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GAS—LIQUID CHROMATOGRAPHY REFERENCE METHOD FOR THE ASSAY OF URINARY CREATININE

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SUMMARY

The gas—liquid chromatographic measurement of urinary creatinine described in this paper employs methylation, the use of a diethylene glycol succinate stationary phase, an internal L-hydroxyproline standard and a temperature of 180°C. The technique, which is specific and reproducible, is shown to be a reference method providing more precise and reliable results than a conventional colorimetric method. In addition, it can be used as a routine method because of its simplicity.

INTRODUCTION

Most techniques for the analysis of blood or urinary creatinine utilize the Jaffe reaction, whose principle is the development of an orange color in the presence of alkaline picrate [1]. The relatively non-specific nature of this reaction can be partially compensated by absorption on Fuller's earth [2] or determination of the kinetics of color development [3]. It thus appeared valuable to develop a specific and precise assay for urinary creatinine using gas—liquid chromatography (GLC).

MATERIALS AND METHODS

Material

Forty-seven urine samples were obtained from healthy subjects without antiseptic in the samples. Immediately after collection, each sample was acidified to pH 2 with 1 *M* HCl and maintained at 4°C overnight. Eventual urate precipitates were eliminated by centrifugation at 1000 *g* for 20 min and the supernatant was removed for assay.

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Instrumentation

A Carlo Erba Fractovap 2300 chromatograph equipped with a flame ionization detector was connected to a recorder. The column was a glass Pyrex tube, 2 m × 4 mm I.D. Hamilton microsyringes of 10 μl (No. 702 N) and 50 μl (No. 1705 N) capacity with crimped needles were used for injection.

Reagents

Standard solutions were freshly prepared: 0.0442 *M* creatinine in 0.1 *M* HCl; 0.076 *M* L-hydroxyproline in distilled water. Also, 1.73 *M* hydroxylamine hydrochloride in 0.1 *M* HCl was freshly prepared. Trimethylaniline hydroxide (MethElute, obtained from Pierce, Rockford, IL, U.S.A.) was 2 *M* in methanol. The stationary phase was Gas-Chrom Q (80—100 mesh) loaded with 15% (w/w) diethylene glycol succinate (DEGS).

OPERATING PROCEDURE

Gas—liquid chromatography

(1) Sample preparation (see Table I).

(2) Methylation: 20 μl of sample or standard were mixed with 40 μl of MethElute.

(3) Chromatography conditions: column temperature, 180°C; injector and detector temperature, 225°C; carrier gas (nitrogen), 50 ml/min at 120 kPa pressure; hydrogen, 45 ml/min at 90 kPa; air, 400 ml/min at 100 kPa; attenuation, 100 × 8; chart speed, 4.16 mm/min; volume injected, 5 μl.

TABLE I

SAMPLE PREPARATION

	Calibration			Test
	11 m*M*	22 m*M*	33 m*M*	
0.0442 *M* creatinine (ml)	0.2	0.4	0.6	—
Urine (ml)	—	—	—	0.8
0.076 *M* L-hydroxyproline (ml)	0.4	0.4	0.4	0.4
1.73 *M* hydroxylamine hydrochloride (ml)	0.2	0.2	0.2	0.2
0.1 *M* HCl (ml)	0.6	0.4	0.2	—

(4) After detector response to the solvent, the L-hydroxyproline peak and the creatinine peak successively appear on the chromatogram 8—10 min after injection (Fig. 1). After measuring peak heights, the creatinine/standard ratio was calculated. After plotting a standard curve, the creatinine concentration in a urine sample can be read directly off the abscissa. Within a 33 m*M* creatinine concentration, diluting the urine sample is not necessary.

(5) Repeatability was determined by successively injecting the same urine sample nineteen times.

(6) Reproducibility was determined by injecting ten successive preparations of the same urine.

Fig. 1. Typical chromatogram obtained for the assay of urinary creatinine. Peaks: 1 = L-hydroxyproline standard (110 nmol); 2 = creatinine (38 nmol).

(7) Statistics were applied to the results obtained with both GLC and colorimetry. The means were compared with standard tests for paired data and correlations were calculated. The recovery rate mean is given with its confidence interval whereas that of repeatability and reproducibility trials are presented with the standard deviation.

Colorimetry

The method of Hare [2, 4], involving the Jaffe assay after absorption on Fuller's earth, was adopted.

