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# Automated screening of urine samples for carbohydrates, organic and amino acids after treatment with urease[a]

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ABSTRACT

Eighty-five clinical urine samples and nineteen urine samples previously found by other laboratories to suggest genetic metabolic defects were prepared for trimethylsilylation by treatment with urease, followed by azeotropic dehydration. The "Target Analyte Search" program provided with the VG Trio 2 gas chromatograph–mass spectrometer required 6 min to quantify 103 compounds relative to endogenous urinary creatinine. This technique has been used to confirm diagnoses including cystinuria, lysinuria, medium-chain acyldehydrogenase deficiency, ornithine transcarbamylase deficiency, aspartylglucosaminuria, methylmalonic, propionic and glutaric acidurias.

## INTRODUCTION

Silylating agents including trimethylsilyl (TMS) and *tert.*-butyldimethylsilyl donors can stabilize a wide variety of compounds for volatilization during gas chromatography (GC) [1]. However, organic extraction of biological fluids is generally directed to a single hydrophilic or hydrophobic moicty: carboxylic acids, amines or hydrophobic groups, for example [2]. Column procedures using various solid phases [3,4] have been used to clean-up urinc and amniotic fluid prior to derivatization. Solvent extraction is cumbersome and time-consuming and column clean-up procedures cannot be used if the chemical nature of the compounds of interest is unknown or unanticipated. We now describe a method for preparing metabolites of urine or amniotic fluid for derivitization without solvent extraction or column clean-up. The method depends on the use of the enzyme urease to remove urea, the major organic constituent from the samples, which then renders the minor components accessible to the derivatizing agent. The number of metabolites detectable by this method is large enough to require

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highly automated interpretation of the mass spectral data and depends on methods developed by Horning and Horning [5] and Jellum *et al.* [6]. Single ion chromatograms [7] must be used to resolve overlapping peaks. Rapid confirmation of peak identities requires a condensed library of compounds of interest such as that published by Markey *et al.* [8]. Our procedure included two steps: first, during the GC run, the mass spectra of total ion current (TIC) peaks were matched against a library of 1700 mass spectra (TMS derivatives from the 42 000-entry NBS library plus compounds we have derivatized). Second, after the GC run, 130 target peaks (from 103 compounds) were identified by retention time and their eight major mass spectral peaks, then quantified by single ion chromatograms. This program required approximately 6 min after the GC run of 67 min or 1900 scans.

## EXPERIMENTAL

### *Materials*

Authentic standards of the compounds listed in Table I, crystalline urease (Type C-3) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Sigma (St. Louis, MO, U.S.A.). Carbon dioxide, ultra-purity helium and nitrogen gasses were purchased from Matheson (Joliet, IL, U.S.A.). Trideuterated creatine [(methyl)-$d_3$-creatine] was purchased from MSD Isotopes (Montreal, Canada). All solvents were purchased from Fisher (Fairlawn, NJ, U.S.A.) and were HPLC grade. Hydrogen sulfide gas was produced from sodium sulfide by addition of concentrated sulfuric acid. Unassembled disposable syringes were purchased from Crown Distributing (Bridgeton, MO, U.S.A.). Anotop 0.2-$\mu$m filters were purchased from Alltech Assoc. (Deerfield, IL, U.S.A.).

### *Urine samples*

Over a six-month period, 85 urine samples were received from a local pediatric hospital. Prior to this, urine samples from 19 previously confirmed cases of genetic metabolic abnormalities were provided by other laboratories. The diagnoses, confirmed by our procedure, included: methylmalonic aciduria (three), dicarboxylic aciduria (five) two of which were due to medium-chain acyl dehydrogenase deficiency, propionic, glutaric (two) and hydroxymethylglutaric aciduria, ornithine transcarbamylase deficiency, lysinuric protein intolerance (two), glycinuria, maple syrup urine disease, isovaleric aciduria and aspartyl-glucosaminuria. Urine was kept at $-20$°C until processed.

### *Urease treatment*

After defrosting the urine sample at 37°C, an aliquot of 0.5–1.0 ml was transferred to a 2-ml vial by passage through a 0.20-$\mu$m Anotop filter using a glycerol-free disposable syringe. To this were added 2 $\mu$mol/ml trideuterated creatine as a 0.1 *M* aqueous solution. The vial was capped with a siliconized rubber septum. Carbon dioxide gas was injected into the vial with a needle through the septum

and the cap was tightened maintaining positive $CO_2$ pressure. The gas was allowed to equilibrate for 5 min, then the vial was uncapped. Approximately 125 U/ml of urine (1–2 crystals per sample) of urease were added and mixed by vortexing. The vial was immediately recapped, $CO_2$-positive pressure again established and the vial maintained at 37°C for 30 min. If concavity of the septum indicated negative internal pressure, $CO_2$ was again added as needed. After 30 min, another 125 U of urease per ml of urine were added, the vial was recapped and the $CO_2$ pressure established. After 15 min, the vial was uncapped, acetone was added to a concentration of 50% and the vial was chilled in an ice bath for 15 min to precipitate urease and other proteins.

The sample was again passed through a 0.20 μm filter into a 2.0-ml vial. Acetonitrile was added to the top of the vial and 40 μl of triethylammonium trifluoroacetate, a clear liquid, were added. The solution was concentrated under a nitrogen stream at 70°C. When the volume reached 50–70% of starting volume, acetonitrile was again used to 'top-off' the samples. As precipitate began to appear, methylene chloride was added to remove the last traces of water as an azeotropic mixture under the nitrogen stream at 70°C.

