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MEASUREMENT OF PLASMA OXALATE BY CAPILLARY GAS CHROMATOGRAPHY AND ITS VALIDATION BY ISOTOPE DILUTION MASS SPECTROMETRY

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SUMMARY

A capillary gas chromatographic method for plasma oxalate and an isotope dilution mass spectrometric reference method, both using the same *tert* -butyldimethylsilyl derivatives, are described. Similar reference ranges for both were found (4.93 ± 1.48 and 4.70 ± 1.44 μmol/l, respectively), together with a close correlation for results covering a wide range of oxalate concentrations.

INTRODUCTION

Direct chemical analysis methods for plasma oxalate, which include early reduction of the plasma pH, minimize oxalogenesis in vitro from ascorbic acid and other precursors present in plasma [1,2]. As the contribution to the measured oxalate from this source can result in a ten- to twenty-fold increase in the normal range [1,3], but is thought to be negligible in uraemic plasma [3], failure to suppress it reduces the diagnostic efficiency of the assay.

In the capillary gas chromatographic (GC) technique of Wolthers and Hayer [4], oxalate in acidified plasma is extracted into ethyl acetate before trimethylsilylation and analysis. This was one of the first direct methods to obtain a range of normal oxalate values (1.3–5.3 μmol/l) close to that deduced indirectly by the in vivo isotope dilution technique (0.75–2.11 μmol/l) [3]. The remaining differ-

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ence might arise if an analyte or contaminant with the same retention time as oxalate contributed to the oxalate peak recorded by the non-specific flame ionization detector [4].

In this paper two plasma oxalate methods using essentially identical sample preparation are described. The first method (GC), intended for routine use, is based on that of Wolthers and Hayer [4], but uses the *tert.*-butyldimethylsilyl (t-BDMS) derivative also suitable for isotope dilution mass spectrometry (MS). The reference method, also intended for studying oxalate turnover in vivo, has an advantage over previous methods using GC–MS [5] in not requiring chemical ionization conditions.

EXPERIMENTAL

Samples

For determination of analytical performance, lyophylized unassayed quality control serum (Ortho Diagnostics, Raritan, NJ, U.S.A.) was reconstituted to 25 ml, acidified with 1 ml of 4 *M* hydrochloric acid (to obtain a pH of ca. 3.0) and frozen in portions. Reference intervals were obtained on heparinized plasmas collected from outpatients without renal disease and with normal creatinine concentrations. Red blood cells were removed at 4 °C (by centrifugation at 200 *g* for 5 min) within 10 min of collection, and 4 ml of plasma were added to a tube containing 150 µl of 4 *M* hydrochloric acid, mixed by inversion and stored at −20 °C until analysed. Uraemic specimens for the method correlation study were sera which had been analysed for renal function tests. They were acidified and stored as above within 6 h of sampling.

Extraction

The method of Wolthers and Hayer [4] was followed with minor changes. To 0.5 ml plasma in polypropylene tubes, ca. 0.25 g of sodum chloride and 250 µl of 0.1 *M* hydrochloric acid were added. The tubes were vortex-mixed and their contents extracted twice with 2 ml of ethyl acetate by vortex-mixing for exactly 2 min. The phases were separated by centrifugation (10 min at 1500 *g*) and the two ethyl acetate extracts pooled and dried over anhydrous sodium carbonate. Malonic acid (Sigma, St. Louis, MO, U.S.A.), used as internal standard for the GC method, was added to all plasma samples (to obtain a concentration of 40 µmol/l) prior to extraction. Sodium [$^{13}C_2$] oxalate (90% atom excess; MSD Isotopes, Pointe Claire, Canada), used as an internal standard for the GC–MS method, was also added to a duplicate plasma sample prior to extraction to give a concentration of 40 µmol/l. A 0.5-ml portion of this ethyl acetate extract was taken for GC–MS analysis; the remainder served as the oxalate-enriched sample for response factor calculation in the GC method. Aqueous standard solutions at 1000 µmol/l in 0.1 *M* hydrochloric acid were stored in portions at −20 °C.

Derivatization

For GC, 1.5-ml portions of each extract were evaporated at 40 °C under nitrogen. A 25-µl aliquot of N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide

(MTBSTFA; Pierce, Rockford, IL, U.S.A.) was added to the dry residues, and the tubes were mixed, stoppered and left for 24 h at 26°C before transfer to 1-ml autosampler vials. For GC–MS, 0.5-ml portions of [$^{13}C_2$] oxalate-enriched ethyl acetate extract were reduced to ca. 50 μl under nitrogen in polyethylene vials. MTBSTFA (30 μl) was added and the mixture heated at 50°C for 30 min [6]. Derivatives were stable for 24 h at 26°C or up to one week at −20°C.

