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Journal of Chromatography B, 755 (2001) 287–295

JOURNAL OF CHROMATOGRAPHY B

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Measurement of nephrolithiasis urinary markers by capillary electrophoresis☆

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Received 8 November 2000; received in revised form 30 January 2001; accepted 6 February 2001

Abstract

A previously developed method for screening organic acidurias by capillary electrophoresis has been validated for oxalate and citrate measurement in urine. Sample pretreatment is minimum, just acidification and centrifugation. Detection is by direct UV. Validation parameters of the method can be considered adequate. Response is linear for both analytes in standards and samples. The assayed ranges were 200–1000 mg/l for citrate and 10–200 mg/l for oxalate. Recoveries ranged from 99.4±3 to 101.7±2.4%, maximum imprecision in oxalate concentration was of 7.6% RSD and limits of detection in samples were 0.67 mg/l for oxalate and 25.9 mg/l for citrate, both lower than the measured values in samples. Identification of increased glyoxylic (oxoacetic acid) and glyceric acids (2,3-dihydroxy propanoic) are also included to facilitate the diagnosis.

Keywords: Oxalate; Citrate; Oxoacetic acid; 2,3-Dihydroxypropanoic acid

1. Introduction

Nephrolithiasis is a common disorder, affecting about 0.2% of the US population annually. Between 5 and 15% of Americans will suffer from renal calculi sometime during their lifetime [1], and the same rates are reached in other industrialised countries.

The majority of stones, 70–80%, are composed mainly of calcium oxalate crystals; the rest are composed mainly of calcium phosphate salts, uric acid, struvite (magnesium and ammonium phosphate), or the amino acid cystine [2].

A stone can form only when urine is supersaturated with respect to its constituent crystals. Apart from overexcretion, supersaturation can be increased by abnormal interactions between urine ions. Urine citrate forms a complex with calcium that normally reduces free calcium ion levels appreciably, so low urine citrate can raise calcium oxalate supersaturation and promote stones [3,4]. Moreover, nucleation on the surfaces of other crystalline phases is important in the formation of stones in the urinary tract and hyperuricosuria can promote stones that are pure uric acid or mixed calcium oxalate–uric acid stones [2].

☆Presented at the 29th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques, Alcalá de Henares, Madrid, 12–14 July 2000.

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00123-2

Rare genetic disorders increase endogenous oxalate production; there are two types of primary hyperoxaluria. They are characterised by elevated urinary oxalate excretion. In hyperoxaluria type I, a defect in glyoxylate metabolism is found, leading to increased oxalate synthesis. Excessive quantities of glyoxylic and glycolic acid urinary excretion occur. Type II is rare; it is characterised by excessive excretion of oxalic and L-glyceric acids with normal excretion of glycolic acid [5].

Comprehensive reviews of early methods for determining oxalate in biological fluids have been published [6–8] and only a brief account will be presented here.

The majority of the earlier methods for measuring oxalate required preliminary separation with the corresponding consumption of time and increased in potential errors.

Current methodologies for analysis of oxalate and citrate include enzymatic techniques [9–11], ion chromatography [12,13] and HPLC [14]. The last two techniques have the additional advantage of obtaining simultaneous measurement of other closely related organic acids [13,15,16]. In a comparison of five methods for oxalate analysis [17] suppressed and unsuppressed ion chromatography, as well as the enzymatic Sigma-Kit, achieve comparable results with respect to reliability and accuracy, but the enzymatic methods require more manual work.

Capillary electrophoresis has become a powerful analytical technique with an increasing impact in the clinical laboratory. The value of an analytical method is measured by how well it can be applied to ''real world'' samples. CE is proving to be especially suited for demanding analyses involving the separation of small organic molecules in complex sample matrices without pre-treatment.

Minimal sample volume and solvent consumption, coupled with high sensitivity, specificity, resolution, reproducibility, flexibility, and speed are key assets of CE.

Oxalic, together with other seven organic acids of clinical interest, was separated by CE by Willetts et al. [18] but quantitation was not evaluated. Moreover, oxalate was not detectable in the samples probably due to the noisy baseline caused by the surfactant. Indirect UV detection of short chain organic acids including oxalic and citric has been reported [19,20]. In Holmes' method [20] urine samples need 100-fold dilution, which affects limits of detection, and this dilution may be variable depending on the sample concentrations. Indirect UV detection has also been employed to determine oxalate in amniotic fluid, but in spite of amniotic fluid being cleaner than urine, it needs prior clean-up with Ag^+ resins to avoid chloride interference.

