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Short communication

Mass spectrometric–selected ion monitoring assay for an oxalate absorption test applying [$^{13}C_2$]oxalate

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Abstract

In order to assess the significance of the intestinal absorption of oxalate from food for the hyperoxaluria of the individual patient, an oxalate absorption test has been developed using doubly ^{13}C-labelled oxalate and a gas chromatographic selected ion monitoring mass spectrometric assay. This test has been applied to volunteers and patients with urinary stones. The percentage of dose absorbed (range 1–48%) could be determined with a coefficient of variation of 15.2%. The assay to measure doubly ^{13}C-labelled oxalate in the presence of unlabelled oxalate in urine, using the homologue malonic acid as internal standard, is described. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The gastrointestinal hyperabsorption of oxalate is an important cause for the formation and growth of calcium oxalate urinary stones. A number of studies applying adequate methodology showed that a large fraction – but not all – of the patients with recurrent stones were hyperabsorbers of oxalate [1,2]. The aim of our investigation was to develop a procedure to evaluate the extent of oxalate absorption for the individual patient and the response to changes in diet or treatment.

Although there have been severe problems with older assays for total oxalate in urine [3], reliable assays have become available in recent years [4–8]. Total urinary oxalate is always the sum of oxalate absorbed as such and oxalate synthesized within the body (endogenous oxalate) from different precursors [9–11]. Previously, three different methods have been used to estimate the amount of oxalate absorbed in the gastrointestinal tract: (i) the isotopic method (applying ^{14}C-oxalate), (ii) the load method (applying unphysiologically large amounts of oxalate) and (iii) the daily excretion method (comparing the total amount of oxalate excreted as a function of oxalate in food, preferentially applying ''oxalate-free'' formula diet as the reference).

The data obtained so far and the validity of these methods have been reviewed [8]. All these methods were not suited for routine use.

An oxalate absorption test should fulfil the following requirements: (i) reproducible determination of the individual oxalate absorption; (ii) easy to perform, no hazard to patients or staff, minimal incon-

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venience to the patient; (iii) with the same patient, it must be repeatable as often as required to reach (and monitor) a sufficient and successful therapy; (iv) representative for the daily life situation of the patient.

Therefore, we apply oxalate doubly labelled with the stable carbon isotope ^{13}C in a dose representative of the normal food intake [9,10]. Unabsorbed oxalate will be excreted with the faeces or partly digested by intestinal bacteria. All the labelled oxalate absorbed will be excreted rapidly via the kidneys [9,10]. In an aliquot of the urine, the concentration of the labelled oxalate can be measured. The amount and fraction absorbed can be calculated from the ingested dose, the concentration of labelled oxalate in urine and the urine volume.

The use of stable isotope labelled oxalate in low doses requires a mass spectrometric detection of the labelled compound. In this paper, we describe the use of the conventional quantitative mass spectrometric technique of selected ion monitoring using a benchtop gas chromatography–mass spectrometry (GC–MS) system. Studies using an alternative analytical technique, gas chromatography/combustion/isotope ratio mass spectrometry (GC–C–IRMS), are under way – promising higher sensitivity.

2. Experimental

2.1. Chemicals

Sodium [$^{13}C_2$]oxalate was made from [$^{13}C_2$]oxalic acid (99+ atom% ^{13}C), Promochem (Wesel, Germany) by titration with sodium hydroxide (Titrisol, Merck, Darmstadt, Germany). The internal standard [2-^{13}C]malonic acid was obtained by saponification from [2-^{13}C]malonic acid diethyl ester (99.6 atom% ^{13}C), Aldrich (Steinheim, Germany). Unlabelled oxalic acid 99+% was from Aldrich. Oxalate standards (0.25, 0.5 and 1 mmol l^{-1}, catalogue No. 591-11) were from Sigma (Deisenhofen, Germany). *N* -Methyl-*N* - *tert*.-butyldimethylsilyltrifluoroacetamide (MBDSTFA) was from Macherey–Nagel (Düren, Germany). Acetic acid ethyl ester and hydrochloric acid 25% analytical-reagent grade were from Merck.