APPLICATION

Both techniques were used to assay creatinine in 47 urine samples. In addition, extreme values were determined by diluting twenty samples with distilled water and by overloading fifteen samples with 0.0442 *M* creatinine.

The mean values obtained and their confidence intervals for each technique and each urine class are presented in Table II. The results obtained with GLC are slightly higher than those provided by colorimetry, but the confidence interval and standard deviation are of the same order. A comparison of the two assay methods with the paired data method is shown in Table III. The mean of the differences, which is significant for each batch of urine samples, increases with increasing creatinine concentration. The correlation coefficients and slopes of the regression lines, however, are of the same order (Figs. 2—4).

TABLE II

ASSAY RESULTS WITH GLC AND COLORIMETRY FOR NORMAL, DILUTED AND OVERLOADED URINE SAMPLES

Values are given in m*M*.

Native urines (n = 47)		Diluted urines (n = 20)		Overloaded urines (n = 15)	
GLC	Col.*	GLC	Col.	GLC	Col.
19.82	17.60	17.17	16.01	29.20	27.61
26.37	25.48	19.47	15.93	36.80	34.69
5.75	5.66	4.86	5.22	34.33	33.27
27.43	24.24	13.89	14.51	55.30	54.87
7.70	9.73	13.80	13.09	16.28	17.70
24.51	20.79	5.97	5.13	28.76	25.66
7.08	6.63	1.32	0.97	28.93	20.35
7.52	6.63	3.31	4.07	14.16	13.27
18.76	15.57	5.08	5.48	19.64	19.64
13.89	11.68	5.08	4.07	12.83	13.80
12.92	12.65	14.07	13.62	18.05	13.80
5.57	5.31	11.94	11.50	19.02	16.10
6.01	5.48	6.41	6.19	19.64	19.64
13.50	11.59	4.20	4.16	28.76	30.97
17.87	15.93	5.53	5.39	10.84	9.91
3.10	3.36	6.41	6.19		
9.55	7.69	4.20	4.15		
2.65	2.56	5.53	5.39		
6.19	6.10	14.60	13.80		
8.32	6.90	13.89	13.80		
8.40	10.00				
21.24	18.85				
12.12	10.79				
2.21	1.77				
10.97	9.73				
5.48	4.71				
11.94	10.79				
8.14	6.63				
15.04	12.83				
6.63	5.75				
4.16	3.54				
8.40	8.54				
13.89	11.68				
18.32	15.66				
10.79	11.15				
20.26	20.53				
10.79	9.91				
13.45	12.21				
11.24	9.46				
15.66	13.80				
15.66	15.48				
7.08	7.69				
25.39	19.47				
6.37	6.19				
4.16	4.15				
5.48	5.39				
14.60	13.80				
Mean (m*M*)	11.75	10.68	8.84	8.43	24.84	23.41
Confidence interval (5%)	±0.22	±0.20	±0.29	±0.26	±0.75	±0.75
Standard deviation (± 2σ)	±13.10	±11.74	±10.70	±9.80	±23.20	±23.20

*Col. = colorimetry.

TABLE III

STATISTICAL COMPARISON FOR PAIRED DATA BETWEEN THE GLC AND COLORIMETRIC ASSAY METHODS FOR URINARY CREATININE AMONG THE THREE CLASSES OF SAMPLES ASSAYED

	Native urines (n = 47)	Diluted urines (n = 20)	Overloaded urines (n = 15)
Mean of differences (mM)	1.13	0.52	1.99
Reduced deviation	5.08	2.96	2.69
Difference (S = significant at 5% level)	S	S	S
Correlation coefficient	0.96	0.99	0.98
Slope of regression line	1.07	1.08	0.98

