Improvement and Validation of D-Xylose Determination in Urine and Serum as a New Tool for the Noninvasive Evaluation of Lactase Activity in Humans

Carmen Hermida,^{1,2†} Oscar H. Martínez-Costa,^{1‡} Guillermo Corrales,³ Cristina Teruel,⁴ Valentina Sánchez,¹ José J. Sánchez,⁵ Dolores Sarrión,⁴ María J. Ariza,⁴ Rosa Codoceo,⁴ Idoia Calvo,⁶ Alfonso Fernández-Mayoralas,³ and Juan J. Aragón^{1*}

¹Departamento de Bioquímica and Instituto de Investigaciones Biomédicas Alberto Sols, Facultad de Medicina de la Universidad Autónoma de Madrid, Universidad Autónoma de Madrid-Consejo Superior de Investigaciones Científicas, Madrid, Spain

²Venter Pharma SL, Madrid, Spain

³Instituto de Química Orgánica General, Consejo Superior de Investigaciones Científicas, Madrid, Spain

⁴Servicio de Bioquímica, Sección de Gastroenterología y Nutrición, Hospital Universitario La Paz, Madrid, Spain

⁵Departamento de Medicina Preventiva y Salud Pública y Microbiología, Facultad de Medicina de la Universidad Autónoma de Madrid, Madrid, Spain

⁶CibNavarra, Pamplona, Spain

Background: The phloroglucinol assay is the current method for D-xylose determination in urine/plasma/serum. However, its sensitivity is limited when low amounts of D-xylose are to be measured, such as in the noninvasive evaluation of intestinal lactase with 4-galactosylxylose (gaxilose). An improved assay was therefore needed. **Methods:** We developed and validated a modified version of the phloroglucinol-based assay for quantification of D-xylose in urine/serum samples. A method for gaxilose determination by gas chromatography (GC) was also optimized. **Results:** Linearity ranged from 0.125 to 5.0 mg/l (5–200 mg/l in original sample). Accuracy at LOQ (0.125 mg/l) was 0.97/2.49% in spiked urine/serum; for other quality controls (QC), it was <1.27%.

Intra- and interassay precision at LOQ were 6.02% and 6.45% for urine, and 8.86% and 10.00%, respectively, for serum; for other QC, precision was <2.15%. Linearity of gaxilose determination by GC was 3.90–195.17 for urine and 9.75–195.17 mg/l for serum with acceptable sensitivity and reproducibility. The method proved adequate for the D-xylose determination in healthy and hypolactasic subjects after oral administration of gaxilose. **Conclusions:** The modified method provides high sensitivity and robustness for D-xylose quantification in urine/serum for routine clinical use especially in the noninvasive diagnosis of intestinal lactase deficiency with the gaxilose test. J. Clin. Lab. Anal. 28:478–486, 2014.

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†Correspondence to: Carmen Hermida, Departamento de Bioquímica, Facultad de Medicina de la Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain. E-mail: carmen.hermida@uam.es

‡Correspondence to: Oscar H. Martínez-Costa, Departamento de Bioquímica, Facultad de Medicina de la Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain. E-mail: oscar.martinez@uam.es

*Correspondence to: Juan J. Aragón, Departamento de Bioquímica, Facultad de Medicina de la Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain. E-mail: juanjose.aragon@uam.es

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INTRODUCTION

D-Xylose testing is of clinical value for the diagnosis of small intestine diseases, principally intestinal malabsorption (1,2). The test involves an oral dose of D-xylose with determination of urinary D-xylose after an appropriate collection time (usually 5 hr), and/or D-xylose measurement in a blood sample taken 1–2 hr after ingestion. Several methods have been reported in the literature for the determination of D-xylose in urine, plasma, and serum (3–8). Of these, the most widely used is the phloroglucinol colorimetric method reported by Eberts et al. (6) due to its simplicity and reliability. In addition, the equipment required is available in almost all clinical laboratories.

