoes hydrolytic decomposition [29,30]. All such events suggest potentially reactive *N*-oxide configurations prone to intramolecular migration and rearrangements.

The causation of the outlying male cluster from the population data is explained easily by suggesting that the 6 subjects ate larger amounts of foods containing these substances. Examination of their diet sheets provided confirmation, as this group of students had ingested a large meal of fish during the evening of the urine collection period. The failure of ethylamine to match the significant increases observed for the other 4 compounds alludes to the metabolically unrelated nature of ethylamine and, indeed, its comparatively low levels in fish [31].

The reason for the outlying cluster of female subjects, who only displayed a significant increase in trimethylamine levels, is more difficult to explain. The percentage of trimethylamine-related material (trimethylamine plus trimethylamine *N*-oxide) excreted as *N*-oxide in these 6 female subjects was 82.1% $\pm$ 19.0% with a comparable value of 97.8% $\pm$ 2.1% for the male outliers; in the majority of the population this value is greater than 90% [5,6]. By comparing the female/male ratios for the amounts of trimethylamine and trimethylamine *N*-oxide excreted by the entire population (101 women, 102 men) with those obtained for the 6 female subjects in the outlying group (6 women, 102 men), it became apparent that the trimethylamine ratio for the female outliers was higher (outliers, 1.25; population, 0.67) and that the trimethylamine *N*-oxide ratio was lower (outliers, 0.24; population, 0.59). This refining of mathematical comparison suggested that the outlying female cluster was producing relatively more trimethylamine and less *N*-oxide when compared with the overall female cohort, and it follows that this relative excess of trimethylamine may have occurred owing to its decreased conversion to the *N*-oxide. The activity of the flavin monooxygenase system undertaking this N-oxidation is known to be influenced by testosterone levels in rat and mouse [32,33], and the isoenzyme responsible in humans (flavin monooxygenase 3) has been reported to be under the influence of steroid hormones [34-36]. In pregnancy, the activity of flavin monoxygenase 3 appears to be induced [37], and healthy women around menstruation have been shown to exhibit decreased trimethylamine N-oxidation [36,38]. Further questioning revealed that all of these 6 female subjects within the outlying cluster were approaching or had entered the menstrual phase of their cycle. Subsequent examination of their urine samples showed that this feature was not due to blood contamination.

The advantage of statistical analyses of parallel data sets and the use of cluster analysis with its inherent capability in offsetting the influences of compounding variables is evident within this study. The ability to separate and identify subgroups with characteristics that differ from the majority lessens previous stringent requirements on test subjects, such as maintaining populations on a constant diet or hormonal cycle. Provided the consequences of such variations are appreciated, multivariate analyses can provide valuable insights, such as with suggested global metabonomic analyses of body fluids [39].

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