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Application of gas chromatography–mass spectrometry with selected ion monitoring to the urinanalysis of 4-pyridoxic acid

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ABSTRACT

An analytical protocol has been developed for the analysis of urinary 4-pyridoxic acid (4-PA) by gas chromatography–mass spectrometry (GC–MS) for use in metabolic studies. Aliquots of urine were deproteinised and fractionated by isocratic reversed-phase high-performance liquid chromatography. The eluent fraction containing the 4-PA was collected, freeze-dried and silylated using N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide. Derivatisation produced the mono-*tert.*-butyldimethylsilyl derivative of 4-PA lactone. This derivative was readily amenable to GC–MS analysis in the electron ionisation (70 eV) mode, yielding a prominent fragment ion at *m/z* 222 ($[M - 57]^+$; base peak). A heavy isotope-labelled derivative of pyridoxine [dideuteriated pyridoxine; 3-hydroxy-4-(hydroxymethyl)-5-[hydroxymethyl-$^2H_2$]-2-methylpyridine] has been synthesised and is being employed to determine the kinetics of labelling of the body pools of vitamin B_6. Kinetic measurements are based on the determination of the relative proportions of metabolically produced deuterium-labelled and non-labelled 4-PA in urine, obtained from stable isotope ratios determined by low-resolution selected ion monitoring using a bench-top quadrupole GC–MS system.

INTRODUCTION

Vitamin B_6 comprises pyridoxine, pyridoxal, pyridoxamine and the 5′-phosphate esters of these vitamer forms. 4-Pyridoxic acid (4-PA) is a metabolically irrecoverable form of vitamin B_6 and is the principal urinary excretion product of vitamin B_6 metabolism. Urinary 4-PA excretion is considered to be a short-term indicator of vitamin B_6 status because the excretion of 4-PA changes rapidly in response to a change in vitamin B_6 intake. Moreover, if a defined diet is consumed, urinary 4-PA excretion can provide valuable information on the long-term vitamin B_6 status [1]. In controlled studies with adult humans, urinary excretion of 4-PA reflects vitamin B_6 intake [2,3] and accounts for 91–98% of excreted vitamin B_6 [4]. Measurement of urinary

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4-PA can therefore be used to assess vitamin B_6 nutritional status.

Previous studies on the kinetics of vitamin B_6 assimilation following a single oral or subcutaneous dose of radiolabelled pyridoxine yielded multiphasic labelling curves [5]. A simple metabolic model for the overall metabolism of pyridoxine was proposed in which a large pool with slow turnover was in equilibrium with a small pool with rapid turnover with additions and losses only occurring from the small pool [5]. From this model the elimination rate of vitamin B_6 from the body reservoir could be calculated and the total body vitamin B_6 pool could be estimated [5]. Muscle contains 70–80% of the vitamin B_6 in the body [6], the majority of which is bound to glycogen phosphorylase (EC 2.4.1.1). Pyridoxal phosphate (PLP) is an essential cofactor of this enzyme and in mouse muscle 75–96% of vitamin B_6 is bound to phosphorylase in this vitamer form [7]. The buried hydrophobic environment of the cofactor [8] suggests that it would only be released when the enzyme is degraded. We have postulated that the large, slowly exchanging pool is the vitamin B_6 associated as PLP with muscle glycogen phosphorylase.

The low rate of exchange and the size of the muscle vitamin B_6 pool isolate this pool in kinetic terms. We have administered ^{3}H-labelled pyridoxine to mice and have monitored the decay of muscle phosphorylase-bound label as a measure of the rate of degradation of the enzyme [9–12]. As our research is now focussing on human subjects we are attempting to develop methodologies using non-radioactive labels and non-invasive techniques such as urinanalysis to measure the turnover of glycogen phosphorylase. The key feature of the analysis is the use of selected ion monitoring (SIM) to measure the isotope ratio of the deuteriated to non-deuteriated 4-PA. The change in isotope ratio with time permits calculation of the kinetics of labelling of glycogen phosphorylase. The methodology can be used to measure 4-PA concentration in urine in conjunction with an internal standard. We present here a protocol for the urinanalysis of 4-PA in urine that is directly applicable to studies using stable isotope derivatives of vitamin B_6.