A new simple method for the evaluation of intestinal lactase activity *in vivo* has been developed and optimized in experimental animals (9) and recently used in humans (10), with potential advantages over current tests for the noninvasive diagnosis of lactase deficiency or hypolactasia. This disorder, which can lead to symptoms of lactose intolerance, is mainly genetically determined, affecting more than half of the adults in the world (11–13). The new methodology assesses intestinal lactase activity *in vivo* by using the synthetic compound 4-galactosylxylose (from here on referred as to gaxilose) (14, 15), a disaccharide analogue of lactose. Following oral administration, gaxilose is hydrolyzed by intestinal lactase into the physiological products galactose and D-xylose. D-Xylose is passively absorbed from the small intestine (16) and its levels can be determined either in blood or urine as a measure of total lactase activity *in vivo* (9). Eberts et al.'s procedure (6) is, however, not sensitive enough to determine accurately the low amounts of urine and serum D-xylose derived from gaxilose (9, 17). Therefore, an improvement of the colorimetric method for D-xylose quantification was needed to conduct clinical studies with the gaxilose test as well as for its use in clinical practice.

In this work, we have modified and validated the traditional phloroglucinol-based method for D-xylose measurements in biological fluids. The validated assay was shown to be specific, linear, sensitive, precise, and accurate in the range of concentrations needed for the determination of the D-xylose derived from gaxilose in human urine or serum samples. Additionally, we also developed and optimized a sensitive and precise method for the quantification of gaxilose by gas chromatography (GC). Furthermore, results of using these methods in normolactasic and hypolactasic subjects after orally administered gaxilose are shown.

MATERIALS AND METHODS

Chemicals and Reagents

D-Xylose and pyridine were from Panreac (Barcelona, Spain). Phloroglucinol (1,3,5-trihydroxybenzene) was

from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid was from Prolabo (Fontenay-sous-Bois, France). *N*-trimethylsilyl-imidazol was from Fluka (Buchs, Switzerland). Concentrated hydrochloric acid and trichloroacetic acid were from Merck (Darmstadt, Germany). All other reagents were of analytical grade. Gaxilose (4-*O*-β-D-galactopyranosyl-D-xylopyranose) was provided by Derivados Químicos S.A. (Murcia, Spain). The experimental details of gaxilose synthesis have been previously reported (15). For GC analysis, an internal standard solution (ISTD) containing 80 mg sucrose in 200 ml pyridine was used.

Equipment and Instrumental Method for Colorimetric and GC Measurements

The absorbance in the colorimetric assay of D-xylose was read using a spectrophotometer (Model Hitachi 150/20 data processor) set at 554 nm with a 1-cm light path using 1 ml glass OS-cuvettes (Hellma Analytics, Hellma GmbH & Co. KG, Germany). GC measurements were performed on a DB-1701 column (30 m) with 0.32 mm id and 0.25 μm film, using a Hewlett-Packard GC 5890 equipped with a flame detector. Helium was used as carrier gas (15 psi in the head, 1 ml/min). Three microliters of sample were introduced by splitless injection (split ratio, 80:1). The temperatures of injector and detector were set at 220°C and 260°C, respectively. The column temperature program was from 170°C (hold 9 min) to 210°C at 20°C/min (hold 40 min).

Sample Collection and Preparation

For quality controls (QC), 25 human urine and venous blood samples were obtained from healthy subjects who attended La Paz University Hospital (Madrid, Spain). Serum was immediately separated from blood samples by centrifugation at $2,500 \times g$ for 10 min at 4°C and stored at –20°C until use. QC of urine and serum were prepared with the same D-xylose or gaxilose solution used for the calibration curves. For all assays, a fresh stock solution of D-xylose and the corresponding working dilutions were prepared. Serum samples to be used in D-xylose determination and for GC analysis of gaxilose required a previous deproteinization step with 10% trichloroacetic acid.

D-Xylose and gaxilose determination in urine and serum after oral gavage of gaxilose was performed in six hypolactasic subjects (with lactase activity in small intestine biopsy <10 U/g protein (18)) enrolled in a phase IIb–III multicenter, open label, nonrandomized trial (EUDRA-CT:2006–002793–21), designed to address the diagnostic performance of the gaxilose test (to be published elsewhere). The subjects were given either 0.45- or 2.7-g gaxilose dose for urine or blood analyses,

respectively, essentially as described previously in a phase I, dose-finding clinical trial (EUDRA-CT:2005-001899-12), which included 12 healthy subjects (10). These studies were conducted in accordance with the Declaration of Helsinki and approved by the corresponding ethics committee.