A volume of MSTFA equivalent to 25–100% of the starting volume of the urine sample was added to the residue. Derivatization proceeded under nitrogen at 70°C for 1 h. A 1–2 μl volume of supernatant was injected into the gas chromatograph, using splitless mode and a valve time of 2.5 min. The temperature was held at 80°C for 1 min, then programmed to increase from 80 to 130°C at 2°C/min, 130 to 200°C at 3°C/min and 200 to 280°C at 6°C/min, then held at 280°C for 10 min. The column was a 30 m × 0.32 mm I.D. OV-5 capillary, with a coating thickness 0.5 μm, purchased from Ohio Valley Specialty Chemical (Marietta, OH, U.S.A.). The carrier gas was ultra-purity helium kept at a head pressure of 1 bar.

*Mass spectrometry and automated searching*

The VG Trio 2 gas chromatograph–mass spectrometer–computer (VG Masslab, Altrincham, U.K.) was used for the analyses. Spectra were obtained in positive electron-impact ($EI^+$) mode by scanning from 50 to 800 $m/z$ in 1.95 s with an interscan interval of 0.05 s. A custom program started the GC run and after 4 min began to identify scan numbers associated with TIC peaks, calculate their areas and find the best match for the scan within a custom library of 1700 TMS derivatives. The library was comprised of TMS derivatives from the 42000-entry NBS library of mass spectra supplied by VG Masslab, plus 200 additional compounds we have derivatized. When the program had found the best library match for the first batch of compounds, the rest of the TIC chromatogram acquired up to that point was analyzed and the areas and identification data were added to the previous list. Generally, the 50–60 largest peaks were identified by the end of the 67 min GC run. A proprietary program supplied by the manufacturer was then used to search for 'target' compounds and quantify them. The data used by the pro-

TABLE I

INFORMATION REQUIRED FOR AUTOMATED QUANTIFICATION OF 103 COMPOUNDS FROM URINE SAMPLES

Missing row numbers are due to removal of duplicate information supplied in the program for cases in which double peaks may occur. The quantification ion shown is not necessarily the ion of highest intensity, rather it is a fragment ion unique to the target compound within the vicinity of its elution. It is the ion used to create the single ion chromatogram for quantification, shown graphically in Fig. 2B. RF = Response factor (molar) of the quantification ion relative to mass 118 in $d_3$-creatinine, the internal standard. RRT = Relative retention time compared to retention time standard 1 ($d_3$-creatinine) or 2 (pseudouridine).

| Row No. | Target compound[a] | RRT | RF | Mass:intensity (*versus* 1000) of quantification ion |
|---|---|---|---|---|
| 1 | $d_3$-Creatinine $TMS_3$ | 1.000 | 1.000 | 118:1000 |
| 3 | Lactic acid $TMS_2$ | 0.194 | 0.152 | 191:162 |
| 4 | Hexanoic acid TMS | 0.200 | 0.701 | 173:652 |
| 5 | Glycolic acid $TMS_2$ | 0.207 | 0.095 | 205:60 |
| 6 | Pyruvic acid $TMS_2$ | 0.219 | 0.049 | 217:162 |
| 7 | Alanine $TMS_2$ | 0.237 | 1.089 | 116:1000 |
| 8 | Glycine $TMS_2$ | 0.256 | 1.058 | 102:1000 |
| 9 | Oxalic acid $TMS_2$ | 0.269 | 0.016 | 190:35 |
| 10 | Sarcosine $TMS_2$ | 0.278 | 1.992 | 116:1000 |
| 11 | $\beta$-Hydroxybutyric $TMS_2$ | 0.312 | 0.431 | 191:594 |
| 12 | $\beta$-Alanine $TMS_2$ | 0.382 | 1.152 | 102:1000 |
| 13 | $\beta$-Aminoisobutyric $TMS_2$ | 0.348 | 0.420 | 102:1000 |
| 14 | Methylmalonic $TMS_2$ | 0.392 | 0.026 | 247:66 |
| 15 | Valine $TMS_2$ | 0.395 | 1.354 | 144:1000 |
| 16 | Ketoleucine $TMS_2$ | 0.449 | 0.045 | 259:94 |
| 17 | Octanoic acid TMS | 0.463 | 0.665 | 210:540 |
| 18 | Keto valine $TMS_2$ | 0.459 | 0.100 | 245:114 |
| 19 | Ethanolamine $TMS_3$ | 0.479 | 1.883 | 174:1000 |
| 20 | Leucine $TMS_2$ | 0.490 | 1.530 | 158:1000 |
| 21 | Ethylmalonic $TMS_2$ | 0.500 | 0.031 | 261:21 |
| 22 | Phosphate $TMS_3$ | 0.502 | 0.320 | 314:152 |
| 23 | Proline $TMS_2$ | 0.522 | 1.964 | 142:1000 |
| 24 | Isoleucine $TMS_2$ | 0.528 | 1.456 | 158:830 |
| 25 | Maleic $TMS_2$ | 0.535 | 0.171 | 245:67 |
| 26 | Glycine $TMS_3$ | 0.544 | 1.058 | 174:1000 |
| 27 | Ketoisoleucine $TMS_2$ | 0.549 | 0.111 | 259:96 |
| 28 | Succinic acid $TMS_2$ | 0.552 | 0.167 | 247:81 |
| 29 | Ketoisoleucine $TMS_2$ | 0.564 | 0.111 | 259:96 |
| 30 | Fumaric acid $TMS_2$ | 0.618 | 0.520 | 245:1000 |
| 31 | Pipecolic acid $TMS_2$ | 0.645 | 3.769 | 156:1000 |
| 32 | Serine $TMS_3$ | 0.664 | 1.345 | 204:1000 |
| 33 | Threonine $TMS_3$ | 0.716 | 0.460 | 218:446 |
| 34 | Glutaric $TMS_2$ | 0.731 | 0.206 | 261:133 |
| 35 | Propionylglycine $TMS_2$ | 0.771 | 1.252 | 158:815 |
| 36 | $\beta$-Aminoisobutyric $TMS_3$ | 0.833 | 1.152 | 174:1000 |