EXPERIMENTAL

Preparation of dideuteriated pyridoxine

Dideuteriated pyridoxine ([$^2H_2$]PN) was synthesised essentially according to the method described by Coburn *et al.* [13]. The oxidation step for the conversion of 4,3-O-isopropylidenepyridoxine hydrochloride to isopropylidene-5-pyridoxic acid hydrochloride was only partially successful (as determined by mass spectrometry); the mass spectrum indicated the presence of a significant amount of the aldehyde intermediate. A homogeneous product was ensured by a second oxidation. The precipitate from the first oxidation was re-dissolved in water by adjusting to pH 7

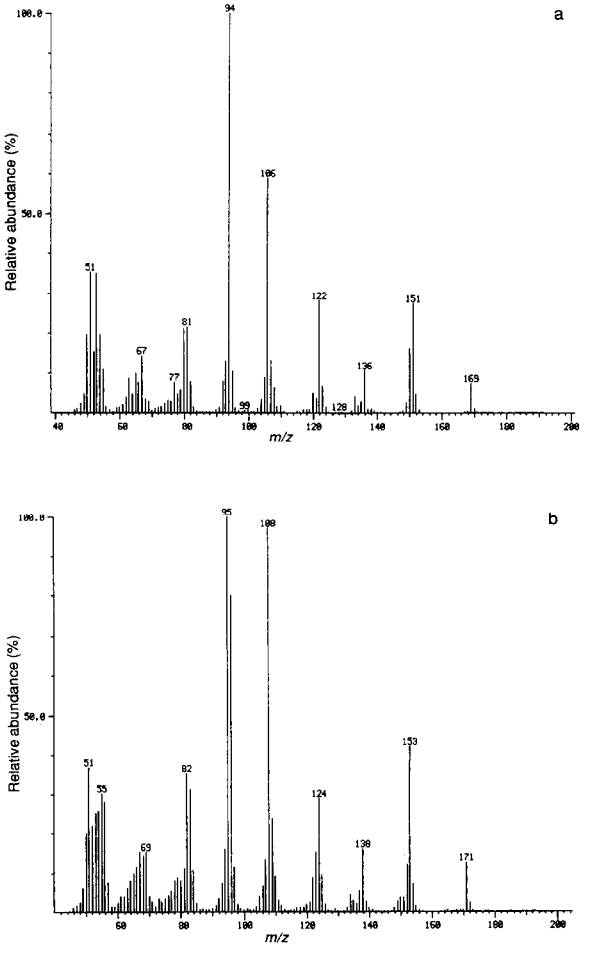

Fig. 1. Direct insertion probe electron ionisation (70 eV) mass spectra of (a) authentic pyridoxine and (b) the dideuteriated pyridoxine synthesised for this work.

with 10 *M* NaOH. After addition of a saturated solution of $KMnO_4$ the pH was adjusted to pH 5.5 for oxidation to occur. After stirring the solution for 15 min the pH was raised to 7.3 to precipitate manganese dioxide. After stirring for a further 15 min the fine precipitate was collected on a 0.45-μm nitrocellulose filter, the filtrate was concentrated *in vacuo* and the pH adjusted to 5.3 with 10 *M* HCl to precipitate isopropylidene-5-pyridoxic acid hydrochloride. The solid was filtered and lyophilised with a yield of 64%. The re-oxidation step resulted in no significant loss of product and increased the isotopic purity of the desired dideuteriated pyridoxine compared to that reported by Coburn *et al.* [13]. The isotopic composition following $LiAl(^2H)_4$ reduction was calculated from the electron-impact (EI) mass spectrum: $^2H_0$ 0.6%, $^2H_1$ none detected, $^2H_2$ 96.5%, $^2H_3$ 2.1%. Fig. 1 shows the EI (70 eV) direct insertion probe mass spectra for authentic pyridoxine (a) and the dideuteriated pyridoxine (b) prepared as described above.