Measurement of D-Xylose

D-Xylose determination was carried out using a modification of the method reported by Eberts et al. (6). The changes were (a) final reaction volume was reduced from 5 to 2 ml; (b) the calibration curve range was changed from 1.035–19.6 mg/l to 0.125–5.0 mg/l; (c) serum samples were deproteinized; and (d) phloroglucinol reagent was freshly prepared. Samples of 50 μ l urine, serum, or aqueous solutions containing D-xylose were mixed with 50 μ l of deionized water in disposable test tubes, and 1.9 ml of phloroglucinol color reagent was added. All tubes were incubated for 4 min at 100°C, and then cooled to room temperature in water. After mixing, the absorbances were read at 554 nm. Calibration curves of D-xylose were obtained by appropriate serial dilution of 100 mg/l stock solution. D-Xylose assay concentrations of standard curve were 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 5.0 mg/l.

Analytical Performance of D-Xylose Determination

All assay validation procedures were carried out according to current guidelines (19, 20), which include linearity, specificity, selectivity, intra- and interassay precision and accuracy, lower limit of detection (LOD), lower limit of quantification (LOQ), and recovery.

Comparison of Methods

Six urine and six deproteinized serum samples spiked with 5–400 mg/l of D-xylose were simultaneously measured by the currently used method for D-xylose determination (6) in clinical laboratory, and by using the modified method. Linearity, range of D-xylose concentration, and LOQ were calculated for both methods.

Stability of D-Xylose Samples and of Phloroglucinol Color Reagent

Stability of D-xylose in urine and serum was studied at -70°C , -20°C , 4°C or room temperature, and after freeze/thawing cycles at -70°C and -20°C . Stability of D-xylose stock solution (100 mg/l) at -20°C was investigated weekly during 1 month. Stability of the phloroglucinol color reagent was assessed over 4 days.

Gaxilose Measurement by GC

Calibration mixtures for GC measurements were obtained from solid gaxilose, and from solutions of gaxilose in urine and serum. Standard stock solutions were prepared in triplicate by dissolving gaxilose (19.5 mg) in 50 ml of ISTD solution containing the internal standard sucrose. Calibration curves for solid gaxilose were obtained by appropriate dilution from standard stock solution to yield concentrations ranging from 0.39 to 195.17 mg/l. An aliquot (20 μ l) of the resulting solution was treated with *N*-trimethylsilyl-imidazole (20 μ l) at 60°C for 30 min and analyzed by GC. The spiked urine and serum samples were prepared by appropriate dilution of an aqueous solution of gaxilose (100 mM) with the corresponding media. An aliquot (50 μ l) was lyophilized and stored at -70°C until use. The residue was treated with ISTD and analyzed by GC as described above. Final concentrations (mg/l) refer to the amount of gaxilose in the silylating mixture.

Statistical Analysis

Results of continuous variable are presented as the mean (SD). Groups (calibration curves, and intra- and between-day, intra- and interassay, and stability data) were compared by Student's *t*-test. A *P*-value (two-tailed) ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 (Chicago, IL). The linear equation, the Pearson coefficient (*r*), and the determination coefficient (R^2) were calculated.

