Journal of Chromatography, 525 (1990) 265–275
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5066

SIMULTANEOUS DETERMINATION OF CREATINE, URIC ACID AND CREATININE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIRECT SERUM INJECTION AND MULTI-WAVELENGTH DETECTION

G. WERNER, V. SCHNEIDER and J. EMMERT*

Forschung Schnelldiagnostica, Boehringer Mannheim GmbH, Sandhoferstrasse 116, D-6800 Mannheim 31 (F R G)

(First received May 22nd, 1989; revised manuscript received October 4th, 1989)

SUMMARY

An isocratic high-performance liquid chromatographic separation of creatine, uric acid and creatinine with direct on-column application of the serum sample has been developed. The pre-column, used for sample clean-up, is switched out of the eluent flow after a definite time. Simultaneous three-wavelength detection with a diode array detector allows a very precise quantitation of the three compounds at the optimum wavelength for each (210, 234 and 290 nm) with a coefficient of variation of less than 2% (creatine 3–5%). Good linearity and 100% recovery are obtained over a wide range of concentration in biological material. No interferences are observed caused by medication with lidocaine or dobesilate (Ca), or high bilirubin concentrations. This makes the method useful for clinical purposes or even as a reference for other methods.

INTRODUCTION

Creatinine and uric acid are important parameters in clinical chemistry, e.g. for recognition of renal diseases. In some cases of muscle catabolism, elevated serum concentrations of creatine can be observed. Photometric methods such as the alkaline picrate reaction according to Jaffe [1] (creatinine) or the reduction of phosphotungstate (uric acid), are usually used for determination of these compounds, but various endogeneous or exogeneous substances, such as bilirubin or drugs, can interfere with the analysis [2,3]. Enzymic assays, such as the creatinase-*p*-aminophenazon (PAP) method for creatinine [4], are more specific, but still suffer from interference problems with bilirubin [3] and med-

0378-4347/90/$03.50

ication with dobesilate (Ca). Endogeneous creatine has to be eliminated by performing a two-step reaction [5]. However, quantitation of creatine from the first step is not very practicable, and its elimination may also sometimes result in imprecise values for low creatinine concentrations.

High-performance liquid chromatography (HPLC) has the advantage that it separates compounds before quantitation. HPLC methods have been described for creatinine [6,7], creatinine and neopterine [8], uric acid and creatinine [9], creatine and creatinine [10] and urea, creatinine and uric acid [11]. Sample preparation is done off-line by protein precipitation with acid [6,10,11] or by using solid-phase extraction [8,10]. Direct injection of the serum sample, diluted with the mobile phase, using a pre-column, is discussed in refs. 7 and 9, one of which [7] refers to column switching.

This paper describes a method for the direct injection of the undiluted serum sample using an online pre-column and a column-switching technique for simultaneous routine determination of creatine, uric acid and creatinine. With a diode array detector each compound is quantitated at its optimum detection wavelength, and this results in very good precision and accuracy over a wide concentration range.

EXPERIMENTAL

Chemicals and solutions

The following chemicals were used: creatine monohydrate p.a., creatinine p.a. and uric acid p.a. (Serva, Heidelberg, F.R.G.); dipotassium hydrogenphosphate trihydrate, lithium carbonate p.a., methanol p.a., phosphoric acid 85% p.a. and hydrochloric acid 37% p.a. (Merck, Darmstadt, F.R.G.); sodium azide (Riedel-de Haen, Seelze, F.R.G.) and Milli-Q-purified water (Millipore, Eschborn, F.R.G.).

Reagent solutions were prepared as follows: 3 *M* phosphoric acid by diluting 20 ml of phosphoric acid (85%) to 100 ml with water; sodium azide solution (0.5%, pH 7.0) by dissolving 5 g of sodium azide in 1 l of water, and then adjusting the pH to 7.0 with 3 *M* phosphoric acid; 0.03 *M* dipotassium hydrogenphosphate solution by dissolving 6.85 g of dipotassium hydrogenphosphate trihydrate in 1 l of water.