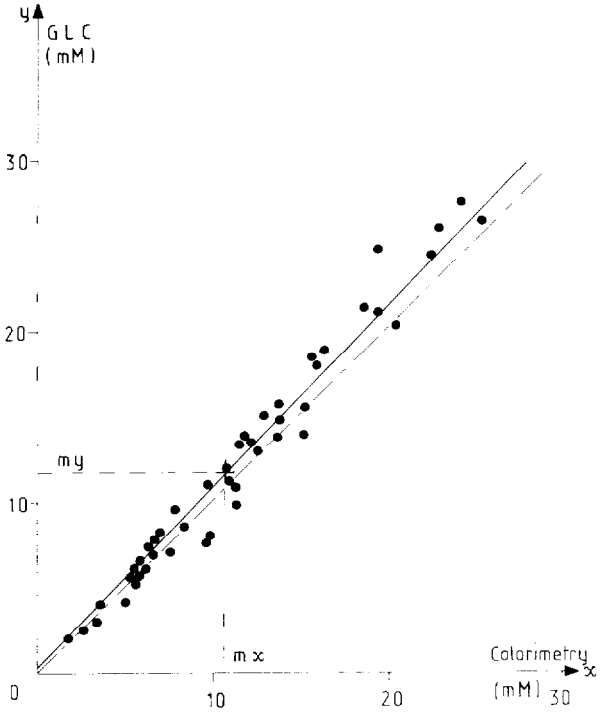

Fig. 2. Correlation between urinary creatinine results obtained with GLC (y-axis) and colorimetry (x-axis). Regression line (——), $Y = 1.07\ X + 0.03$, $r = 0.96$, $\alpha < 1\%$. Bisector (- — · — -).

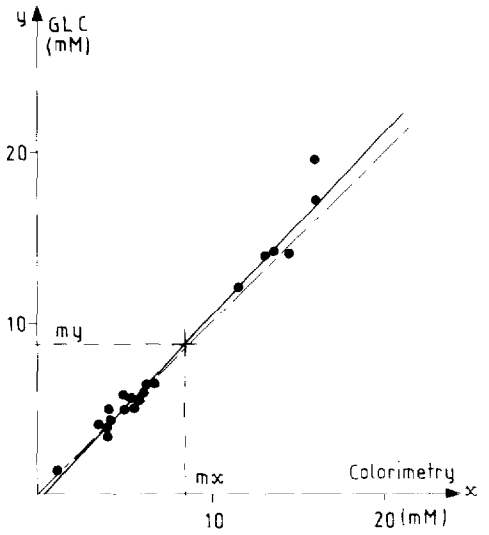

Fig. 3. Correlation between creatinine results for diluted urines measured with GLC (y) and with colorimetry (x). Regression line (——), $Y = 1.08\ X - 0.03$, $r = 0.99$, $\alpha < 1\%$. Bisector (- — · — -).

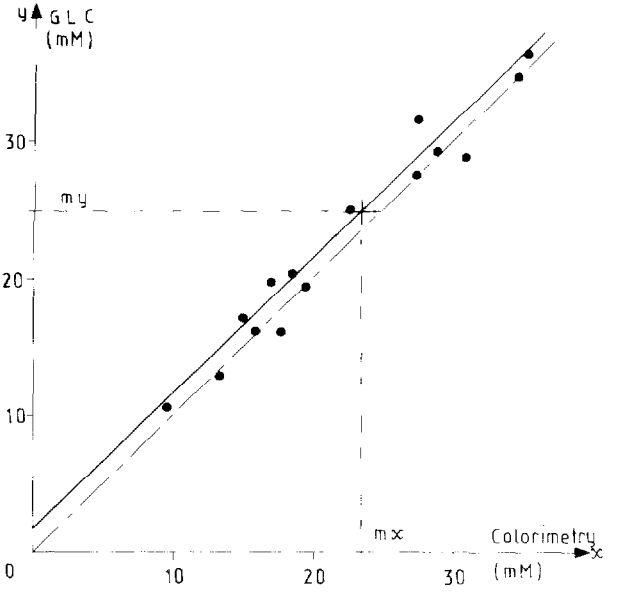

Fig. 4. Correlation between creatinine results in overloaded urines measured with GLC (y) and with colorimetry (x). Regression line (——), $Y = 0.98\ X + 0.214$, $r = 0.98$, $\alpha < 2\%$. Bisector (- — · — -).

The results from the repeatability and reproducibility trials are presented in Table IV.

Finally, 31 urine samples were subjected to GLC before and after a carefully weighed creatinine overload was achieved. Relevant results are given in Table V.

They correspond to a mean recovery rate of 98.09 ± 0.96%, for a 5% confidence interval.