Instrumental

GC. A Hewlett-Packard (Avondale, PA, U.S.A.) Model 5880 gas chromatograph equipped with a flame ionization detector was used. Two 12 m×0.25 mm I.D. fused-silica (FSOT) columns coated with 1-μm OV-101 phase gave similar results. For the first (Hewlett-Packard), 1-μl injections were made in splitless mode, with helium as carrier gas at a head pressure maintained at 70 kPa. The second (SGE, Ringwood, Australia) gave similar resolution and peak shapes with 10-μl split injections and a column head pressure at 60 kPa. The split ratio was 50:1 and the helium flow-rate through the column 1 ml/min in each case. Injector and detector temperatures were 240 and 260°C, respectively. The oven was programmed from 70 to 140°C at 2°C/min and thereafter at 20°C/min to 260°C and maintained at 260°C for 10 min. Injections were made using a Hewlett-Packard Model 7671A autosampler. To check the linearity of the oxalate response, oxalic and malonic acids (Sigma) were made up to 1000 μmol/l in ethyl acetate. Varying proportions of these were derivatized and chromatographed.

GC–MS. A Kratos MS80RFA mass spectrometer (Spectros, Manchester, U.K.) was used in the selected-ion monitoring (SIM) mode with electron-impact (EI) ionization (30 eV) and a source temperature of 220°C. GC separations were performed using a Carlo Erba Mega gas chromatograph equipped with a 0.17-μm OV-1 coated FSOT column (25 m ×0.32 mm I.D., Hewlett-Packard). On-column injections (1 μl) were made with a carrier gas (helium) head pressure of 25 kPa. The GC oven temperature was maintained at 70°C for 1 min following injection, then programmed at 12°C/min to 180°C. Oxalate(t-BDMS)$_2$ had a retention time of 6.3 min. The GC–MS transfer line was maintained at 250°C. The ions at 261.096 and 263.104 mass/charge ratio were monitored using a cycle time of 500 ms and an electric sector settling time of 30 ms. No lock mass was used. For the calibration curve, a set of mixed [^{12}C]- and [^{13}C]sodium oxalate standards were extracted into ethyl acetate, derivatized and analysed. Measured peak-area ratios, *m/z* 261 to *m/z* 260, were corrected according to the method of Colby and McCaman [7].

To determine oxalate extraction recovery, [U-^{14}C]oxalic acid (1.85 MBq and 0.57 μmol/ml; Amersham, Sydney, Australia) was diluted 1:10 in saline. A 20-μl sample of this dilution was added to 2 ml of acidified pooled plasma. Duplicate 25-μl samples were taken for scintillation counting, while 250-μl samples were extracted into 2 ml ethyl acetate in the usual way. Samples of extract (200 μl) were then again taken for counting. We used an LKB-Wallac Model 1214 Rackbeta and 10 ml aqueous counting scintillant (Amersham).

The linearity of the methods was checked using pooled plasma to which

[^{12}C]sodium oxalate (BDH, Poole, U.K.) had been added to obtain oxalate enrichments varying from 0 to 80 μmol/l.

RESULTS

A representative flame ionization detector chromatogram for the capillary GC method is shown in Fig. 1A. Retention times shown for the t-BDMS derivatives of oxalic and malonic acids were reproducible. Although the chromatograms were complex, the analyte peaks were free of obvious interferences such as incomplete resolution from other components. Fig. 2 shows EI mass spectra for the t-BDMS