Eluent A, 0.03 *M* dipotassium hydrogenphosphate–methanol (99.5:0.5, v/v), pH 7.5, was prepared by diluting 5 ml of methanol and 0.5 ml of 0.5% sodium azide solution (pH 7.0) to 1 l with 0.03 *M* dipotassium hydrogenphosphate solution; the solution was degassed, and its pH was adjusted to 7.5 with 85% phosphoric acid (ca. 0.5 ml). Eluent B was methanol and eluent C was 0.5% sodium azide solution (pH 7.0).

Standard solutions were prepared as follows: creatine stock solution by dissolution of 22.4 mg of creatine monohydrate in 20 ml of water; creatinine stock solution by dissolution of 50 mg of creatinine in ca. 10 ml of water, to which

0.5 ml of hydrochloric acid (37%) was added and then made up to 50 ml with water; uric acid stock solution by dissolution of 50 mg of uric acid and 37.5 mg of lithium carbonate (for increased dissolution speed and solution stability) in 50 ml of water.

The standard solution contained 1 ml of creatine stock solution, 3 ml of creatinine stock solution and 3 ml of uric acid stock solution, made up to 50 ml with water. Concentrations were then: creatine, 150 μmol/l (1.97 mg/dl); creatinine, 6.00 mg/dl; uric acid, 6.00 mg/dl.

Samples

Serum samples were obtained from healthy volunteers and clinical patients. Standard solution as well as serum samples were injected directly into the HPLC system without any pretreatment; the injection volume was 5 μl.

Columns

The analytical column was Hyperchrome (250 mm × 4.6 mm I.D., Bischoff, Leonberg, F.R.G.), filled with Partisil ODS 3, 5 μm particle size (Whatman, Clifton, NJ, U.S.A.); the pre-column was Hyperchrome (40 mm × 4.6 mm I.D., Bischoff), filled with Partisil ODS 3, 5 μm particle size, or with Nucleosil 5 NH_2, 5 μm particle size (Macherey & Nagel, Düren, F.R.G.).

Apparatus

The HPLC system was an HP 1090 liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.) equipped with a DR 5 ternary solvent-delivery system, autosampler and autoinjector, thermostatted column compartment and column-switching valve and a diode array detector. Control of the instrument, data storage, evaluation, integration and reporting were performed by an HP Series 300 computer (Hewlett-Packard, Boeblingen, F.R.G.).

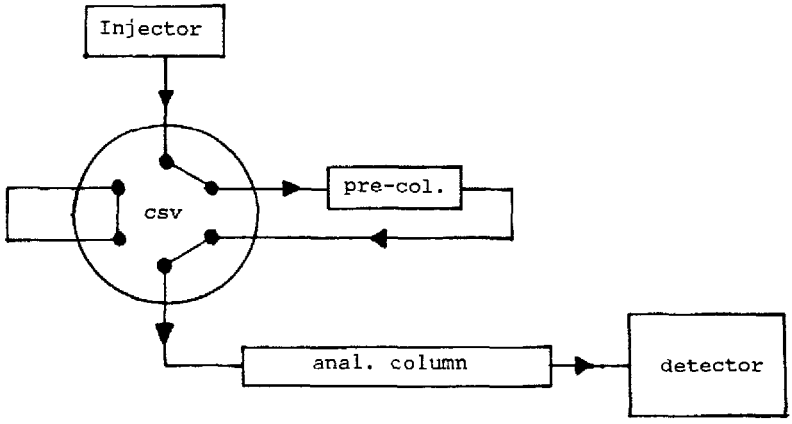
Separation conditions

The columns were connected to the column-switching valve (csv) as shown in Fig. 1. The basic position was 1, with the pre-column disconnected; 0.01 min after the turn of the injection valve that indicates the start of the run, the column-switching valve was switched to position 0, allowing the eluent to flow through the pre-column for 3 min. The column-switching valve was then switched back to position 1 for the rest of the analysis time.

The mobile phase for separation was eluent A for 8 min, followed by a linear gradient to 20% B (methanol) from 8 to 10 min. The post-elution time for reconditioning to eluent A was 4 min; this gave a total run time of 14 min.