TABLE I *(continued)*

| Row No. | Target compound[a] | RRT | RF | Mass:intensity (*versus* 1000) of quantification ion |
|---|---|---|---|---|
| 37 | Homoserine $TMS_3$ | 0.834 | 1.541 | 218:1000 |
| 38 | Niacinamide TMS | 0.837 | 0.461 | 179:1000 |
| 39 | Niacinamide $TMS_2$ | 0.852 | 0.461 | 151:433 |
| 40 | Butyrylglycine $TMS_2$ | 0.893 | 0.997 | 172:565 |
| 41 | Malic acid $TMS_3$ | 0.915 | 0.778 | 233:185 |
| 42 | Adipic acid $TMS_2$ | 0.923 | 1.208 | 111:788 |
| 43 | Hydroxyproline $TMS_2$ | 0.937 | 2.233 | 156:1000 |
| 44 | Methionine $TMS_2$ | 0.940 | 0.992 | 176:1000 |
| 45 | Isovaleryglycine $TMS_2$ | 0.942 | 0.424 | 288:266 |
| 46 | Aspartic acid $TMS_3$ | 0.966 | 1.170 | 232:700 |
| 47 | γ-ABA $TMS_2$ | 0.967 | 2.944 | 174:1000 |
| 48 | Hydroxyproline $TMS_3$ | 0.971 | 2.233 | 230:1000 |
| 50 | Cysteine $TMS_4$ | 0.991 | 0.540 | 220:1000 |
| 51 | Creatinine $TMS_3$ | 1.000 | 1.000 | 115:901 |
| 52 | Cysteine $TMS_3$ | 1.012 | 0.540 | 220:1000 |
| 53 | Tiglyglycine $TMS_2$ | 1.017 | 0.625 | 286:537 |
| 54 | 2-Oxoglutarate | 1.105 | 0.076 | 347:135 |
| 55 | Phenylalanine $TMS_2$ | 1.110 | 0.922 | 218:906 |
| 56 | Ornithine $TMS_3$ | 1.118 | 3.377 | 142:1000 |
| 57 | Glutamic acid $TMS_3$ | 1.121 | 1.060 | 246:957 |
| 58 | Suberylglycine $TMS_3$ | 1.155 | 4.477 | 188:1000 |
| 59 | Hexanoylglycine $TMS_3$ | 1.160 | 0.651 | 200:433 |
| 60 | Homocysteine $TMS_3$ | 1.176 | 0.947 | 234:902 |
| 61 | Ribose $TMS_4$ | 1.187 | 5.825 | 217:1000 |
| 62 | Asparagine $TMS_3$ | 1.195 | 4.766 | 231:727 |
| 63 | Ribose $TMS_4$ | 1.204 | 5.825 | 217:1000 |
| 64 | Suberic acid $TMS_2$ | 1.216 | 0.204 | 187:334 |
| 65 | β-Met Crotonylglycine $TMS_2$ | 1.220 | 0.369 | 170:1000 |
| 66 | Phenylpyruvic acid TMS | 1.225 | 0.429 | 293:264 |
| 67 | Lysine $TMS_3$ | 1.251 | 1.268 | 84:974 |
| 68 | Quinolinic acid $TMS_2$ | 1.254 | 0.209 | 296:198 |
| 69 | Xylose $TMS_4$ | 1.267 | 3.691 | 204:1000 |
| 70 | Xylitol $TMS_5$ | 1.278 | 0.522 | 307:286 |
| 71 | Orotic acid $TMS_3$ | 1.300 | 0.721 | 254:1000 |
| 72 | Homovanillic $TMS_2$ | 1.312 | 1.272 | 209:948 |
| 73 | Glutamine $TMS_3$ | 1.331 | 0.408 | 156:1000 |
| 74 | Xylose $TMS_4$ | 1.341 | 3.691 | 204:1000 |
| 75 | 3-Methylhistidine $TMS_3$ | 1.352 | 0.541 | 96:1000 |
| 76 | Hippuric acid $TMS_2$ | 1.355 | 0.477 | 105:1000 |
| 77 | Hippuric acid TMS | 1.387 | 0.477 | 206:837 |
| 78 | Ornithine $TMS_4$ | 1.397 | 3.377 | 142:1000 |
| 79 | Citric acid $TMS_4$ | 1.400 | 2.158 | 273:1000 |
| 80 | Fructose $TMS_4$ | 1.401 | 0.419 | 437:143 |
| 81 | MHPG $TMS_3$ | 1.406 | 6.717 | 297:1000 |
| 82 | Mannose $TMS_5$ | 1.408 | 4.335 | 204:1000 |