Experimental animals

Inbred adult BALB/C male mice were obtained from the department animal unit. Animals were maintained on a 12 h light–12 h dark cycle and allowed free access to food and water. Dideuteriated pyridoxine hydrochloride synthesised as described above was dissolved in autoclaved tap water (to prevent microbial utilisation of the vitamer) at 100 μ*M*, supplying each animal with between 80 and 100 μg of $[^2H_2]$PN per day depending on water consumption. Water was changed daily. The animals also received unlabelled pyridoxine (15 μg/kg) from the pelleted diet. Urine collected by applying very gentle pressure to the animal's bladder was stored at –20°C (4-PA was stable during storage under these conditions). This method of urine collection causes minimal discomfort to the animal.

Reversed-phase high-performance liquid chromatography (HPLC) of mouse urine

Reversed-phase HPLC was performed on a 125 mm × 4 mm I.D. column packed with 3-μm Spherisorb ODS-2 (Pharmacia, Milton Keynes, UK). An LKB Model 2150 HPLC pump programmed via an LKB Model 2150 controller was used to deliver a mobile phase comprising a formate buffer pH 2.2 (approximately 20 m*M*) containing 7.5% methanol (buffer A) and a 60% solution of acetonitrile (buffer B). Solvents were maintained under helium with an LKB Model 2156 solvent conditioner. Urine samples were chromatographed using 100% A for 10 min, 100% B at 15 min, and between 20 and 30 min 0–100% A. Mouse urine (60 μl) was diluted with buffer A and urinary proteins were precipitated overnight at −20°C by the addition of 12.5 μl of 40% (w/v) trichloroacetic acid (TCA) in a final volume of 100 μl. Overnight incubation was necessary for the slow precipitation of protein. Precipitated material was pelleted by centrifugation and 80 μl of the supernatant fraction were diluted with 160 μl of buffer A; 100–220 μl of this mixture were injected direclty onto the HPLC column. The column eluate was monitored at 315 nm using an LKB Model 2141 monitor; the absorbance of urinary 4-PA was maximal at this wavelength. Urinary 4-PA, which elutes isocratically in buffer A at approximately 7 min, was collected directly into siliconised reaction vials. The eluate (~0.7 ml) was freeze-dried and derivatised for gas chromatographic–mass spectrometric (GC–MS) analysis.

Derivatisation of urinary 4-PA for GC–MS analysis

tert.-Butyldimethylsilyl (TBDMS) ether derivative. Authentic 4-PA, or the urine fractions isolated by HPLC, was treated with 50 μl of N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA; Phase Separations, Deeside, UK) and 50 μl of pyridine (BDH sequenal grade) in the siliconised reaction vials used above. The reaction vial was tightly capped and heated at 100°C for 45 min. The derivatised samples were transferred to autosampler vials fitted with 0.1-ml glass inserts, and the vials sealed with crimp caps.

Trimethylsilyl (TMS) ether derivative. Trimethylsilylation of authentic 4-PA (microgram amounts) was performed by reaction (100°C, 1 h)

with 50 μl of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% (v/v) trimethylchlorosilane (50 μl; Pierce, Chester, UK) and 50 μl of pyridine (BDH sequenal grade). The samples were diluted with hexane for GC–MS analysis.