After a whole series of serum samples (40–70 samples) the instrument was washed with eluent C (0.5% sodium azide solution) for disinfection purposes. The pre-column was in contact only with eluent A and was changed after ca.

position 0



position 1

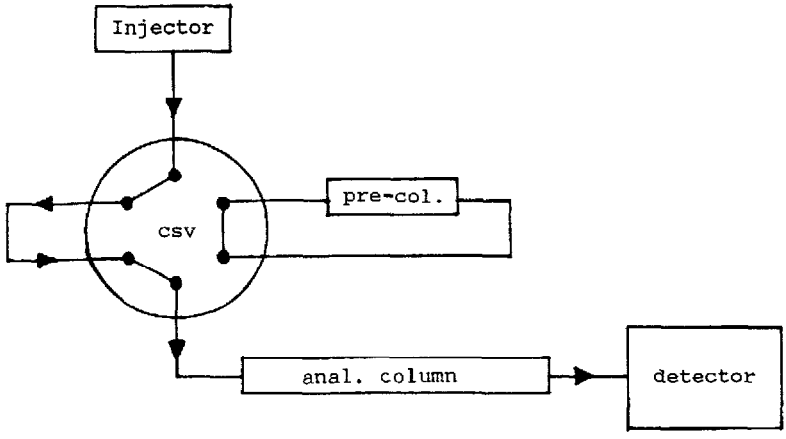


Fig. 1. Flow diagram of the chromatographic system at different positions of the column-switching valve (csv). Position 0, pre-column connected to flow; position 1, pre-column disconnected.

TABLE I

DIODE ARRAY DETECTOR SETTINGS

Substance detected	Detection wavelength (nm)	Detection bandwidth (nm)	Reference wavelength (nm)	Reference bandwidth (nm)
Creatine	210	20	550	100
Creatinine	234	10	550	100
Uric acid	290	10	550	100

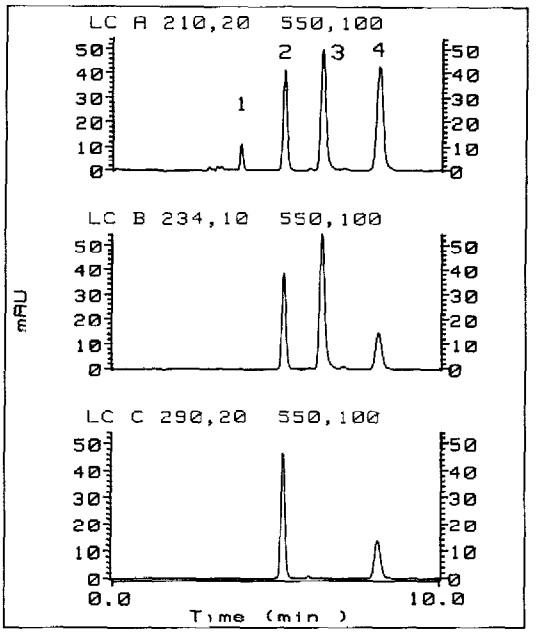


Fig. 2. Standard chromatogram at 210, 234 and 290 nm under the chromatographic conditions described in the text. Peaks: 1 = creatine (150 μmol/l or 1.97 mg/dl), 2 = uric acid (6.00 mg/dl); 3 = creatinine (6.00 mg/dl); 4 = dobesilate (6.00 mg/dl).

70 samples. The temperature was 22°C and the eluent flow-rate was 1.0 ml/min. The diode array detector settings were as shown in Table I.

RESULTS AND DISCUSSION

Fig. 2 shows a standard chromatogram with dobesilate as an artificially added peak, to demonstrate the separation of this drug, which is often found in the serum of kidney patients and interferes with the popular PAP creatinine determination method. The good separation of creatinine and dobesilate allows

a very precise quantitation of creatinine even in the presence of high levels of dobesilate in serum. The detection wavelengths were chosen according to the maxima in the UV spectra of the substances (Fig. 3): creatine at 210 nm, creatinine at 234 nm and uric acid at 290 nm. Fig. 4A shows a chromatogram of a serum sample with the three substances in the normal range; in Fig. 4B creatine is elevated. Whereas creatinine and uric acid can be integrated and quantified easily and with high precision at 234 and 290 nm, respectively (Fig. 5), it can be seen that many disturbances and peaks occur between 3 and 4 min in the chromatogram at 210 nm, where creatine must be detected. This complicates the quantitation of that compound, which often must be integrated with special methods, e.g. as a shoulder or by the tangent skim method. In addition, an unknown peak often co-elutes with creatine, making a quantitative determination almost impossible. For that reason we tried to improve the resolution in this segment of the chromatogram. We succeeded in separating the co-eluting compound from creatine with a pre-column filled with NH_2 packing material instead of C_{18}.