2.2. Equipment

The mass-selective detector Hewlett-Packard HP 5972, connected to an HP 5890 gas chromatograph with HP 7673 autosampler, was from Hewlett-Packard (Waldbronn, Germany). The HP G 1034C software, version B.02.04, for HP ChemStation was run on an HP Vectra VL2 4/50.

2.3. Volunteers and patients

Only volunteers with no previous history of urinary stones and without acute or chronic diseases were allowed to participate. In-patients of the department of urology undergoing a series of tests because of recurrent Ca-oxalate stone formation were given this test, too. The study protocol had been approved by the ethics commission of the Faculty of Medicine of the University of Bonn.

2.4. Application of [$^{13}C_2$]oxalate

The volunteers collected a 24 h blank urine. Thereafter, the disodium salt of [$^{13}C_2$]oxalate [amounts between 400 and 50 mg (2.94 and 0.37 mmol), preferentially 50 mg] dissolved in 100 ml deionized water was ingested, the glass was rinsed with 100 ml deionized water and this water was swallowed also by the volunteers. In later experiments, the labelled oxalate was given in a capsule, soluble in gastric juice. The capsule increased the chance that all the labelled oxalate was swallowed. One hour later, a breakfast consisting of two rolls with 20 g margarine and jelly was taken with mineral water. No food was allowed during the next 5 h. Six hours after the ingestion of the labelled oxalate, the volunteers had lunch. Thereafter, no restrictions on food intake were imposed. The volunteers were advised to drink 2 l during the 24 h after ingestion of the oxalate.

2.5. Sample preparation

2.5.1. Collection of urine

Urine was voided into a clean 500-ml polyethylene bottle and thereafter poured into the storage

bottle. The storage bottles, 1 or 2 l, contained 20 or 30 ml 25% hydrochloric acid to prevent precipitation of calcium oxalate or bacterial growth. All bottles were stored at 4°C until processing within a few days after the collection. The collection periods were 24 h for the blank urine on the day before the labelled oxalate and 6, 6 and 12 h after application of the labelled oxalate. The pH of the urine was measured and adjusted to 1 with 32% hydrochloric acid, if not already that acidic from the hydrochloric acid originally added. The urine volumes were measured and three 1.2 ml aliquots of each fraction were stored in microcentrifuge tubes at −20°C until work-up. Urines could be stored up to three months at −20°C.

2.5.2. Work-up

A set of urine samples from one application (blank, 0–6, 6–12 and 12–24 h) was allowed to thaw at room temperature. Two 0.1-ml aliquots of each urine after application of labelled oxalate were transferred into polypropylene reaction vials. Simultaneously, the calibration samples (containing 0, 5, 10 and 20 mg [$^{13}C_2$]oxalic acid l^{-1} of urine) were worked up in duplicate. To all urine samples, 30 μl of the internal standard solution (0.5 mmol [2-^{13}C]malonic acid l^{-1}) were pipetted. To the analytical samples, 40 μl of water were added. To the calibration samples made from the blank urine of each volunteer or patient, 40 μl of water or the appropriate labelled oxalic acid solution were added. After addition of 200 μl 25% hydrochloric acid, the aqueous phase was extracted with 1 ml ethyl acetate. After 1 min shaking on a vortex mixer and 2 min centrifugation at 6000 *g*, 500 μl of the ethyl acetate phase were transferred into a clean reaction vial and washed with 50 μl of 25% HCl to remove the majority of the remaining urea. After shaking and centrifugation as above, 250 μl of the ethyl acetate phase were transferred in the final reaction vial. The samples were lyophilized. To the dry samples, 100 μl isooctane and 25 μl MBDSTFA were pipetted. Samples were allowed to react overnight at atmospheric pressure in a desiccator containing silica gel with moisture indicator. The solutions were transferred into autosampler vials with so called 100 μl inserts; the vials were capped tightly.