Fu et al. [21] determined oxalic acid in urine by co-electroosmotic capillary electrophoresis with amperometric detection. The paper shows research progress in this area, but it is not useful for routine work, because the detector is not commercially available.

Previous work in our laboratory [22–24] showed that the small organic molecules quoted above could be easily detected and measured by CE with direct UV absorbance.

The aim of the present work was the validation of a method for direct measurement of oxalate and citrate and identification of glyoxylic and glyceric acids. The method was applied to 29 urine samples taken in the nephrology service of Hospital Universitario La Candelaria and analysed by enzymatic methods in the clinical analysis service and statistical tests comparing both methods were made.

2. Materials and methods

2.1. Apparatus

CZE was performed on a Beckman System 5500 (P/ACE, Beckman Instruments, Palo Alto, CA, USA) equipped with a UV detector set at 200 nm, a column cartridge (Beckman Coulter, Madrid, Spain) pretreated with polyacrylamide (PAG) 37 cm in total length, with an internal diameter (I.D.) of 50 μm. The detector window was set at 30.6 cm from the inlet.

All the experiments were carried out at 25°C. Sample injections were made by pressure of 0.5 p.s.i. for 5 s. The separation potential was −10 kV. Injection was made in the cathode and detection in the anode.

2.2. Reagents

Standards were obtained from Sigma (St. Louis, MO, USA). Phosphoric acid 85% was from Merck (Darmstadt, Germany), Acetic acid and sodium hydroxide from Panreac (Madrid, Spain), methanol from Scharlau (Barcelona, Spain) and hydrochloric acid 37% (w/w) was from Carlo-Erba (Milan, Italy)

Buffer solutions and all dilutions were prepared with purified water by a Milli-Q-System (Millipore, Bedford, MA, USA).

2.3. Electrophoretic buffers

The electrophoretic buffer S, pH 6.0, was prepared with 0.2 *M* phosphoric acid adjusted to pH with NaOH to which 10% (v/v) methanol was added [24]. The second buffer employed in that study, called A, was 0.2 *M* phosphoric and 0.01 *M* acetic acids adjusted to pH 4.0 with NaOH and without adding methanol.

2.4. Samples

A pool of urine from ten healthy volunteers in our department was used as control urine.

Twenty-four-hour urine collections were obtained from normal individuals and from those who had formed kidney stones and they were kindly provided by the Laboratory Service of Virgen de la Candelaria Hospital (Spain) in agreement with the ethical committee. They were stored at −20°C.

Prior to analysis, defrosted samples were shaken gently, diluted 1:1 with HCl 0.5% (w/w), vortex-mixed for 1 min and centrifuged for 4 min at 2000 *g*. The supernatant was directly injected in the CE equipment.

2.5. Validation study

Individual stock solutions of each organic acid at 20 m*M* in water were prepared and stored at −20°C; with this concentration, samples can be spiked without appreciable dilution of the urine. The day of the analysis they were diluted to the concentrations described below with purified water.

Linearity of response for standards was tested assaying in triplicate five levels of concentrations, covering all the expected values: 250, 375, 500, 750 and 1000 mg/l for citrate and 10, 25, 50, 100 and 200 mg/l for oxalate. Linearity of response for samples was tested in the same way, but replacing water with urine.

Recovery was estimated comparing the values obtained in the linearity of the calibration urine with the linearity standards, taking into account the endogenous urinary concentrations, which had been previously measured.

Within-day precision was tested to check the constancy of instrumental response to a given analyte, and the reproducibility of concentration and migration time, since the latter is a key parameter for peak assignment. For this purpose, the assay was performed with six solutions of standards and six of urine samples, at the middle concentration of the calibration curve 500 mg/l for citrate and 50 mg/l for oxalate.

2.6. Enzymatic assays

The urine samples were analysed in the clinical laboratory of the Virgen de la Candelaria Hospital following the enzymatic methods: procedure Cat. No. 755 699 for oxalate and Procedure Cat. No. 139 076 for citrate (Boehringer-Mannheim). Prior to oxalate measurement, samples were acidified with hydrochloric acid (25%, w/w) achieving 0.25% as final concentration of HCl and neutralised with potassium hydroxide (10%, w/w). To improve recovery and to avoid oxalate precipitation EDTA was also added (500 mg/l urine).