The working principle of the two different pre-columns is shown in Fig. 6. The C_{18} pre-column shows a good pre-separation of the three compounds, but the interfering and the creatine peak are almost coincident. This remains the same on the C_{18} material of the analytical column, resulting in slightly higher retention times for uric acid and creatinine; however, separation of the unknown peak and creatine is not achieved. With the NH_2 material no pre-separation of the three compounds can be obtained, but the interference is more retained than creatine, which can be found in the front part of the large single peak eluted from the pre-column under these conditions. Because of the similar elution properties of both substances on C_{18} material, sufficient resolution is maintained on the analytical column, whereas uric acid and creatinine are further retained and separated. Without the prior separation on the pre-column, the retention times are slightly shorter than with the C_{18} pre-column.

Fig. 7 shows the chromatogram of a serum sample: the unknown compound is well separated; it can be recognized by a low absorption at 234 nm, which is not observed with creatine.

Concerning the C_{18} material it can be said that, in general, materials with some residual activity of silanol groups are better for this type of separation than the end-capped ones. This was expected, because of the high polarity of substances and the mobile phase. With a strongly polar eluent as used here, the pre-column works as a filter for the serum proteins. Therefore it is important to expose it to the solvent flow for only a short time per run and to change it after ca. 70 serum injections, otherwise protein breakthrough will occur. With these precautions the analytical column can be used for 300–400 injections.

The post-separation gradient is used to clean the column of the more lipophilic compounds, e.g. some drugs. Substances well known to interfere with the photometric or enzymic methods, such as dobesilate, lidocaine and biliru-

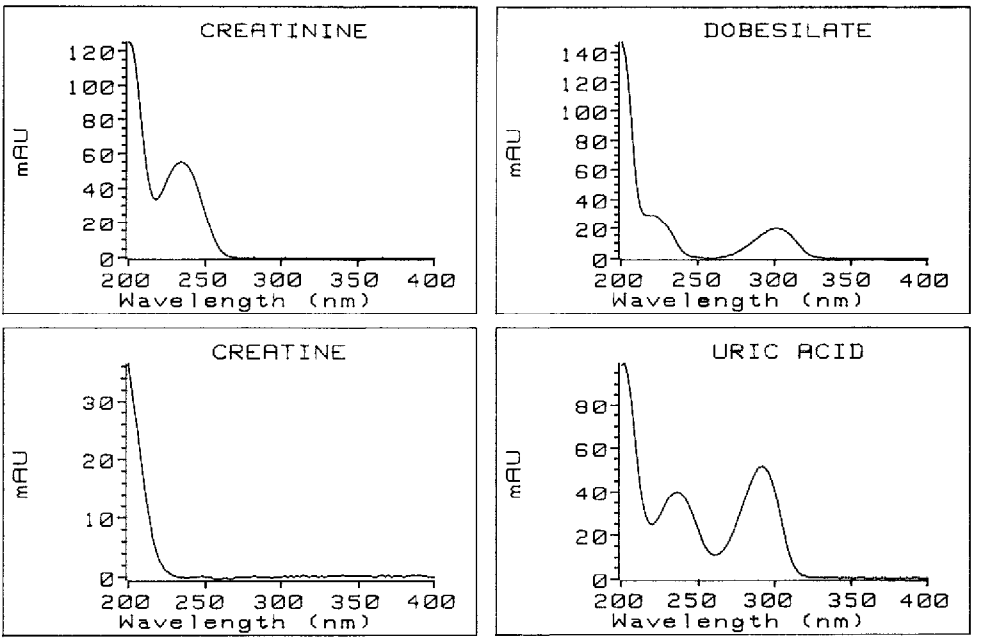


Fig 3. UV spectra of creatinine, dobesilate, creatine and uric acid, taken from chromatographic standard runs with a diode array detector