TABLE IV

REPEATABILITY AND REPRODUCIBILITY TRIALS

	n	Mean (m*M*)	S.D.	C.V. (%)
Repeatability	19	11.2	0.4	3.6
Reproducibility	10	13.7	0.2	1.5

TABLE V

RECOVERY DATA

Trials	Native urines (m*M*)	Overload (m*M*)	Overloaded urines (m*M*)	Recovery (%)
1	7.96	4.42	12.17	95.2
2	11.50	3.45	14.71	93.0
3	5.97	5.40	11.50	102.4
4	8.41	5.58	13.72	95.2
5	9.73	4.37	13.94	96.3
6	5.09	3.36	8.30	95.5
7	5.09	4.57	9.62	99.1
8	14.82	4.11	18.80	96.8
9	11.72	1.88	13.50	94.7
10	11.50	4.46	16.03	101.6
11	12.61	5.13	17.70	99.2
12	3.32	3.89	7.08	96.7
13	8.63	4.19	12.61	94.9
14	12.16	5.00	17.03	97.4
15	20.53	9.38	29.82	99.0
16	7.30	4.44	11.72	99.5
17	2.65	3.25	5.97	102.1
18	4.42	4.23	8.63	99.5
19	4.86	5.22	9.95	97.5
20	4.42	4.32	8.85	102.4
21	18.36	3.36	21.68	98.8
22	9.90	2.67	12.60	101.1
23	12.16	9.73	21.68	97.8
24	5.30	2.65	7.75	92.5
25	4.86	2.76	7.52	96.4
26	3.76	3.55	7.30	99.7
27	4.87	3.40	8.18	97.3
28	10.62	3.58	14.16	98.9
29	13.70	13.26	27.17	101.6
30	14.60	2.32	16.81	95.5
31	6.86	6.44	13.49	103.0

METHODOLOGICAL CONSIDERATIONS

Chemical transformation of creatinine

Before GLC can be performed, creatinine must be converted into a stable and volatile derivative. Initially, silylation with N,O-bis(trimethylsilyl)acetamide (BSA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was attempted, either alone or with small quantities of trimethylchlorosilane (TMCS), used as an acid catalyst. Under these conditions, four to six peaks were obtained, regardless of the stationary phase, temperature and incubation time used.

As recommended by Poole et al. [5], hydroxyl functions were first blocked with hydroxylamine hydrochloride, but improvement was not sufficient.

As previously reported for GLC assays of urea [6], hot nitric acid in ethanol was unsuccessfully attempted, except for very high creatinine concentrations, which gave good results.

Acylation with acetic anhydride was attempted without success. Methylation with 0.2 *M* trimethylaniline hydroxide was chosen. By first reacting samples with a well-defined quantity of hydroxylamine hydrochloride, this reagent generated a single GLC derivative of creatinine.

Choice of internal standard

The criteria for this standard were a cyclic compound with both hydroxyl and amine functions, providing a single peak well separated from the injection and creatinine derivative peaks under the present operating conditions. L-Hydroxyproline was fully in agreement with these requirements. In the normal state, the concentration of this compound in urine is too low to be detected by this technique. In bone diseases giving rise to a significant elevation in urinary hydroxyproline excretion, creatinine assay might be erroneous. In this case, a control injection should be performed without adding hydroxyproline.

Choice of stationary phase and temperature

The stationary phase was chosen by injecting the methylated creatinine derivative with temperature programming from 50°C up to the critical phase temperature. Trials were performed with 3% OV-17 silicone on Gas-Chrom Q, 8% ethylene glycol succinate (EGS) on Gas-Chrom Q, 5% OV-225 silicone on Gas-Chrom P, 5% Apiezon L grease on Chromosorb G, 5% and 15% DEGS on Gas-Chrom Q. Using the latter phase, methylated creatinine could be detected at around 170°C.

At 180°C, 5% DEGS enabled two well-separated peaks of L-hydroxyproline and methylated creatinine to be obtained. When the stationary phase was increased to 15%, the peaks were delayed in the column and so precise quantitative determinations of low creatinine concentrations could be reached.

Interference

Our study only concerned compounds likely to change the Jaffe reaction. Glucose added at a rate of 55 m*M* gives two peaks well separated from that of

creatinine and hydroxyproline. Acetylacetone and phenylpyruvate peaks are hardly different from the solvent one.

Our working conditions did not enable us to study drug interference.

CONCLUSIONS

Urinary creatinine assay with GLC constitutes a reference method on account of its specificity and reproducibility. It is also usable in routine applications as a result of its simplicity. The results obtained are slightly higher than those provided by the colorimetric method. This technique is thus recommended to be used for 24-h diuresis and glomerular filtration rates.

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