*(Continued on p. 130)*

TABLE I *(continued)*

| Row No. | Target compound[a] | RRT | RF | Mass:intensity (*versus* 1000) of quantification ion |
|---|---|---|---|---|
| 83 | Homogentisic acid TMS | 1.411 | 0.783 | 384:489 |
| 84 | Fructose $TMS_4$ | 1.414 | 0.419 | 437:161 |
| 85 | Galactose $TMS_5$ | 1.427 | 4.335 | 217:1000 |
| 86 | Normetanephrine $TMS_3$ | 1.437 | 1.957 | 297:1000 |
| 87 | Formiminoglutamic $TMS_4$ | 1.439 | 0.997 | 241:864 |
| 88 | Hydroxy lysine $TMS_4$ | 1.452 | 0.656 | 232:384 |
| 89 | Sebacic acid $TMS_2$ | 1.460 | 0.579 | 331:366 |
| 90 | Vanillylmandelic $TMS_3$ | 1.463 | 5.221 | 297:1000 |
| 91 | Galactose $TMS_5$ | 1.468 | 4.335 | 204:1000 |
| 92 | Galactonic acid $TMS_5$ | 1.492 | 1.674 | 217:876 |
| 93 | Glucose $TMS_5$ | 1.494 | 5.570 | 204:1000 |
| 94 | Metanephrine $TMS_3$ | 1.503 | 5.239 | 116:1000 |
| 95 | Histidine $TMS_3$ | 1.507 | 1.856 | 154:719 |
| 96 | Lysine $TMS_4$ | 1.519 | 1.268 | 174:1000 |
| 99 | 4-Pyridoxic acid TMS | 0.816 | 0.811 | 309:674 |
| 100 | Tyrosine $TMS_3$ | 0.817 | 2.966 | 218:1000 |
| 101 | Glucitol $TMS_3$ | 0.830 | 2.723 | 319:894 |
| 102 | Ascorbic acid $TMS_4$ | 0.830 | 0.725 | 332:427 |
| 103 | Phenylpropionylglycine $TMS_2$ | 0.842 | 0.160 | 351:403 |
| 104 | Pantothenic acid $TMS_4$ | 0.845 | 0.325 | 291:482 |
| 105 | Glucuronic acid $TMS_6$ | 0.846 | 0.415 | 292:152 |
| 106 | Glucose $TMS_5$ | 0.862 | 5.570 | 204:1000 |
| 107 | Gluconic acid $TMS_6$ | 0.868 | 0.819 | 333:465 |
| 108 | Galactonic acid $TMS_6$ | 0.869 | 0.921 | 292:436 |
| 109 | Glucaric acid $TMS_6$ | 0.876 | 3.337 | 333:1000 |
| 110 | Glucuronic acid $TMS_6$ | 0.881 | 0.415 | 292:156 |
| 111 | N-Acetyl glucosamine $TMS_5$ | 0.897 | 3.884 | 245:1000 |
| 112 | Kynurenine $TMS_3$ | 0.906 | 0.975 | 120:1000 |
| 113 | Inositol $TMS_6$ | 0.909 | 2.335 | 318:330 |
| 115 | Uric acid $TMS_4$ | 0.918 | 1.744 | 441:760 |
| 117 | N-Acetylglucosamine $TMS_5$ | 0.918 | 3.884 | 173:1000 |
| 118 | N-Acetylglucosamine $TMS_5$ | 0.921 | 3.884 | 245:1000 |
| 119 | Kynurenine $TMS_4$ | 0.940 | 0.510 | 307:523 |
| 120 | Tryptophan $TMS_3$ | 0.951 | 4.303 | 202:1000 |
| 121 | 5-HIAA $TMS_3$ | 0.954 | 3.807 | 290:1000 |
| 122 | Cystathionine $TMS_4$ | 0.954 | 1.256 | 128:687 |
| 123 | Xanthurenic acid $TMS_3$ | 0.964 | 2.184 | 406:1000 |
| 125 | Cystine $TMS_4$ | 0.974 | 0.458 | 146:846 |
| 126 | Pseudouridine $TMS_5$ | 1.000 | 3.981 | 217:999 |
| 127 | Lactose $TMS_8$ | 1.091 | 2.690 | 204:796 |
| 128 | Sucrose $TMS_8$ | 1.092 | 0.234 | 437:105 |
| 129 | Maltose $TMS_8$ | 1.096 | 2.409 | 204:1000 |
| 130 | Maltose $TMS_8$ | 1.119 | 2.409 | 204:1000 |
| 131 | Lactose $TMS_8$ | 1.119 | 1.431 | 191:532 |

[a] ABA = aminobutyric acid; HIAA = hydroxyindoleacetic acid; MHPG = 3-methoxy-4-hydroxyphenyl glycol.

gram are provided in Table I. A single-ion chromatogram of a window of ten to fifteen scans on either side of a given relative retention time was searched and the scans of maximum intensity were matched against eight-peak mass spectra stored in a custom library file. The area of the peak yielding the best match was used to calculate the concentration of the compound relative to the internal standard, $d_3$-creatinine, using the relative response factor (RF) listed in Table I. These response factors were calculated from five-point standard curves previously run for each compound. The five concentrations were generally between 25 and 1000 nmol per sample. Standards were run in groups of ten to twenty at a time. A custom program then calculated the concentrations in terms of the endogenous creatinine (target compounds 49 and 51), added together multiple derivative peaks of some compounds and printed the results.

*Verification and statistical methods*

The urea nitrogen and creatinine concentrations of twenty samples were measured by a clinical autoanalyzer, either the Beckman 'Synchron' CX-3 or the Baxter Paramax. Both instruments measured urea by urine conductance. The creatinine procedure utilized the dual-wavelength absorbance ratio of the chromophore produced by picric acid.

The first 21 clinical samples received from out-patients over six months of age which were found to be within previously established normal ranges for excretion of the target compounds were statistically analyzed using the Lotus 123 spreadsheet software. Means and standard deviations were calculated from these 21 subjects and appear in Table II.