RESULTS

Analytical Performance of D-Xylose Measurement in Urine and Serum

The linearity was evaluated by between- and within-day analyses of D-xylose at seven different assay concentrations ranging from 0.125 to 5.0 mg/l. Data were collected from three, four, and three calibration curves for days 1, 2, and 3, respectively. The constructed calibration curve is shown in Figure 1. The responses were linear ($R^2 \geq 0.9998$). No significant difference ($P > 0.4$) was found between the ten calibration curves or between- and within-day assays, with mean coefficient of variation (CV) of 1.65%. Statistic analyses of the calibration curve are given in Table 1. The LOD was 0.034 mg/l and the LOQ determined as the mean plus ten SD of 50 blank replicates was 0.115 mg/l. Almost identical values (0.125 mg/l) were obtained when LOQ was evaluated as the lowest concentration at which the CV was still below 20%, by using a series of low concentration calibrators (ranging from 0.025 to 0.125 mg/l), as well as by analyzing the data of spiked

Fig. 1. Calibration curve for D-xylose. Linearity of D-xylose test was evaluated by intra- and interday analysis of ten calibration curves over a period of 3 days at seven different concentrations spanning a range of 0.125 to 5.0 mg/l. Each D-xylose concentration was prepared and measured five times ($n = 50$). SD values of the means do not exceed the height of the symbols.

urine and serum samples (see below). Thus, 0.125 mg/l was accepted as the lower LOQ of the assay.

The linear range of the assay was shown to be 0.125–5.0 mg/l. This allows, therefore, the determination of D-xylose in a sample at concentrations ranging from 5 to 200 mg/l. Thus, 5 mg/l is the minimal concentration of D-xylose in a given sample that can be adequately detected when a 50 μ l sample is used.

The potential interferences of urine and serum compounds with D-xylose determination were studied. Mean concentration of apparent D-xylose in blank urine samples was 0.007 ± 0.008 mg/l ($n = 50$), corresponding to

5.6% of the assay LOQ value. Thus, urine components did not interfere with the assay. In contrast, we found higher values of apparent D-xylose level in assays of blank sera [0.348 ± 0.071 mg/l ($n = 50$), equivalent to 13.92 ± 2.84 mg/l in serum samples]. As glucose reactivity with phloroglucinol under similar assay conditions has been previously reported (6,21), our findings may result from the basal levels of glucose in serum. To examine this effect, we analyzed aqueous samples containing 5.5 mM glucose (physiological concentration), which yielded an apparent D-xylose assay concentration of 0.908 ± 0.014 mg/l ($n = 15$). Thus, glucose reacted with phloroglucinol to an extent of around 1.83% for a 5.5 mM solution of this sugar, which is in agreement with data reported by others (6,21).

The within- and between-day accuracy and precision were assessed over 3 days in urine and serum spiked with D-xylose at 5, 20, 80, and 160 mg/l to yield assay concentrations of 0.125 (LOQ), 0.5 (low QC), 2.0 (medium QC), and 4.0 (high QC) mg/l, respectively. The mean accuracy ranges were 99.03–99.87% and 97.51–99.84% for spiked urine and serum, respectively (Table 2), while the corresponding bias% oscillated between -0.13 and -2.49 . Remarkably, deproteinization of serum samples by trichloroacetic acid precipitation needed to increase sensitivity was very efficient as D-xylose recovery was unaffected. From intra- and interassay measurements in spiked samples, the mean CVs were not greater than 7 and 10% for urine and serum (Table 2), respectively, and notably these values were observed at the LOQ concentration. D-Xylose concentrations below 0.125 mg/l showed CV values of more than 20% (data not shown). As seen in Table 2, the between-day RSD% values were 0.35–5.29% and 0.45–2.84% for spiked urine and serum samples, respectively.

TABLE 1. Calibration Statistics and Analysis Limits^a

Parameter	
Calibration range, mg/l	0.125–5.0
Linear slope $\pm$ SD, mg/l	0.1221 ± 0.0021
95% CI, mg/l	$0.1205 - 0.1236$
Linear intercept $\pm$ SD	0.0101 ± 0.0031
95% CI	$0.0079 - 0.0124$
R^2	0.9998
LOD, mg/l	0.034
LOQ ($m + 10$ SD ^b /CV < 20% ^c), mg/l	0.115/0.125

CI, Confidence Interval.

^aLinearity of D-xylose assay was evaluated by the analysis of ten calibration curves over a period of 3 days at seven different concentrations spanning from 0.125 to 5.0 mg/l. Each D-xylose concentration was prepared and measured five times ($n = 50$).

^b $m + 10$ SD, LOQ calculated as mean of the 50 replicates of blank samples plus ten times SD.