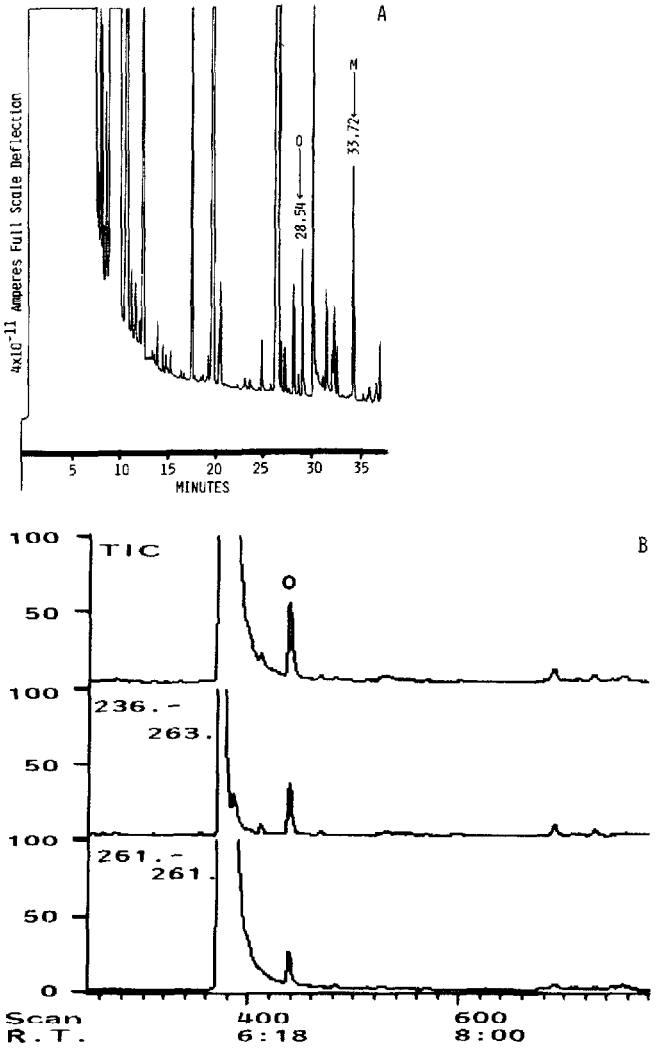

Fig. 1. (A) Sample chromatogram (GC) for the derivatized extract of control serum (17.2 μmol/l oxalate). O = Oxalate(t-BDMS)$_2$; M = malonate(T-BDMS)$_2$. (B) Sample chromatogram (GC–MS) for the derivatized extract of a plasma sample (17.0 μmol/l oxalate).

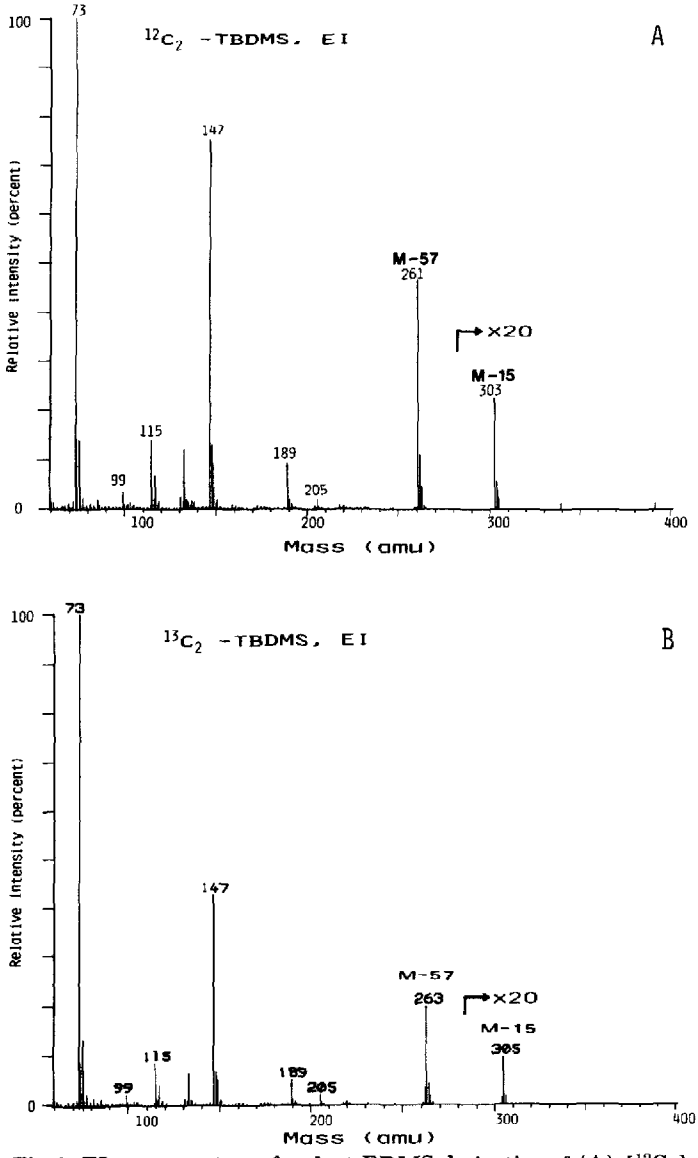

Fig. 2. EI mass spectrum for the t-BDMS derivative of (A) [$^{12}C_2$]oxalic acid and (B) [$^{13}C_2$]oxalic acid.

derivatives of [$^{12}C_2$]oxalic acid and [$^{13}C_2$]oxalic acid. A SIM chromatogram for the GC–MS method is shown in Fig. 1B.