TABLE II

MEANS AND STANDARD DEVIATIONS FOR 103 COMPOUNDS IN URINE

Data were from 21 outpatients, over six months of age, none of whom had values outside established clinical norms. Patients older than 0.5 years: mean creatinine excretion 3.04 μmol/ml of urine.

| Compound | Concentration (μmol/mmol of creatinine) | | Compound | Concentration (μmol/mmol of creatinine) | |
|---|---|---|---|---|---|
| | Mean | S.D. | | Mean | S.D. |
| *Organic Acids* | | | *Neurotransmitters* | | |
| Lactic acid | 50.73 | 53.54 | γ-ABA | 0.09 | 0.10 |
| Pyruvic acid | 9.79 | 9.34 | Homovanillic acid | 4.93 | 3.37 |
| Glycolic acid | 38.10 | 27.35 | Normetanephrine | 0.85 | 3.17 |
| Oxalic acid | 3.26 | 9.04 | Vanillylmandelic acid | 3.67 | 2.12 |
| Hexanoic acid | 0.91 | 1.05 | Metanephrine | 1.08 | 2.95 |
| Octanoic acid | 0.39 | 0.37 | HIAA | 4.75 | 5.38 |
| Succinic acid | 12.60 | 12.13 | MHPG | 0.06 | 0.06 |

*(Continued on p. 132)*

TABLE II *(continued)*

| Compound | Concentration (μmol/mmol of creatinine) | | Compound | Concentration (μmol/mmol of creatinine) | |
|---|---|---|---|---|---|
| | Mean | S.D. | | Mean | S.D. |
| Glutaric acid | 1.08 | 1.24 | Ethanolamine | 71.32 | 33.29 |
| Fumaric acid | 0.95 | 0.93 | | | |
| Maleic acid | 0.00 | 0.02 | *Amino acids and glycine conjugates* | | |
| Adipic acid | 2.87 | 2.50 | Butyrylglycine | 0.24 | 0.36 |
| Suberic acid | 4.34 | 5.99 | Suberylglycine | 0.29 | 0.24 |
| Sebacic acid | 0.60 | 0.78 | Isovalerylglycine | 0.37 | 1.19 |
| Ketoisoleucine | 0.05 | 0.16 | Tiglyglycine | 0.50 | 0.93 |
| Ketovaline | 0.16 | 0.48 | β-Methyl Crotglycine | 0.48 | 0.64 |
| Ketoleucine | 0.00 | 0.00 | Serine | 74.67 | 37.74 |
| β-Hydroxybutyric acid | 2.60 | 3.50 | Glycine | 513.54 | 360.61 |
| Methylmalonic acid | 0.92 | 1.97 | Sarcosine | 1.35 | 1.21 |
| Ethylmalonic acid | 0.14 | 0.27 | Alanine | 113.35 | 69.81 |
| Homogentisic acid | 0.04 | 0.17 | β-Alanine | 0.23 | 0.73 |
| Phenylpyruvic acid | 0.25 | 0.43 | Proline | 4.14 | 2.85 |
| Malic acid | 0.46 | 0.79 | Hydroxyproline | 88.17 | 49.59 |
| Pipecolic acid | 0.03 | 0.07 | Hydroxylysine | 0.35 | 0.62 |
| Hexanoylglycine | 0.41 | 1.72 | Asparagine | 0.96 | 0.65 |
| Phen Prop glycine | 0.00 | 0.00 | Ornithine | 2.19 | 3.21 |
| Propionylglycine | 0.00 | 0.00 | Tryptophan | 13.52 | 7.37 |
| Citric acid | 226.32 | 98.12 | Leucine | 7.58 | 4.10 |
| 2-Oxoglutarate | 43.46 | 53.46 | Valine | 12.30 | 7.69 |
| Hippuric acid | 871.36 | 1024.84 | Isoleucine | 3.75 | 2.75 |
| Uric acid | 118.09 | 114.57 | Lysine | 31.45 | 52.32 |
| Phosphate | 1364.27 | 915.27 | Histidine | 194.90 | 144.31 |
| | | | Homoserine | 0.29 | 0.37 |
| *Nutritionals* | | | Threonine | 36.20 | 25.38 |
| Formiminoglutamic acid | 0.90 | 0.71 | Methionine | 1.21 | 1.20 |
| 4-Pyridoxic acid | 2.99 | 5.25 | Cysteine | 30.32 | 24.58 |
| Pantothenic acid | 11.16 | 8.88 | Homocystine | 0.07 | 0.10 |
| Xanthurenic acid | 0.10 | 0.17 | Cystine | 10.05 | 28.34 |
| Kynurenine | 0.56 | 0.91 | Cystathionine | 0.35 | 0.65 |
| Quinolinic acid | 0.67 | 1.34 | Aspartic acid | 1.36 | 1.06 |
| Orotic acid | 1.05 | 1.22 | β-Aminoisobutyric acid | 82.35 | 136.96 |
| Niacinamide | 0.30 | 0.51 | Glutamine | 118.96 | 89.94 |
| 3-Methylhistidine | 42.76 | 22.84 | Phenylalanine | 23.59 | 16.02 |
| Pseudouridine | 63.47 | 32.76 | Tyrosine | 27.35 | 13.98 |
| Ascorbic acid | 63.65 | 142.96 | Glutamic acid | 6.87 | 3.51 |
| *Carbohydrates* | | | | | |
| Fructose | 70.38 | 107.54 | Glucuronic acid | 33.82 | 20.95 |
| Mannose | 11.65 | 15.42 | Galactonic acid | 43.25 | 28.14 |
| Galactose | 262.58 | 881.72 | Glucaric acid | 3.91 | 5.53 |
| Glucose | 143.14 | 399.78 | Glucitol | 9.74 | 10.36 |
| N-Acetylglucosamine | 2.96 | 1.85 | Inositol | 6.11 | 6.25 |
| Lactose | 6.08 | 6.07 | Gluconic acid | 35.23 | 29.19 |
| Maltose | 6.14 | 5.12 | Sucrose | 18.79 | 29.28 |
| Ribose | 6.35 | 4.59 | Xylitol | 16.29 | 26.02 |
| Xylose | 26.69 | 23.74 | | | |

## RESULTS AND DISCUSSION

The stepwise reduction in urea concentration achieved by this method is illustrated in fig. 1A–C. The presence of urea did not preclude acquisition of data from the rest of the chromatogram, but large amounts of the silylating agent were consumed by urea and other constituents may not have been completely derivatized in its presence. A typical TIC chromatogram is shown in Fig. 2A.