^cCV < 20%, LOQ measured as the last concentration with CV less than 20%.

Comparison of Methods

Calibration curves and QC of urine and serum samples were analyzed by using the modified method and that described by Eberts et al. (6) simultaneously. Final assay concentrations of D-xylose were 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 5.0 mg/l for our method, and 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 5.0, 7.5, 10.0, and 12.5 mg/l for Eberts et al.'s procedure. Standard D-xylose measurements of six replicates on two different days were linear within the concentration tested in both methods ($R^2 \geq 0.9993$). The CV value was below 10% for the lowest concentration (0.125 mg/l) and did not exceed 4% for the other calibrators in both procedures. In our method, recoveries were 98.00–105.77% and 96.86–103.65% for spiked urine and serum samples, respectively, in agreement with data obtained in the previous section and confirming a LOQ value of 0.125 mg/l. Likewise CV values were below 10%. Conversely, with the

TABLE 2. Assessment of Accuracy and Precision of D-Xylose in Urine and Serum^a

Nominal concentration (mg/l)	Calculated concentration ^b (mg/l)	Accuracy (%)	Bias	Precision (CV)		RSD% Between day
				Intra-assay (%)	Interassay (%)	
Urine						
0.125 (LOQ)	0.124 ± 0.007	99.03	−0.97	6.02	6.45	5.29
0.5	0.495 ± 0.009	99.03	−0.97	1.86	1.83	0.91
2.0	1.997 ± 0.017	99.87	−0.13	0.87	0.97	1.08
4.0	3.982 ± 0.019	99.54	−0.46	0.47	0.48	0.35
Serum						
0.125 (LOQ)	0.122 ± 0.011	97.51	−2.49	8.86	10.00	2.84
0.5	0.494 ± 0.011	98.73	−1.27	2.15	2.15	0.65
2.0	1.997 ± 0.023	99.84	−0.16	1.14	0.97	0.98
4.0	3.953 ± 0.024	98.82	−1.18	0.60	0.63	0.45

^aSix different D-xylose-supplemented urine and serum samples were prepared for each D-xylose concentration over a period of 3 days, which were then split into five independent aliquots. Accuracy and intra-assay precision were assessed by analyzing each of these artificial preparations ($n = 90$). Interassay precision was evaluated by measuring three of the five aliquots ($n = 54$).

^bValues are mean ± SD.

Eberts et al.'s test, 1.0 mg/l was the lowest assay concentration from urine and serum samples, in which CV was below 20% (17.56 and 19.08%, respectively). Thus, this concentration was defined as LOQ. Recoveries were out of the recommended range 80–120% for concentrations below LOQ, and fluctuated from 90.89–112.78% and from 95.61–107.06% for urine and serum samples, respectively, at concentrations 1.0–12.5 mg/l. With Eberts et al.'s method, we were therefore able to measure D-xylose in urine and serum within a concentration range of 50–400 mg/l, compared to a range of 5–200 mg/l with the modified method.

Stability of D-Xylose in Urine and Serum and of Phloroglucinol Reagent

Urine and serum D-xylose samples at 2.0 mg/l were stable at -20°C and -70°C (Fig. 2A and B, respectively) for up to 2 months, with no significant changes in D-xylose recovery and CV of the frozen samples over time 0 ($P > 0.05$). As shown in Figure 2C, 2.0 mg/l D-xylose was stable in urine samples at 4°C for up to 24 hr, but significant variability (CV values $\geq 17\%$) was observed thereafter. In contrast, serum D-xylose was stable during the 3-day test period. At room temperature, urine and serum samples were stable for at least 8 hr (Fig. 2D). Similar results were obtained for D-xylose concentrations of 0.125 and 0.5 mg/l at -20°C , -70°C , 4°C and room temperature (data not shown). We also examined the stability of 0.125, 0.5, and 2.0 mg/l D-xylose in urine and serum stored at -20°C and -70°C after three freeze/thawing cycles. Recovery and CV analyses over 15 days ($n = 9$ for each day and concentration) revealed no significant variation ($P > 0.05$) in any of the samples tested. Stability of D-xylose stock solution (100 mg/l) was minimally affected

after storage at -20°C during 1 month, the mean recovery ranging from 98.86% to 100.07%, 98.34% to 99.22%, and 99.88% to 101.36% for final assay D-xylose levels of 0.125, 0.5, and 2.0 mg/l, respectively ($n = 5$ for each day and concentration tested).