GC–MS analysis of 4-PA

GC–MS analysis of 4-PA-TBDMS were performed on a Hewlett Packard 5890 series II gas chromatograph interfaced with a HP 5971A mass-selective detector. Operation of the GC–MS system was under the control of an HP 59970 MS Chemstation. Automatic tuning of the mass spectrometer, using perfluorobutylamine as calibrant, was carried out each day before proceeding with sample analyses. Derivatised samples were injected on-column using a Hewlett Packard 7673A automatic injector. The analytical column was 12 m × 0.2 mm I.D. BP-1 coated flexible fused-silica polyimide-clad capillary (immobilised dimethylpolysiloxane; 0.25 μm film thickness, S.G.E., Milton Keynes, UK). A "retention gap" consisting of a length of deactivated flexible fused-silica capillary tubing (0.5 m × 0.53 mm I.D., Phase Separations) was connected between the injection port and the analytical column. As well as permitting automated cool on-column injections the retention gap served as a pre-column and extended the life of the analytical column. Helium was the carrier gas with a column head pressure of 0.55 bar being maintained to give a flow-rate of *ca.* 1 ml/min into the mass-selective detector. The GC oven temperature was maintained at 50°C for injection, then programmed rapidly (50°C/min) to 100°C, then at 10°C/min to 250°C. An ionisation potential of 70 eV was maintained throughout. The ion source temperature was maintained at around 180°C. In SIM analyses ions at *m/z* 222 and 224 were monitored for 4-PA and dideuteriated 4-PA, respectively, employing a dwell time of 100 ms per ion and 3.7 cycles/s (automatically estimated from the parameters entered in the SIM program software).

RESULTS AND DISCUSSION

Isolation of urinary 4-PA

A method for the simultaneous analysis of B_6 vitamers in biological samples by isotope dilution mass spectrometry (IDMS) has been described by Hachey *et al.* [14]. The complex procedure that was described used four chromatographic purification steps to resolve the six vitamers found in biological samples. This method was described as necessary to minimise solid residue remaining in the final stages of the isolation procedure. In contrast to the requirements of this latter study, our investigations demanded a simplified analytical protocol compatable with a relatively high throughput of urine samples requiring measurements of the ratio of deuteriated/non-deuteriated 4-PA. As such, our protocol has been optimised for 4-PA, but not for the other vitamers.

The method involves deproteinisation of urine samples followed by a single reversed-phase HPLC purification step prior to GC–MS analysis. A range of mobile phase compositions were

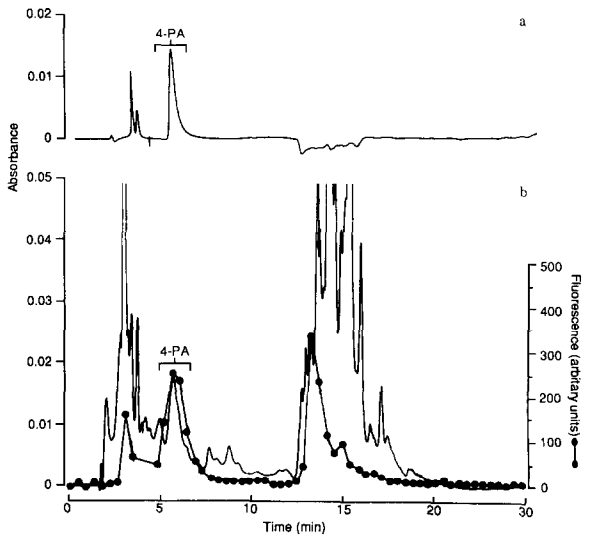

Fig. 2. Reversed-phase HPLC chromatogram obtained from the analysis of (a) authentic 4-PA prepared and chromatographed in the same manner as urine samples and (b) deproteinised mouse urine employing both UV absorbance detection at 315 nm and fluorescence detection. Column eluate passed through a flow cell where UV absorbance at 315 nm was measured and subsequently traced onto chart paper. From the flow cell the eluate was collected into fractions using an LKB Helirac. Fractions were collected at 30-s intervals and 60 fractions of approximately 250 μl were collected. The fluorescence of each fraction was measured using excitation and emission wavelengths of 315 and 425 nm, respectively, in a Perkin Elmer 3000 fluorescence spectrometer. 4-PA elutes isocratically in the first 10 min of the gradient following this the column is regenerated with 60% acetonitrile.

investigated. Mobile phases buffered with organic acids were found to be more suitable for our application since inorganic acids (particularly phosphoric acid, which has been used in several HPLC methods of 4-PA analysis [15–18]) produced interfering peaks in the total ion chromatograms in the subsequent GC–MS analyses of 4-PA.