For citrate measurement, samples were centrifuged and the supernatant diluted for the assay.

3. Results and discussion

In a previously developed method for rapid profiling of organic acidurias by capillary electrophoresis [24] oxalate and citrate plus another 25 organic acids had been separated and identified in urine. When we were training clinical analysts in that method, there was interest in the use to the measurement of

nephrolithiasis markers. To simplify the number of working buffers we tried to validate the assay with the same conditions as the previous method.

In one preliminary attempt samples were analysed directly without hydrochloric acid addition, but oxalate recoveries were low. However, when samples were treated as described in Section 2, results were accurate. EDTA, employed in other methods [25] is not necessary as it could be proved with recoveries which are around 100%. Fig. 1 shows the electropherograms of the standards in buffer S including a chloride peak, oxalic (25 mg/l), citric (250 mg/l) and glyceric acids (125 mg/l). Glyceric and uric acids were identified in these conditions, but uric acid partially precipitates in the acidic media, which is necessary to keep oxalate in solution; uric acid is usually measured with automatic analysers, therefore we left it out. In the same Fig. 1 are also included electrophoregrams of control urine, an adult patient with low citrate concentration and an infant patient with high value of the oxalate/citrate ratio. The different profiles can be compared and good resolution for oxalate and citrate can be observed in all the cases. Fig. 2 shows the electropherograms corresponding to the standards, infant urine and this urine co-injected with the standards for identification; the concentrations for oxalate and citrate are the same as above and 250 mg/l for glycerate. Oxalate and citrate peaks increase clearly and uniformly, meanwhile glycerate is in a more complicated zone. It could not be quantified accurately, just tentatively identified. Glyoxylate in buffer A shows a similar

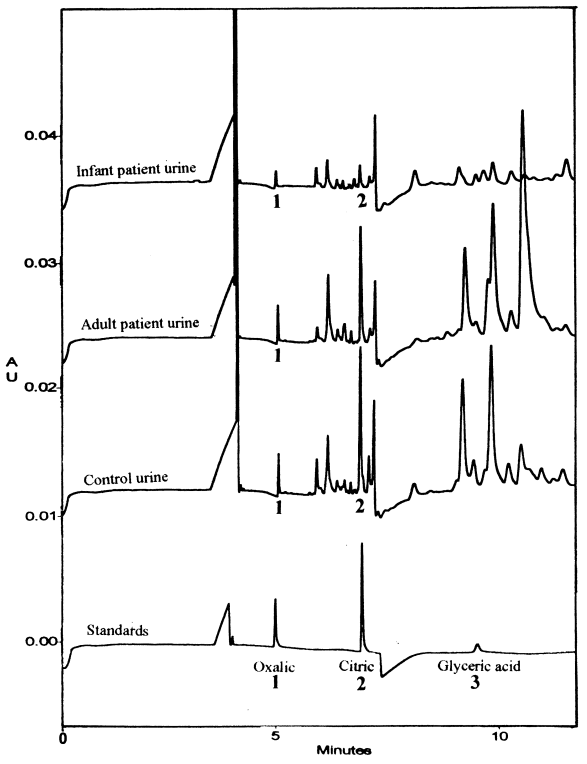

Fig. 1. Electropherograms of standards (oxalic, citric and glyceric acids), control urine, urine from adult and urine from an infant patient analysed by CE using buffer S. Applied voltage, −10 kV; injection for 5 s; pressure, 0.5 p.s.i. (see Section 2).