2.6. Measurement

The autosampler was programmed to perform two sample pumps, inject 1 μl and wash six times with isooctane.

2.6.1. GC conditions

The carrier gas was helium, purity 99.999%, inlet pressure 180 kPa. The column was a HP 5, 50 m×0.25 mm I.D., 0.25 μm film thickness, 5% phenylmethylsilicone, crosslinked, fused-silica capillary from Hewlett-Packard. Injector temperature 240°C, purge on 0.4 min, interface temperature 280°C. The temperature program was 1 min isothermal 90°C, increase to 150°C with 30°C/min, increase to 230°C with 10°C/min, 2 min isothermal, increase to 300°C with 30°C/min, 5 min isothermal.

2.6.2. MS condition

The $(M-57)^+$ ions (M-*tert*.-butyl radical) from the bis-(*tert*.-butyl-dimethylsilyl) derivatives were measured after 70 eV electron impact ionization: m/z 261.1, 263.1 and 276.1 for oxalic, doubly-labelled oxalic and singly-labelled malonic acid. Dwell time per ion was 0.1 s, solvent delay was 9.6 min, measurement of masses m/z 261.1 and 263.1 lasted from 9.6 to 10.6 min, the internal standard (I.S.) mass was measured from 10.6 to 11.4 min.

Prior to the start of each series, the MS was tuned and one blank sample was run with the temperature program in scan mode. Scans were repeated until no more interferences from previous measurements of other analytes were detected. Thereafter, a selected ion monitoring run with spaced masses was performed to localize the maximal sensitivity for the analytes on the mass scale. The analysis series was started with parameters adjusted, if required. The results from the first three injections were always excluded from calculations. These injections served as further equilibration runs.

2.7. Calculation of absorption

The amounts of [$^{13}C_2$]oxalate excreted in the urines collected 0–6, 6–12 and 12–24 h after ingestion of the labelled dose were calculated from the concentrations measured and the volumes of the

corresponding fractions. The sum of these three amounts was expressed as percent of the dose.

3. Results

3.1. Sensitivity

For a GC–MS assay, oxalate was always present in high concentrations, about 0.1–0.5 mmol l^{-1}, in patients up to maximal 1.3 mmol l^{-1} of urine. So the sensitivity for unlabelled oxalic acid was of no concern. Because of the natural isotope content of carbon, oxygen and especially silicon there was always about a 10% signal on the mass for the doubly labelled oxalate even in the absence of labelled oxalate. The limit of detection was therefore determined by the standard deviation (S.D.) of the ratio of the signals at masses m/z 263 and m/z 276. This ratio had a S.D. of ± 0.0013 [$n=20$, concentration of unlabelled oxalate was 17.0 mg (0.19 mmol) l^{-1}, data from the patient reported in Table 2]. Three S.D.s above the mean gave a limit of detection of 0.07 mg (0.78 pmol) l^{-1}. If 50 mg of the disodium salt of [$^{13}C_2$]oxalate corresponding to 33.8 mg (0.37 mmol) [$^{13}C_2$]oxalic acid were given and only 1% were excreted in the 24 h urine, this amount could go undetected. However, low absorptions are of no clinical concern. Only in one case, an absorption of 0% was actually measured. After an extended discussion on the reliability of the measurement, the volunteer was visited at home. After intensive questioning, the unused capsule with the labelled oxalate was recovered (this volunteer did not belong to the laboratory crew).

3.2. Interferences

In measurements performed on blank urines of volunteers and patients with different diseases measured in the scan mode to check for interferences, there were no interferences at the retention time and mass for the oxalic acids and the internal standard.