Fig. 2b shows the reversed-phase HPLC chromatogram for the TCA-soluble fraction of a mouse urine sample obtained using a mobile phase of aqueous formate buffer, pH 2.2, containing 7.5% (v/v) methanol, with UV detection at 315 nm. The chromatogram is complex and shows a peak which elutes after 7 min and has a retention time congruent with that of authentic 4-PA, under identical HPLC conditions (Fig. 2a). Fractions from the urine sample were collected from the HPLC column at 30-s intervals and the fluorescence of each fraction measured using excitation and emission wavelengths of 315 nm and 425 nm, respectively. The peak of fluorescence at fractions 12 and 13 includes 4-PA, as identified by chromatography with authentic material, and by GC–MS after derivatisation. The peaks eluting prior to 4-PA in Fig. 2a are present in the TCA added to the sample. In routine urinanalyses simply collecting the HPLC fraction corresponding in retention time to authentic 4-PA, followed by freeze-drying to remove the solvent, yielded an isolate suitable for derivatisation and capillary GC–MS analysis.

A full recovery of 4-PA is obtained from HPLC as determined by chromatography of a series of authentic 4-PA standards, and also of a series of urine samples (collected from an individual animal) with increasing amounts of authentic 4-PA added. A linear response was obtained when fluorescence (corresponding to the 4-PA peak) was plotted against amount of 4-PA applied to HPLC or 4-PA added to urine samples ($r = 0.99$). The intercept value on the plot of spiked urines can be interpolated from the standard curve to give the amount of 4-PA originally in the sample. A small amount of 4-PA-lactone may be present in urine samples, however, in this HPLC system the lactone co-chromatographs with 4-PA so no losses occur through lactonisation.

Derivatisation of 4-PA

A range of derivatives were considered for the GC–MS analysis of 4-PA. Hachey *et al.* [14] prepared the acetate derivative by treatment of 4-PA with a pyridine–acetic anhydride mixture (1:1). The spectrum of the 4-PA-lactone acetate derivative is relatively complex being characterized by a large number of low- and high-mass fragment ions. The production of an array of fragment ions is an undesirable feature of the acetate derivatives in this instance as this would reduce absolute sensitivity in trace analyses employing SIM. A recent report [19] described the use of trifluoroacetyl derivatives of B_6 vitamers in conjunction with negative ion chemical ionisation. As chemical ionisation is renowned for problems of reproducibility it was decided to explore an alternative analytical strategy. Our preferred approach was to use trialkylsilylation prior to GC–MS analysis. Trimethylsilylation was investigated, but produced a mixture of derivatives of differing silylation state in spite of a range of derivatisation conditions that were tested. However, a recent report described the use of a TMS derivative for the analysis of covalently bound pyridoxal phosphate in enzymatic proteins [20].

In contrast, silylation of 4-PA with MTBSTFA using pyridine as solvent generated the mono-TBDMS derivative with simultaneous

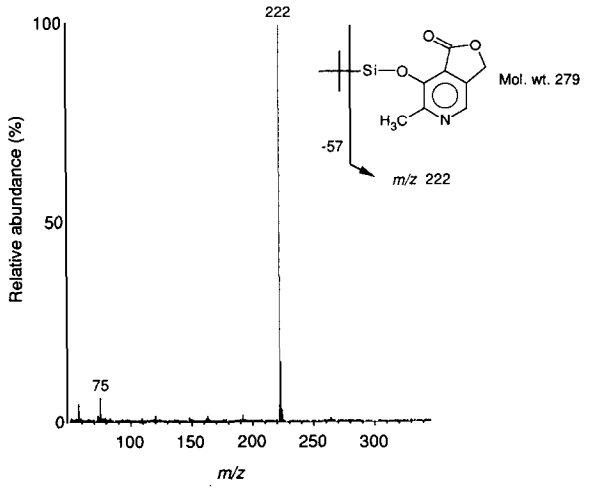

Fig. 3. EI (70 eV) mass spectrum of authentic TBDMS-4-PA-lactone obtained by GC–MS. The inset structure shows the origin of the major fragment ion.