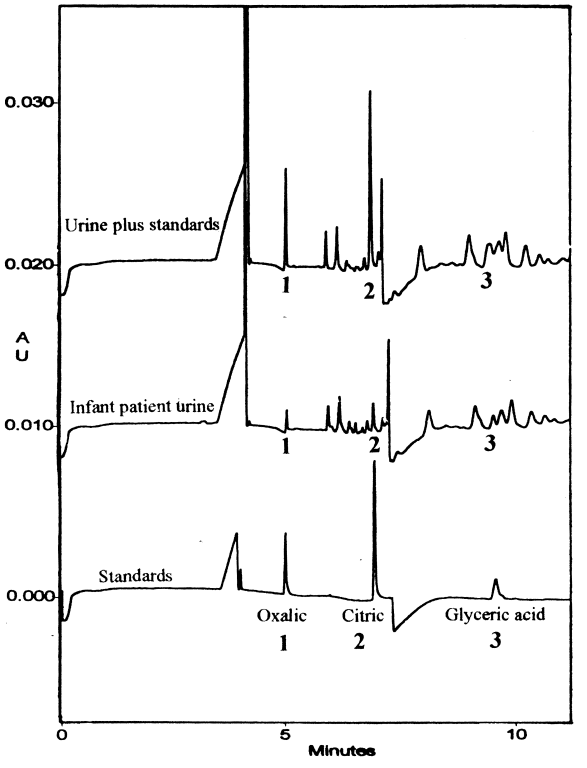

Fig. 2. Electropherograms of standards (oxalic, citric and glyceric acids), infant urine and this urine co-injected with the standards for identification. Analysis by CE using buffer S. Applied voltage, −10 kV; injection for 5 s; pressure, 0.5 p.s.i. (see Section 2).

situation, it is not well resolved and only detectable when it presents a clear increase, which often happens with metabolites related to inborn errors. Fig. 3 shows the standard with chloride and glyoxylate peaks (140 mg/l), an infant urine and the same urine co-injected with the standard.

Main validation parameters of the method for oxalate and citrate are included in Table 1. As shown there, standards fit the linear model ($r>0.999$) for both organic acids and no bias was found.

Samples also showed a good linearity, with correlation coefficients over 0.999. A small bias was found for citrate in samples, which has statistical significance, as the intercept with its limits of confidence did not include the zero value. Nevertheless, it can be explained by the good fit of the points to the regression line which makes the limits of confidence very narrow and it has no practical consequences as can be seen in the recoveries that are near 100% in the whole range.

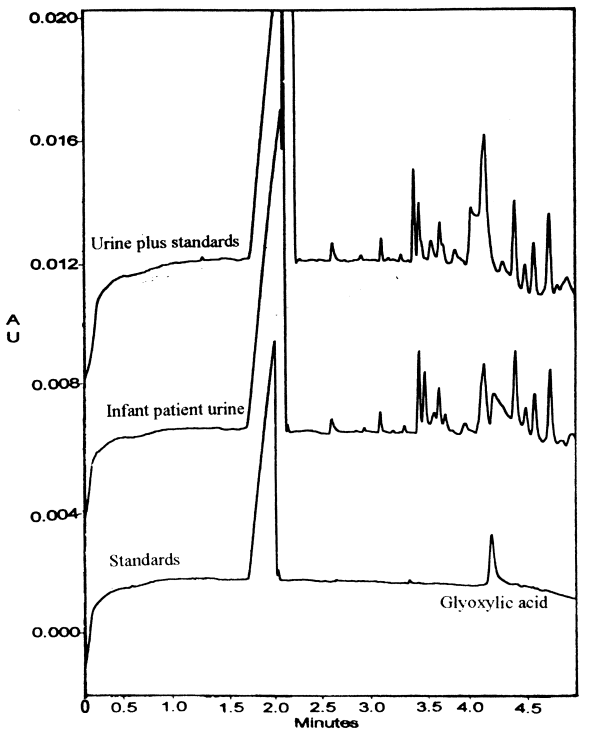

Fig. 3. Electropherograms of standard with chloride and glyoxylate, a urine from an infant patient, and the same urine co-injected with the standard. Analysis by CE using buffer A. Applied voltage, −15 kV; injection for 5 s; pressure, 0.5 p.s.i. (see Section 2).

For accuracy, recoveries were from 100 to 101% for citrate and from 99.4 to 101.7% for oxalate and they did not statistically differ from 100% when a Student *t*-test was applied ($P>95\%$).

Running 10 runs per day of the same standard and sample, daily instrumental precision gave RSDs under 1.8% for citrate and of 3.3% for oxalate concentrations. Migration times showed RSDs under 0.26% for citrate and under 0.10% for oxalate, low enough to consider the method acceptable. Meanwhile, when processing 10 different standards and samples, each of which has been independently prepared according to the method procedure, RSDs for the method were under 3.7% for citrate and under 7.6% for oxalate concentrations. For migration times RSDs were under 1.5% for citrate and under 1.0% for oxalate. The value of RSD 7.6% obtained for oxalate concentration in day 1 corresponds to measurements performed with an older instrument (Beckman P/ACE 2000) and may be related to poorer optics which affects mainly to the lower peaks. The same measurements in a different day with another equipment (Beckman P/ACE 5500) gave a RSD of 3.1%. Anyway, we have maintained all the values to have a wider study of precision.