Three of the 85 clinical samples retained significant amounts of urea after the treatment described. Two of these samples were found to have been acidified below pH 1, apparently in preparation for amino acid analysis by liquid chromatography. Neutralization of these two samples allowed removal of urea. The third sample was presumed to contain heavy metal ions known to inhibit urease [9] and was therefore pretreated by bubbling hydrogen sulfide gas through it for a few seconds until a slight precipitate appeared, then filtering and proceeding as above. This permitted removal of urea. The nature of the inhibitor is not known, but unusual concentrations of lead, iron or calcium were not found in the sample by atomic absorption spectrophotometry.

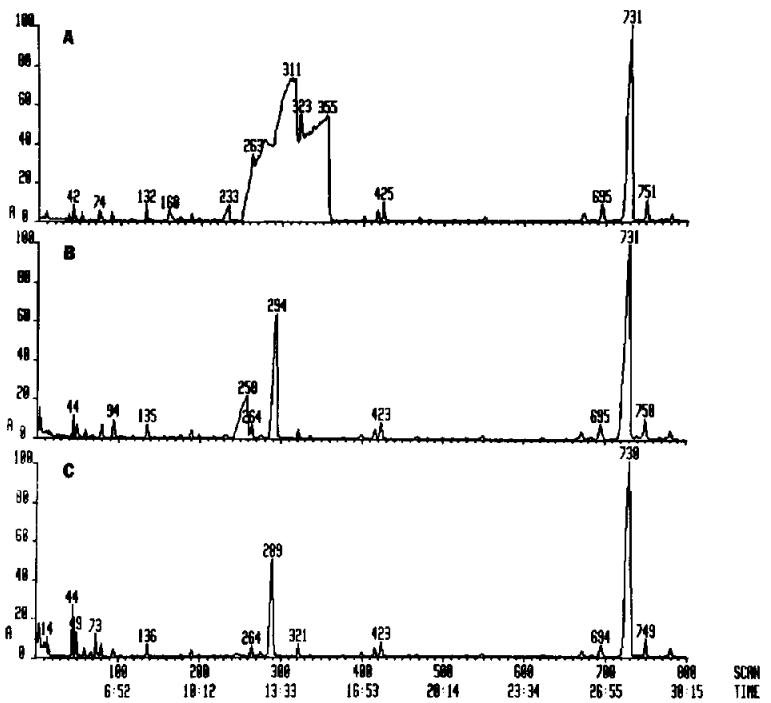



Fig. 1. Disappearance of urea from urine sample treated with urease. Trace A is prior to treatment: peak 160 is urea tri-TMS, and peaks 263–355 are due to urea di-TMS. Trace B shows urea remaining after 30 min of incubation with urease, under a $CO_2$ atmosphere. The area of the single ion chromatogram of mass 189 for peak 258 is approximately 5% that of peaks 263–355 in trace A. Trace C shows elimination of the urea peak after another addition of urease and incubation under a $CO_2$ atmosphere for 15 min.