3.3. Reproducibility of the assay

Over the last three years, more than a dozen determinations of the coefficient of variation (C.V.) have been performed. Typical values were about 10% for high concentrations and 15% for normal concentrations of labelled oxalate (data not shown). The best values are shown in Table 1. One spiked urine sample representative for a high absorption and one for a normal absorption were worked up ten times each. ''High'' urine was representative for a

Table 1
Urines representative for a hyperabsorbing patient and a normal volunteer were prepared from blank urine

	''High'' urine		''Normal'' urine	
	$^{12}C_2H_2O_4$	$^{13}C_2H_2O_4$	$^{12}C_2H_2O_4$	$^{13}C_2H_2O_4$ found (mg l^{-1})
	87.49	10.06	8.54	1.00
	90.31	10.43	8.09	0.94
	82.28	9.52	8.21	0.93
	80.01	9.21	8.50	0.95
	84.96	9.72	7.99	0.89
	84.56	9.68	7.77	0.89
	83.03	9.40	8.36	0.96
	84.66	9.83	7.86	0.87
	86.99	9.79	8.12	0.91
	86.35	9.73	7.62	0.88
Mean	85.06	9.74	8.11	0.92
C.V. (%)	3.44	3.54	3.82	5.02

''High'' urine contained 89 mg (0.99 mmol) unlabelled oxalate l^{-1} and 10 mg (0.109 mmol) [$^{13}C_2$]oxalate l^{-1} and ''normal'' urine contained 8.6 mg (95.6 nmol) unlabelled oxalate l^{-1} and 1 mg (10.9 nmol) [$^{13}C_2$]oxalate l^{-1}.
Values are means of two injections from each of the 20 vials.

patient with short bowl syndrome, ''normal'' urine was typical for a healthy volunteer after a 50 mg $Na_2[^{13}C_2]$oxalate dose. The results of the within-run reproducibility are shown in Table 1. These data also show a slight drift of the instrument resulting in lower mean values. The poor reproducibility, as compared to an assay with a stable isotope labelled internal standard, is the result of the unavoidable use of malonic acid as the internal standard. There was a retention of 54 and 60±5% of the oxalic and malonic acid in the ethyl acetate phase during the wash step with 50 μl hydrochloric acid, i.e., a fractionation of analyte and I.S. In the same series, where we found a C.V. of 15% for the ratio labelled oxalate to I.S., we found a C.V. of typically 1.5 to 2.5% for the ratio of labelled oxalate to unlabelled oxalate.

3.4. Comparison with other assays

Although it was not intended to use this assay merely for the measurement of oxalate in urine, these measurements were always performed. Aliquots of the urines were also measured by the two methods described in Ref. [15], an enzymatic method with oxalate oxidase and an HPLC–enzyme reactor method. The results of these measurements were compiled in Table 3 for 12 volunteers.

Table 3
Amounts of unlabelled oxalate in 24 h urines (mmol/24 h) from 12 female volunteers from the day before the labelled oxalate was given, measured by three procedures (GC–MS=this assay, enzymatic=Sigma kit No. 591 and HPLC–ER=HPLC with enzyme reactor as described in Ref. [15])

Volunteer No.	GC–MS	Enzymatic	HPLC–ER
1	0.341	0.353	0.291
2	0.294	0.303	0.273
3	0.327	0.344	0.295
4	0.350	0.361	0.319
5	0.226	0.230	0.248
6	0.344	0.365	0.386
7	0.484	0.458	0.466
8	0.280	0.269	0.271
9	0.360	0.397	0.365
10	0.380	0.412	0.414
11	0.321	0.336	0.392
12	0.313	0.369	0.307
Mean	0.335	0.350	0.336
S.D.	0.062	0.062	0.068
C.V. (%)	18.6	17.6	20.1

3.5. Reproducibility of the absorption test measurement

Urines from one absorption test with a patient who was a low oxalate absorber were worked up ten times in duplicate, two work-ups on one working day. The data are shown in Table 2. The C.V. for the calculated oxalate absorption was 15.2%.