Intermediate precision evaluated in different days and equipments with a total of 20 runs provided RSD values slightly superior to intra-assay precision (4.4% for citrate and 7.4% for oxalate concentrations, and 0.8% for citrate and 1.2% for oxalate migration times), as could be expected. Repeatability of migration times is very important, because peak identification is performed mainly with this criterion and it has proved to be very good with this type of neutral capillary.

Detection limits ranged from 3.6 to 25.9 mg/l for citrate and from 0.17 to 0.67 for oxalate, the first value being calculated for standards and the second for samples. Calculation have been performed following IUPAC recommendation for chromatographic methods [26]. Mean values described in bibliography as normal for these acids vary in a wide range even working with the same technique. Anyway, detection

Table 1
Main validation parameters of CE method[a]

	R	*a*±C.L.	*b*±C.L.	Range (mg/l)
Standards linearity and range				
Citrate	0.9992	−718±1607	95±2	250–1000
Oxalate	0.9998	−212±357	303±3	10–200
Samples linearity and range				
Citrate	0.9993	646±84	93±2	179–1004
Oxalate	0.9998	144±294	299±3	8–201

Precision RSD (%)			
Concentration	Repeatability (n=10)		Intermediate precision (n=20)
Instrumental	Day 1	Day 2	Day (1+2)
Citrate	1.2	1.8	1.7
Oxalate	3.3	3.3	3.4
Method			
Citrate	3.7	2.2	4.4
Oxalate	7.6	3.1	7.4
Migration time			
Instrumental			
Citrate	0.26	0.16	1.7
Oxalate	0.10	0.08	1.1
Method			
Citrate	1.5	0.57	1.2
Oxalate	1.0	0.41	0.82

Standard accuracy (%) (n=15)	Mean recovery (%)	RSD
Citrate	100	3.0
Oxalate	99.4	3.0
Sample accuracy	Mean	RSD
Citrate	101	2.3
Oxalate	101.7	2.4
Limits of detection (mg/l)	Standards	Samples
Citrate	3.6	25.9
Oxalate	0.17	0.67

[a] *a*, intercept; *b*, slope; C.L., confidence limits.

limits provided for the present method are under these values and, more important, under the lower values found in the randomly assayed samples and in the practice in the clinical laboratory. Therefore, it would be applicable for measuring all kind of samples following the evolution of treated patients.

A comparison of the CE measurement of urinary oxalate and citrate with the corresponding enzymatic methods is shown in Fig. 4. For the least-square fitting, a very good correlation appears in citrate with $r>0.99$, moreover, the intercept of the regression line with its confidence limits ($P>95\%$) includes the zero value and the slope with its confidence limits ($P>95\%$) ranges from 0.92 to 1.00, which means that both methods give results which are statistically equal. Oxalate correlation shows $r>0.70$, the inter-

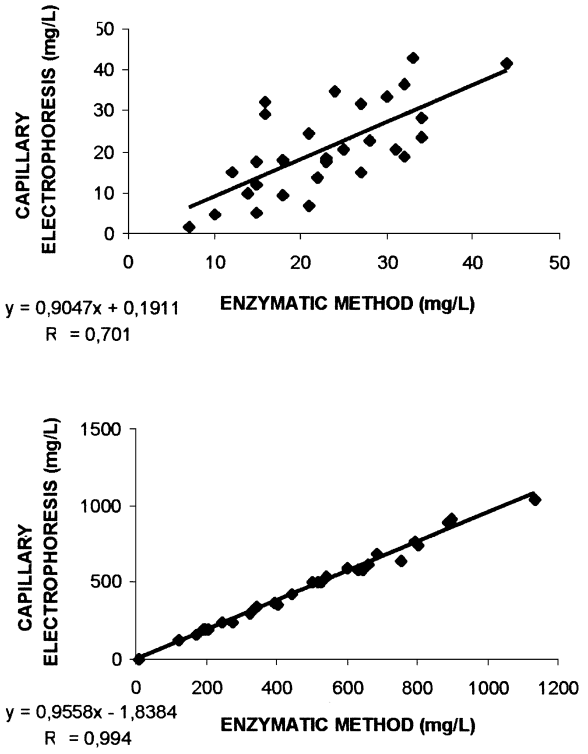

Fig. 4. Comparison of analysis of urinary oxalate and citrate by CE versus enzymatic determinations.