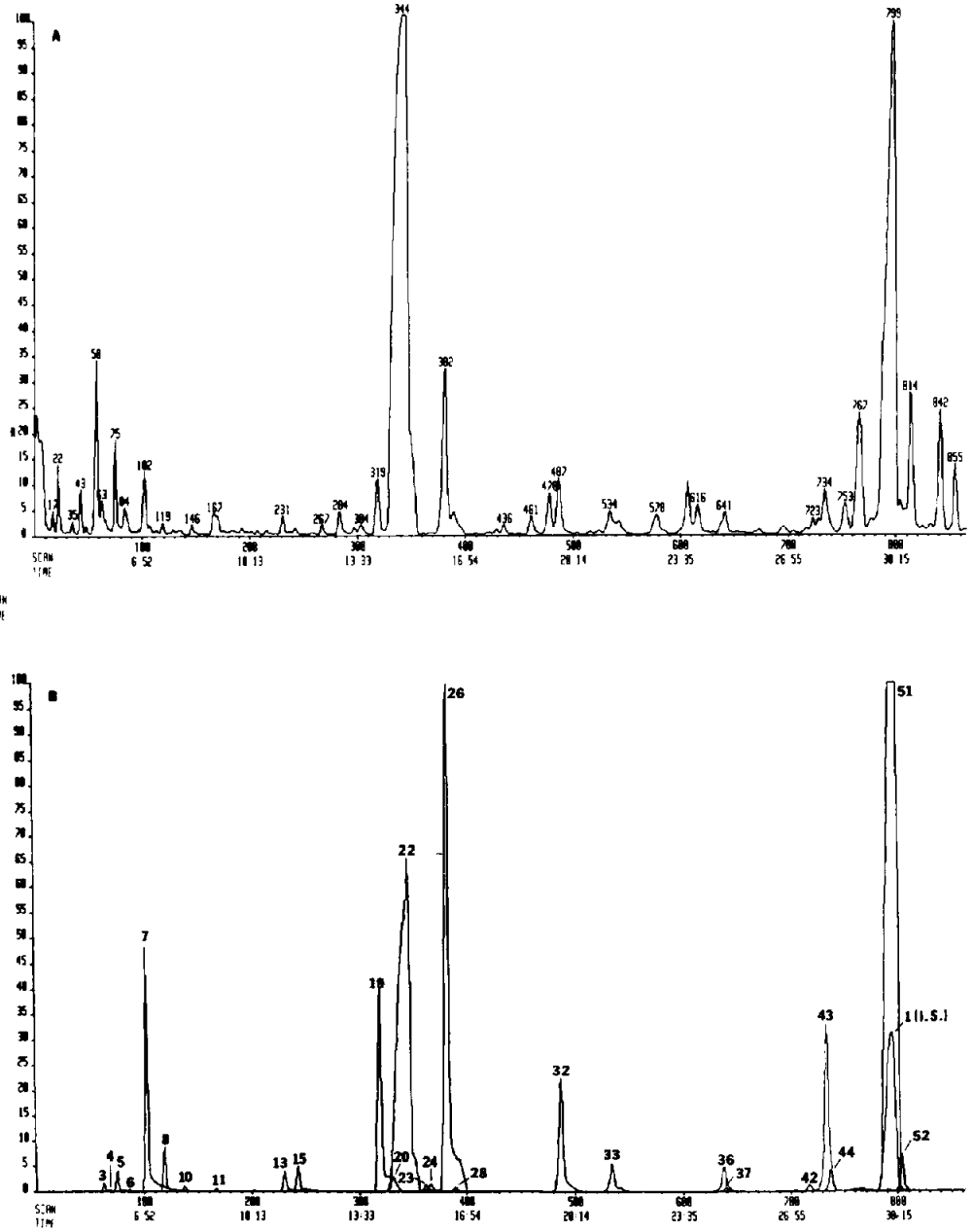
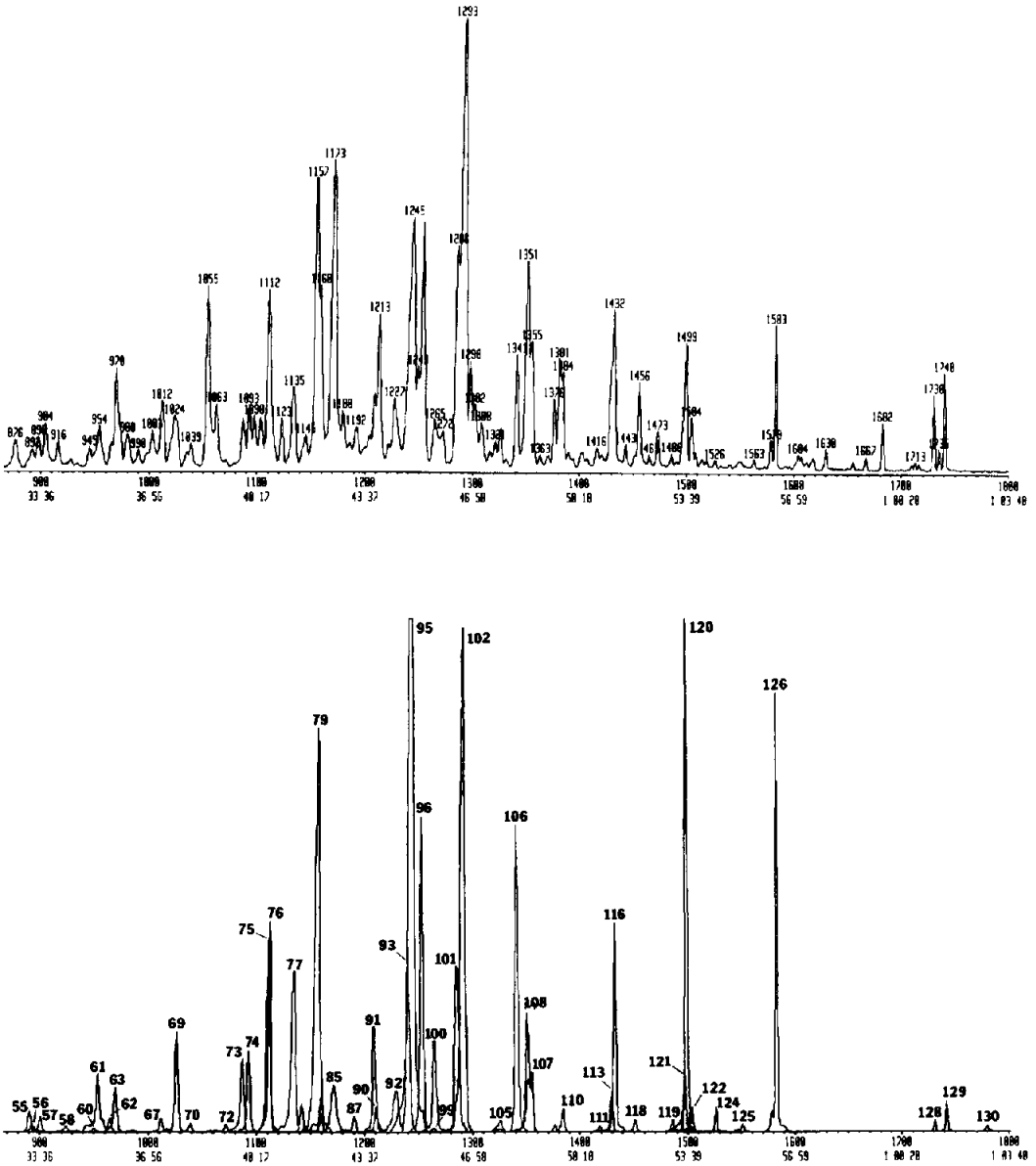