3.6. Time requirements

A technician could work up in duplicate the urines of two absorption tests in half a working day. Starting the mass spectrometric measurements including tuning and scans for quality control required 2 h. The measurements including two reruns of one calibration sample with 5 mg l^{-1} labelled oxalic acid at the end of the series took 36 h. Control of the

Table 2
Results from five work-ups in duplicate on five different days from urine samples from the same absorption test with 50 mg $Na_2[^{13}C_2]$oxalate, corresponding to 33.8 mg or 0.37 mmol $[^{13}C_2]$oxalic acid, patient C.W., a recurrent calcium oxalate stone former but a low oxalate absorber

	Work-up No.										Mean±S.D.	C.V. (%)
	1 and 2		3 and 4		5 and 6		7 and 8		9 and 10			
$[^{13}C_2]$oxalic acid												
Amount (mg) 0–6 h	0.76	0.60	0.89	0.52	0.98	0.81	0.82	0.76	0.92	0.83	0.789±0.140	17.8
Amount (mg) 6–12 h	0.10	0.19	0.05	0.16	0.11	0.12	0.12	0.13	0.22	0.13	0.133±0.05	35.8
Amount (mg) 12–24 h	0.03	0.04	0.00	0.03	0.00	0.00	0.02	0.02	0.11	0.05	0.030±0.033	110
Sum 0–24 h	0.89	0.83	0.94	0.71	1.09	0.93	0.96	0.91	1.25	1.01	0.952±0.146	15.3
% Absorption calculated	2.6	2.5	2.8	2.1	3.2	2.7	2.8	2.7	3.7	3.0	2.81±0.428	15.2

measurements, transfer into an especially prepared excel sheet, calculation of concentrations, amounts, and the percentage of dose absorbed and printing the results took another 2 h.

4. Discussion

The *tert*.-butyldimethylsilyl-derivatives used allowed the measurement on the general available electron impact ionization mass spectrometers. They produced positive ions ($M^+ - tert.$-butyl radical) containing both carbons of the oxalic and all three carbons of the malonic acid. The same derivatives could be used in an GC–C–IRMS assay allowing comparisons of both assays with identical samples. The use of the higher homologue of oxalic acid, malonic acid, is an undesirable but unavoidable choice. The monolabelled form of malonic acid was used to minimize any interference from endogenous malonic acid.

The long analysis time is undesirable. The drift of the mass spectrometer produces deviations proportional to time. But as we were using the GC–MS together with the owner, who needed the 50 m capillary column, the obvious choice of a shorter capillary could not be realized. The data on reproducibility in Table 2 represent realistic and not selected good data. Runs 3 and 4 were interrupted during the night for unknown reasons. The runs were restarted the next morning.

With the method described it was possible to make the measurements required to asses the extent of gastrointestinal absorption of oxalic acid [12]. The urines of 30 patients and 12 volunteers undergoing the oxalate absorption test were measured with the assay described. The range of absorption was between 1 and 48%.

The decisive advantage of the use of ^{13}C-labelled oxalic acid as compared to ^{14}C-labelled oxalic acid was the repeatability of this absorption test. After two doses of ^{14}C-labelled oxalate the total dose of radioactivity allowed for volunteers was almost exhausted [13]. Two of our volunteers have taken ^{13}C-labelled oxalate more than 20 times, several times in higher doses. The advantage of this test compared to the loading test (even if the loading test was performed with [$^{13}C_2$]oxalate [14]) was the more physiological dose of oxalate in our absorption test. As an alternative to the standardized test – in which type and amount of liquid, type and amount of food, calcium content of food and liquid and a set of less obvious parameters were kept constant – the absorption test could also be performed easily in the patients home under the normal diet of the patient. Differences between the standardized hospital conditions and the normal life conditions may reveal dietary causes of hyperoxaluria. The oxalate absorption test could further monitor the success of an adjusted diet and success or failure of therapy. An additional advantage of the oxalate absorption test as compared to the ''oxalate free diet'' is the fact, that our test measured only the absorption. The oxalate free formula diet may also not contain certain components of the normal food that were substrates for endogenous oxalate synthesis, resulting in an overestimation of the absorption.

Further research is needed to clarify the effects of food components and possibly other factors on the absorption of oxalate. Also, the use of GC–C–IRMS should allow the application of smaller (that is as well cheaper as even safer) doses of labelled oxalate.

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