We then examined the stability of the phloroglucinol reagent. Calibration curves were performed at 0, 1, 2, 3, 4, 5, 6, 8, 24, 48, 72, and 96 hr using the reagent prepared at 0 hr ($n = 10$ and 5, for 0 mg/l and other calibrators, respectively). Regression analyses revealed that, although linear, the coefficients of determination and slopes decreased over time (from 0.9999 and 0.1235 to 0.9971 and 0.0639 at time 0 and 96 hr, respectively). LOQ for each calibration curve showed values of 0.115, 0.117, 0.131, 0.181, 0.311, and 0.418 mg/l at 0, 5, 8, 24, 48, and 96 hr, respectively, pointing to decreased sensitivity after 5 hr.

The effect of delaying the absorbance reading of the reaction mixtures was analyzed using three urine and serum samples spiked with D-xylose concentrations of 0.125 (LOQ), 0.5 and 2.0 mg/l at 0, 1, 2, 3, 4, 5, 8, 24, and 48 hr ($n = 9$ at each time and concentration). Recoveries were 86.33–101.29% during the first 3 hr at 0.125 mg/l and below 80% from 4 hr. At 0.5 and 2.0 mg/l, recoveries remained above 85% during the first 4- or 5-hr period, respectively, but were shown to be significantly different with respect to time 0 ($P \leq 0.03$).

Development and Optimization of the Method for Gaxilose Measurement by GC

We have developed and optimized a GC method to analyze gaxilose in urine and serum samples. Typical chromatograms for urine and serum samples spiked with gaxilose are shown in Figure 3. Gaxilose elutes in 45 min,

Fig. 2. Analysis of D-xylose stability in urine and serum at -20°C (A), -70°C (B), 4°C (C), and room-temperature (D). Three different urine and serum samples were prepared at a D-xylose concentration of 2.0 mg/l. These preparations were then divided into aliquots and stored at different temperatures for the indicated time, whereupon three aliquots of each condition were analyzed ($n = 9$, except for the 24-hr urine samples at room temperature, the 24-hr and 7 days serum samples at room temperature and 4°C [$n = 6$], and the 48-hr serum samples at room temperature [$n = 3$]). Microbial contamination of the missing samples prevented a reliable determination of D-xylose content and, therefore, were not included.

well-separated from other urine and serum components that have lower retention times. In the case of the serum samples, we could identify the peaks assigned to physiological glucose (see Fig. 3B).

Linearity was analyzed from data of three replicates at seven gaxilose concentrations (0.39–195.17 mg/l). Excellent linearity was found from 3.9 to 195.17 mg/l. The linear equation was $y = 3.2122x - 0.0019$ ($R^2 = 0.9999$) for solid gaxilose, $y = 3.4131x - 0.0018$ ($R^2 = 1.000$) for gaxilose in urine, and $y = 3.7027x - 0.0078$ ($R^2 = 0.9993$) for gaxilose in serum (where x indicates the concentration of gaxilose and y denotes the ratio of the peak area of gaxilose to internal standard sucrose).

As shown in Table 3, for all mean values (peak area ratio of gaxilose to internal standard) in urine samples at concentrations higher than 3.90 mg/l, the recovery values were above 80% and the coefficients of variation were below 15%. In the case of serum samples, recovery values were above 80% and the coefficients of variation were below 15% for all serum samples at concentrations higher than 9.75 mg/l. Consequently, the LOQ value of gaxilose

in urine and serum samples by GC was established at 3.90 and 9.75 mg/l, respectively.