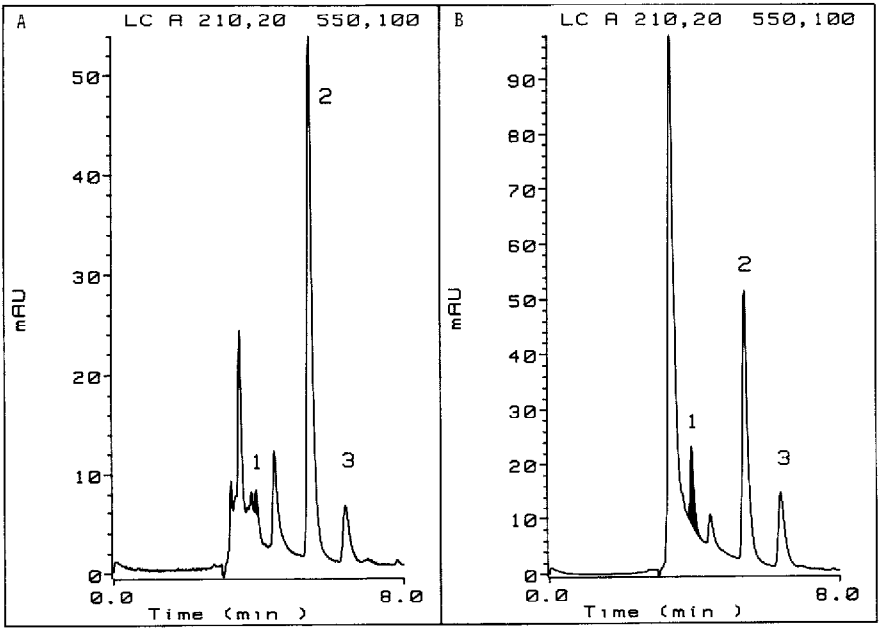


Fig. 4. Chromatograms of serum samples at 210 nm with a C_{18} pre-column. (A) Peaks: 1 = creatine (32 μmol/l), 2 = uric acid; 3 = creatinine. (B) Peaks. 1 = creatine (187 μmol/l); 2 = uric acid; 3 = creatinine.

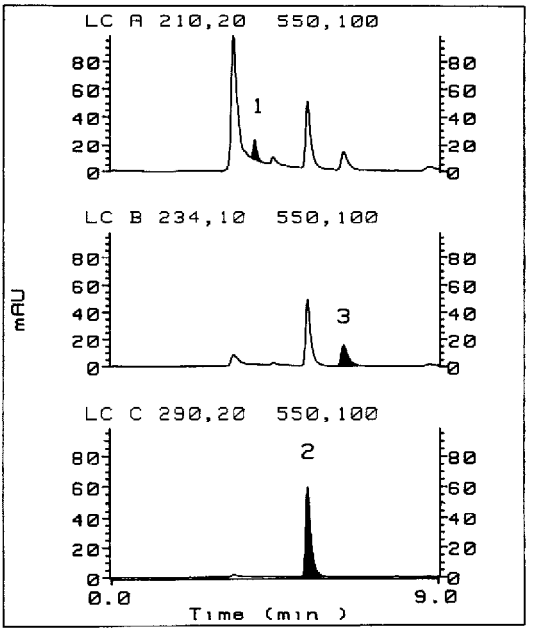


Fig 5. Three-wavelength quantitation of the sample in Fig. 4B. Peaks: 1=creatine (210 nm, 187 μmol/l); 2=uric acid (290 nm, 4.70 mg/dl); 3=creatinine (234 nm, 1.75 mg/dl).