Fig. 2. Typical TIC chromatogram (A) and the corresponding set of single ion chromatograms scaled proportionately to endogenous creatinine (peak 51) from the same data (B). Major peaks in the TIC chromatogram were identified by the library search program as follows, listed by scan number in trace A: 63, lactate; 75, glycolate; 102, alanine; 167, 2-methyl-3-hydroxypropionic acid; 231, 3-hydroxyisovaleric acid; 319, ethanolamine; 344, phosphate; 382, glycine; 487, serine; 534, threonine; 616, 2-deoxytetronic



acid; 641, 2-pentenedioic acid; 734, hydroxyproline; 753, threitol; 767, tetrose; 799, creatinine; 814 and 842, tetronic acids; 970, deoxypentonic acid; 1055, ribitol; 1112, 3-methylhistidine; 1134, hippuric acid; 1157, citric acid; 1245, histidine; 1254, lysine; 1293, ascorbic acid; 1341, glucose; 1351, galactonic acid; 1381, glucuronic acid; 1432, uric acid; 1499, tryptophan; 1583, pseudouridine; 1730, sucrose. The peak numbers in B correspond to the row numbers in Table I.

The success or failure of urea removal can be estimated from the area of a single-ion chromatogram for mass 189 in the region of scans 200–400, as this is a major ion in the mass spectrum of the most common TMS derivative of urea, urea di-TMS. When urea remains in very large amounts, the tri-TMS derivative, containing masses 245, 261 and 276 all at about 20% of base-peak intensity, may also be observed at earlier retention times (Fig. 1A, peak 160).

Urease itself was found to add few contaminants to the samples, even if left in the mixture during dehydration and derivatization by omission of the acetone precipitation and filtration steps. Citric acid was the only significant contaminant and was found to add 11.8 μmol citrate per mmol creatinine when creatinine was present at 10 μmol/ml. The range of urinary urea concentration in twenty samples from children analyzed by an automated clinical analyzer was 32–596 mg urea nitrogen per dl (11–210 mmol/l). Samples from adults may contain higher concentrations of urea and require dilution prior to urease treatment.

Fig. 3 shows the mass spectra for native and trideuterated creatinine. Creatine is quantitatively converted to creatinine during the procedure described above, as long as alkaline conditions are maintained throughout the aqueous steps. This is in contrast to a previous report [10] stating that with mineral acids and bases, acidity favored creatinine and alkalinity creatine formation. A creatine tri-TMS derivative elutes near citric acid tetra-TMS, but is not detectable when the present procedure is used.

The agreement between the two methods of urinary creatinine measurement (isotope dilution in the method above and the automated clinical analyzer method based on picric acid) depended on the concentration. In twenty samples analyzed by both methods, the correlation coefficient was 0.923 ($P < 0.001$) and the calculated linear equation was $y = 0.673x + 0.275$ where $y =$ μmol of creatinine per ml of urine by the automated picric acid method and $x =$ isotope dilution. Thus in the high range, such as 10 μmol/ml, picric acid underestimated the isotope dilution value by 30%, but at the lower range of 0.5 μmol/ml the picric acid method overestimated by 22%.

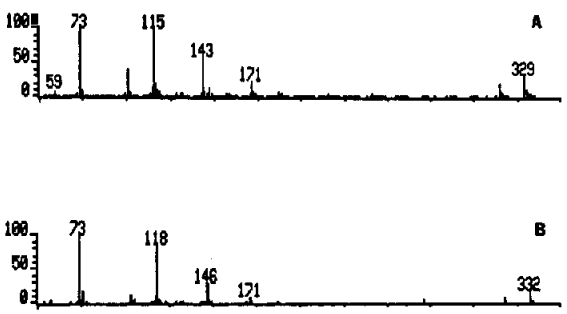



Fig. 3. Mass spectra of TMS derivatives of native (A), and trideuterated (B) creatinine. The molecular ions are at 329 and 332, respectively, for the tri-TMS derivatives of creatinine-enol.

Table II lists the means and standard deviations for 103 compounds found in the urine of 21 outpatients, over six months of age, calculated as described above. Values for the imino acids proline and hydroxyproline may be higher than those derived from ninhydrin-based detection systems. Volatile acids such as lactate, pyruvate and hexanoate may show higher values due to lower losses on evaporation compared to acidification–extraction methods [2]. In our procedure, solvent evaporation takes place at neutral pH and the "volatile" acids are in a less volatile, ionized state.

Twenty amniotic fluid samples, ten from 20 ± 4 weeks and ten from 38 ± 4 weeks of gestation, showed greatly increased lactate, pyruvate and amino acids per unit of creatinine, but less creatinine per ml than urine samples from infants and children.

Trideuterated creatine was the sole internal standard used in these studies, so different classes of compounds had widely different molar response factors, as shown in Table I. Carbohydrates produced more ions per mole than keto acids, for example. These differences were not due to "recovery" *per se*, since no fractionation of the samples took place. The differences in response relative to creatinine may have been due to efficiency of derivatization, enol formation, chromatographic characteristics or molecular structure. Response factors were fairly consistant for each compound: the coefficients of variation were 10–50% over the range of five concentrations studied. More exact quantitation would result if stable isotope analogues were used for each compound of interest. An advantage of the present procedure is that it can be applied to compounds poorly recovered by other methods and possibly obviate the need for stable isotope analogues in routine metabolic screening.

## CONCLUSION

Treatment of urine and amniotic fluid samples with urease prior to derivatization is an alternative to other clean-up methods such as solvent extraction and column elution. Automated search procedures can reduce the time necessary for interpretation of mass spectral data to about the same as that required to interpret a chromatogram by retention times alone, yet greatly increase the certainty of identification and quantitation.

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