D-Xylose Determination in Urine and Serum Samples Following Oral Administration of Gaxilose to Human Subjects

Figure 4 shows the determination of urine and serum D-xylose after oral administration of 0.45- and 2.7-g dose of gaxilose (the doses recommended for the gaxilose urine and blood test, respectively) to six hypolactasic adult subjects from the phase IIb–III study compared with mean values obtained from 12 normolactasic subjects (10). Gaxilose ingestion led to progressive elimination of D-xylose in the urine (Fig. 4A). The amounts of D-xylose in 4- and 5-hr urine (the collection periods chosen for gaxilose urine test (10)) of hypolactasic subjects after gaxilose administration ranged from 12.84 to 24.53 and from 13.98 to 27.25 mg, respectively, these values being significantly lower than the mean value determined in normolactasic subjects (57.24 ± 4.81 and 65.05 ± 5.10 , respectively), and

Fig. 3. Gas chromatograms of derivatized samples from human urine (A) and serum (B) solutions containing gaxilose (195.17 mg/l) and internal standard (sucrose, 200 mg/l).

TABLE 3. Recoveries of Gaxilose in Urine and Serum Samples

Gaxilose added (mg/l)	Urine Recovery (%)	CV (%)	Serum Recovery (%)	CV (%)
0.39	58.40	56.32	90.06	58.70
0.98	172.40	53.80	48.75	47.77
1.95	135.50	28.66	61.37	21.81
3.90	105.73	10.41	53.67	7.49
9.75	113.67	2.95	99.59	10.81
19.52	120.97	7.12	79.95	9.66
195.17	106.73	0.89	113.59	9.68

Each value represents the average of three independent samples.

represented 46.6–88.9% and 36.9–72.0%, respectively, of the cut-off values determined in the phase Ib study (27.58 and 37.87 mg for the 4- and 5-hr gaxilose urine test, respectively (10)). In agreement with urinary results, D-xylose increased in serum following the oral dose of gaxilose (Fig. 4B), reaching a maximum in around 90 min (which corresponds to the selected time for gaxilose blood test (10)) and diminishing thereafter. The increase from baseline in serum D-xylose in 90 min in hypolactasic subjects ranged from 1.33 to 6.14 mg/l, in contrast to normo-

Fig. 4. Elimination of D-xylose in urine (A) and D-xylose rise in serum (B) in normolactasic and hypolactasic subjects after oral administration of gaxilose. A 0.45-g (A) or 2.7-g (B) dose of gaxilose was orally administered to the subjects. Results represent means (SE) of D-xylose in cumulative urine (A) and serum D-xylose rise (B) in 12 normolactasic subjects (■; (10)), and in each of the six hypolactasic subjects (▲, ◇, □, ▽, ◆, and ●). See text for the need for correction in serum for baseline glucose levels.

lactasic subjects (21.43 ± 1.56 mg/l), and corresponded to 13.7–63.3% of the cut-off value (9.7 mg/l (10)). Of note, no gaxilose was detected in the urine and serum samples of normolactasic and hypolactasic subjects by using the optimized GC method.

DISCUSSION

The gaxilose test, based on the determination of D-xylose in urine and serum after cleavage by intestinal lactase of orally administered gaxilose, has been proposed as a new noninvasive method for the diagnosis of lactase deficiency (9, 17). D-Xylose in urine and serum samples can be accurately detected by the method described by

Ebert et al. (6), but within the range of 70–300 mg/l (6, 22) or even between 50 and 400 mg/l as observed in this work (see Comparison of Methods section). Because of the need for higher sensitivity in the gaxilose test, the basic equipment required in Eberts et al.'s procedure was retained (thereby keeping the method simple), but assay performance was enhanced by (a) a decrease in final reaction volume to 2 ml; (b) adjustment of the concentration range of the calibration curve and the sample volume; (c) precipitation of serum proteins; and (d) fresh preparation of phloroglucinol color reagent and its use within the first 5 hr. Here, we show that these modifications noticeably improved the phloroglucinol assay for D-xylose determination in urine and serum, and thus this method was validated, following the criteria of the Food and Drug Administration (19) and the European Medicines Agency (20).