cept of the regression line with its confidence limits ($P>95\%$) includes the zero value and the slope with its confidence limits ($P>95\%$) ranges from 0.54 to 1.27, including the one value too. It can be concluded that there is no statistical difference ($P<95\%$) between both methods for oxalate and citrate. Nevertheless, when $r=0.99$ or greater, linear regression calculations will provide reliable estimates of errors. When $r<0.975$, it is better to use the paired data calculations [27].

Therefore, the paired Student *t*-test was also performed for both analytes and methods; results are shown in Table 2 together with the individual values of 29 urine samples from different patients. As there can be seen, in spite of the good correlation, citrate test is near out of limits, but it is mainly due to the narrower confidence interval of values because of the low standard deviation.

Concerning stability, stock standards are stable at -20°C over 6 months and at $+4$°C over 2 weeks. Samples were frozen after the enzymatic assay and measured by CE 2 months later. Therefore, the results above mentioned include the effect of freezing and thawing on samples. On the other hand, duplicates of the same samples kept in the freezer with and without hydrochloric acid previously added were measured by CE and no difference was found. Finally, five randomly selected samples kept at -20°C were measured by CE 6 months later. Considering the initial value of 100% in each case the mean recovery, after this time was $107\pm18\%$ for oxalate and $102\pm8\%$ for citrate.

To ensure oxalate accuracy, which showed a higher deviation when representing CE values versus the enzymatic ones, the CE method was tested with control urines from Sigma in two different levels of oxalate concentration, normal and elevated (Cat. Nos. O6627, O6502). The results obtained with nine replicates for each level were 20.6 ± 1.6 and 86.2 ± 3.2 mg/l and the certified values are 26.5 ± 5.5 and 97.5 ± 13.5 mg/l, respectively. Therefore, in view of these results there is not statistical difference and the CE proposed method can be considered accurate and reliable.

4. Conclusion

A method has been validated for measuring oxalate and citrate in urine samples by CE. The method is rapid (analysis time<10 min), automated, simple and inexpensive because of the low cost of capillary and electrolyte materials. The simplicity of the whole process and the demonstrated good performance and high throughput make it highly recommendable in routine clinical analysis.

Acknowledgements

The present study was supported by Comunidad Autónoma de Madrid project No. 4392 and Universidad S. Pablo CEU project No. 3/99. We are also grateful to all the people who have provided urine samples for this work.

Table 2
Measurements of oxalate (A) and citrate (B) in 29 urine samples from patients[a]

(A) Oxalate			(B) Citrate		
No.	Enzymatic method (mg/l)	CE (mg/l)	No.	Enzymatic method (mg/l)	CE (mg/l)
m1	21	7	m1	207	194
m2	34	24	m2	804	742
m3	34	29	m3	688	690
m4	15	12	m4	529	505
m5	31	21	m5	348	343
m6	21	24	m6	793	773
m7	7	2	m7	9	4
m8	30	34	m8	602	593
m9	15	18	m9	445	418
m10	15	5	m10	394	372
m11	10	5	m11	195	191
m12	14	10	m12	517	503
m13	18	10	m13	342	335
m14	23	18	m14	503	504
m15	25	21	m15	897	917
m16	22	14	m16	276	245
m17	16	29	m17	634	588
m18	28	23	m18	123	121
m19	27	15	m19	249	236
m20	32	19	m20	171	157
m21	23	18	m20	196	179
m22	24	35	m22	406	360
m23	33	43	m23	888	898
m24	12	15	m24	325	293
m25	18	18	m25	646	581
m26	32	37	m26	1136	1047
m27	44	42	m27	542	537
m28	16	32	m28	758	641
m29	27	32	m29	661	614

[a] Paired *t*-test: (A) $t_{cal}=1.345$, t_{tabl} (95%)=2.048; (A) $t_{cal}=4.370$; t_{tabl} (95%)=2.048.

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