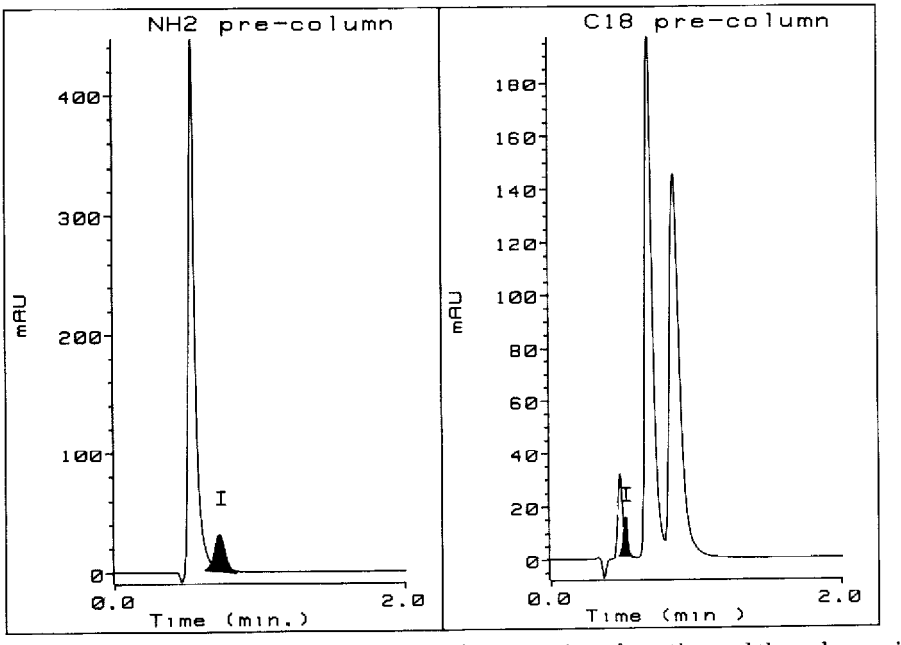


Fig. 6. Influence of different pre-columns on the separation of creatine and the unknown interference. These elution profiles are from the pre-column only: with the NH_2 pre-column the interfering peak (I) is separated from the unresolved creatine, uric acid and creatinine peak; with the C_{18} material peak I co-elutes with the pre-separated creatine.

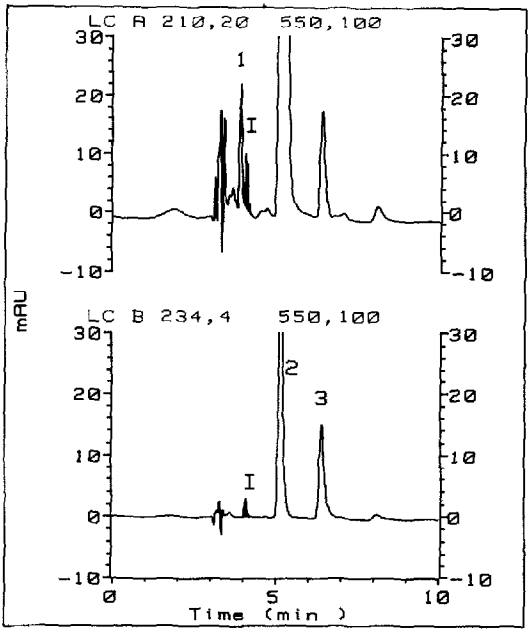


Fig. 7. Two-wavelength chromatogram of a serum sample containing the interfering substance, obtained with the NH_2 pre-column. Peaks: 1 = creatine (162 μmol/l); 2 = uric acid (5.2 mg/dl); I = interference (unknown, small absorption at 234 nm); 3 = creatinine (1.55 mg/dl).

bin, do not interfere with the quantitation of creatinine. A recovery of 100% and good linearity were found in biotest human serum by spiking with 0.2–20 mg/dl creatine (15–1500 μmol/l), 0.2–40 mg/dl creatinine and 0.2–20 mg/dl uric acid. The day-to-day precision for creatinine and uric acid had a coefficient of variation (C.V.) of 1.5%; for creatine the C.V. was 3–5%, depending on interfering impurities.