The modified method demonstrated suitable linearity between 0.125 and 5.0 mg/l of D-xylose with outstanding statistical performance (Fig. 1, Table 1). The assay showed a high analytical sensitivity in aqueous, urine, and serum samples (LOQ value of 0.125 mg/l), and accuracy and precision analyses were highly satisfactory (Table 2).

Nonxylose interfering constituents of urine and serum in the determination of D-xylose using the phloroglucinol test have been described previously (6, 21). Nevertheless, we did not find any significant interference in urine samples. Even though serum proteins were precipitated without affecting D-xylose reproducibility and recoveries, glucose still constituted a major source of background signal (6). Therefore, basal serum blank needs to be subtracted, as proposed previously (6, 21) for serum-based D-xylose measurement to avoid this drawback and guarantee reliable D-xylose determinations.

From validation data on the stability of D-xylose in aqueous solution, urine, or serum, it was shown that sample storage at -20°C and -70°C did not significantly affect D-xylose measurements at least for 2 months, or upon at least three freeze/thawing cycles. By contrast, it is worthwhile recommending that storage at 4°C and at room temperature should not exceed more than 24 and 8 hr, respectively, to avoid interference from potential microbial contamination. Although the phloroglucinol reagent has been reported to be stable at room temperature for 4 days (6), our results indicated that this solution can only be used up to 5 hr after preparation without significant impairment in sensitivity and reliability of the method.

The validated method can accurately and precisely determine a D-xylose concentration in urine and serum samples from 5 to 200 mg/l, and so is more sensitive than Eberts et al.'s procedure by almost one order of magnitude. The method has been applied for monitoring D-xylose levels in serum and urine after oral administration

of gaxilose to determine optimal dose and cut-off values of D-xylose in healthy subjects (10). In agreement with previous data obtained in rats (9), the pattern of D-xylose increase in serum and its elimination in urine after gaxilose administration to healthy and hypolactasic subjects corresponded to the cleavage of gaxilose by intestinal lactase (Fig. 4). The cut-off value of serum D-xylose (defined as the increment in 90 min from baseline) for normal lactase was reported to be 9.7 mg/l for the gaxilose blood test (10). This concentration can only be accurately determined by using the validated method, and not by Eberts et al.'s procedure. As shown in Figure 4B, even in a hypolactasic subject, whose serum D-xylose level was as low as 13.7% of the cut-off value, D-xylose could still be confidently quantified. The 2.7 g dose for gaxilose blood test could be decreased to 0.45 g for 4- and 5-hr gaxilose urine test (10), because of the high sensitivity of the modified method.

In addition to the most commonly used colorimetric assay, other methods to measure D-xylose in biological fluids have been reported (7, 8, 17, 23, 24). Thus, we (17) and others (7) have developed GC procedures for urine and serum D-xylose determination, which offered good sensitivity when compared to other methods. However, GC assays may be too time consuming for use clinically, and the analytical facilities required may hamper their widespread diagnostic application. Enzymatic methods have been reported (8, 23, 24) and have yielded high specificity but have proved less sensitive and reliable than the method validated in this study for determination of D-xylose in urine and serum (unpublished results).

For the application of the gaxilose test in clinical studies, a method for the determination of this compound in biological samples was needed. Therefore, we have also developed and optimized a sensitive and precise GC assay for gaxilose in both urine and serum within the range 3.90–195.17 mg/l for urine and 9.75–195.17 mg/l for serum, which represent 10–500 and 25–500 μM in urine and serum samples, respectively. No gaxilose was detected in urine or serum after the oral administration of the synthetic disaccharide to normolactasic (10) or hypolactasic subjects. Therefore, gaxilose does not permeate the intestinal mucosa when orally administered, as suggested previously in rats (9).

In conclusion, the modified method for D-xylose measurement is simple, rapid, accurate, sensitive, precise, and suitable for routine analysis as required for the diagnosis of hypolactasia with the gaxilose test in clinical practice.

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CONFLICT OF INTEREST

Carmen Hermida is employee of Venter Pharma SL, Madrid, Spain. Alfonso Fernández-Mayoralas and Juan J. Aragón are shareholders of Venter Pharma SL, Madrid, Spain.

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