GC response factors, corrected ion ratios and the procedures as a whole were linear up to 100 μmol/l [$^{12}C_2$]oxalate. Eqn. 10 of Colby and McCaman [7] gave the best linear relationship for the GC–MS method. The equations of the regression lines for total oxalate found in pooled plasma (y) versus oxalate added (x) were $y=0.950x+21.90$ for GC and $y=0.878x+23.93$ for GC–MS; the correlation coefficients were 0.995 and 0.997, respectively. The mean extraction recovery of

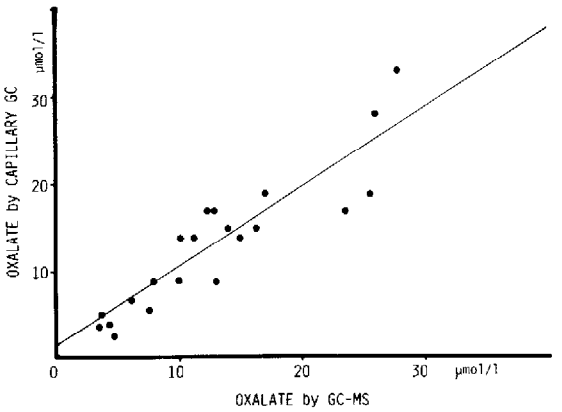

Fig 3. Relationship between oxalate results obtained by capillary GC and GC–MS.

[^{14}C]oxalic acid from pooled reference plasma was 57.9%. Inter-assay precision obtained following six analyses of control serum was 15.9 ± 1.2 (mean ± S.D.) for GC and 14.9 ± 2.1 (mean ± S.D.) for GC–MS. Normal ranges found were 1.5–6.6 μmol/l (mean ± S.D. 4.93 ± 1.48, μmol/l, n = 14) for GC and 2.8–7.5 μmol/l (mean ± S.D. 4.70 ± 1.44 μmol/l, n = 11) for GC–MS.

Fig. 3 shows the relationship between the plasma oxalate measured by both methods for the range of oxalate concentrations encountered clinically (n = 20, r = 0.938). The equation of the regression line was (GC) = 0.919 (GC–MS) + 1.28.

DISCUSSION

Wolthers and Hayer [4] used trimethylsilyl (TMS) derivatives in their capillary GC oxalate method. The t-BDMS derivatives show increased sensitivity using flame ionization detection and improved stability against hydrolysis. Nonetheless our GC normal range was close to that reported by Wolthers and Hayer [4] and slightly elevated compared with those obtained using the in vivo isotope inferential technique [3] and the enzymatic technique of Kasidas and Rose [2] applied to ultrafiltrate at pH 3.6.

t-BDMS derivatives produce simple EI mass spectra with loss of a *tert.*-butyl group giving the base peak. This ion contains both carbons (unlike the major ions in the EI spectrum of TMS-oxalate) and facilitates the use of [$^{13}C_2$]oxalate as an internal standard for GC–MS. The stable isotope-labelled oxalate will more closely parallel the behaviour of native [^{12}C]oxalate during extraction and chromatography, eliminating any variation due to use of the homologous malonic acid internal standard. Results obtained by the GC method showed no positive bias when compared with the reference method, however. It is, therefore, unlikely that the normal range elevation common to GC methods is due to non-specific interference. Low level but variable oxalogenesis might account for part of the relatively high coefficient of variation observed in both methods. Other contributing factors are variable GC column performance, particularly after extended use, and for GC–MS, uncertainty in ion-ratio determination arising from the peak areas

having to be skimmed on the tail of the component eluting just before the oxalate (see Fig. 1B).

We conclude that the GC method is specific and suitable for routine determination of oxalate in plasma. The GC–MS method is at present being applied to the determination of oxalate biosynthetic rates in patients with impaired renal function.

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