formation of the 4-PA lactone. The electron ionisation mass spectrum of this compound displays a major fragment ion at *m/z* 222 corresponding to loss of the *tert.*-butyl radical, $[M - 57]^+$ (Fig. 3). This fragment ion carried the bulk of ion current and sub-nanogram sensitivities were routinely achieved in urinanalyses for 4-PA using SIM. Silylation using MTBSTFA appeared to proceed equally smoothly in the case of the authentic 4-PA and freeze-dried urine fractions collected from the HPLC column. As can be seen from the GC–MS ion chromatogram shown in Fig. 4a the TBDMS-4-PA-lactone displays excellent GC behaviour on the dimethylpolysiloxane-coated column employed in this study.

Automated analyses used on-column injection into a wide-bore (0.53 mm) deactivated fused-silica retention gap. Although no hexane-insoluble residue was apparent after derivatisation of the HPLC fractions peak tailing was found to develop after GC–MS analysis of *ca.* 70 samples. This presumably resulted from the accumulation of an involatile deposit, as optimal chromatographic performance was restored by renewing the retention gap. The lifetime of the analytical column was *ca.* 800 analyses of urine for 4-PA.

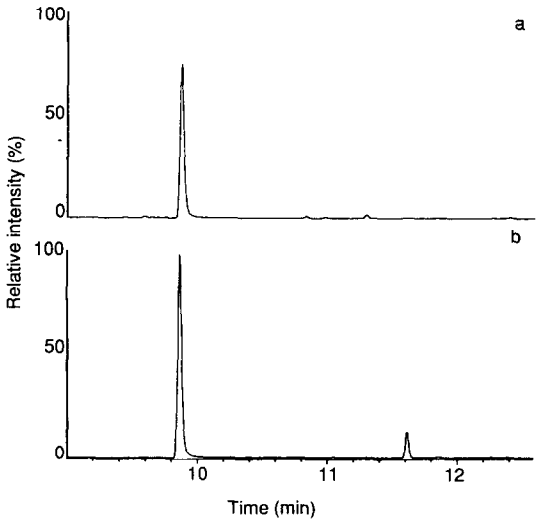

Fig. 4. Partial ion chromatograms obtained from SIM GC–MS analysis of the TBDMS-derivatised 4-PA peak collected from reversed-phase HPLC (see Fig. 2). SIM was at (a) *m/z* 222 and (b) *m/z* 224 corresponding to the major fragment ions of TBDMS-4-PA lactone and TBDMS-$[^2H_2]$4-PA lactone, respectively. The major peak in each of the ion chromatograms corresponds to the 4-PA derivative (see text for further discussion).

Metabolic studies

Fig. 4 shows ion chromatograms obtained by SIM GC–MS analysis of the urine fraction collected from a mouse that was administered an average of 80 μg of dideuteriated pyridoxine daily (two to three times calculated normal daily intake of PN) in drinking water. The ions at *m/z* 222 and 224 correspond to TBDMS-4-PA-lactone and TBDMS-$[^2H_2]$4-PA-lactone, respectively. As expected the deuteriated 4-PA derivative elutes slightly later than its unlabelled counterpart. The relative proportions of labelled and unlabelled metabolites in urine are determined by isotope ratio analysis based on the relative abundances of the *m/z* 222 and 224 responses in the ion chromatograms. As can be seen from Fig. 4 the relative abundances of labelled (*m/z* 224) has exceeded that of unlabelled (*m/z* 222) after ten days of administering the heavy isotope-labelled derivative. Animals that were only administered unlabelled PN showed no peak in the *m/z* 224 ion chromatogram (not shown). These results are in close agreement with those obtained using radiolabelled PN. Current research in this laboratory is now focussing on the use of these techniques to determine the kinetics of the "fast" and "slow" pools of vitamin B_6 in human subjects (results to be presented elsewhere).

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