The accuracy of the method for creatinine was compared with that of an isotope dilution mass spectrometric (MS) method [12]. The slope of 1.06 of the correlation graph indicates a small relative deviation of the two methods (Fig. 8). This may be caused by a standard substance that is less than 100% pure. In fact, the creatinine standard we used was 97.3%, measured against NBS Standard No. 914a. This would result in a slope of 1.03 (HPLC values 3% too high); however, further experiments have to be performed with a higher number of serum samples, to increase the statistical reliability of these results. It cannot be determined yet whether there is a systematic deviation between the two methods. The stability of creatinine and uric acid in serum at room temperature is 20 h minimum and that of creatine is only 6 h. This can be improved by use of a cooled autosampler. The standard solution must be freshly prepared every day from stock solutions.

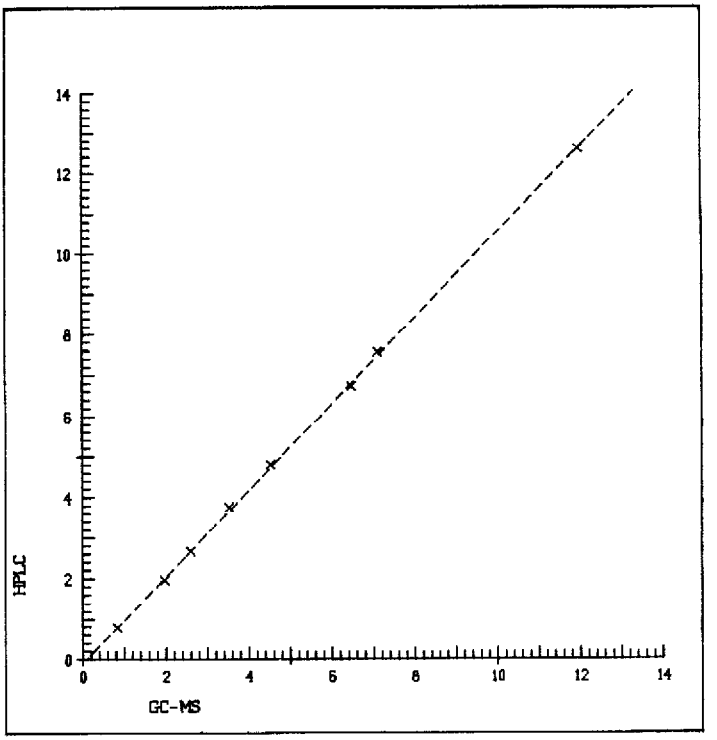


Fig. 8. Comparison of HPLC and GC-MS methods for creatinine; linear regression equation: HPLC = 1.06 GC-MS − 0.09, correlation coefficient = 0.999

CONCLUSION

The presented method for the determination and quantitation of creatine, uric acid and creatinine is useful for routine analysis. It does not require sample pretreatment and avoids therefore analytical errors caused by handling. It can be calibrated with a suitable standard, has 100% recovery, a wide range of linearity and offers good precision in series and from day to day. No interferences by drugs or endogeneous substances have yet been observed. It could therefore be a candidate for a reference method for creatinine and uric acid, as discussed by Stamm [13].

REFERENCES

1 M. Jaffe, Z. Physiol. Chem., 10 (1886) 391.
2 D.S. Young, L.C. Pestaner and V. Gibbermann, Clin. Chem., 21 (1975) 432.
3 F. da Fonseca-Wollheim, Lab. Med., 12 (1988) 317.
4 K. Jung, C. Wesslau, F. Priem, G. Shreiber and A. Zubek, J Clin. Chem. Clin. Biochem., 25 (1987) 357.

5 G. Brivio and G. Torelli, G. Ital. Chim. Clin., 11 (1986) 183
6 G. Schumann and J. Buettner, Z. Anal. Chem., 324 (1986) 284
7 E. Wiedemann, E. Haegele, J. Siedel and J. Ziegenhorn, Z. Anal. Chem , 324 (1986) 333.
8 E.R. Werner, D Fuchs, A. Hausen, G Reibnegger and H. Wachter, Clin. Chem., 33 (1987) 2028.
9 A. Zhiri, O Houot, M. Wellmann-Bednawska and G. Siest, Clin. Chem , 31 (1985) 109.
10 H. Murakita, J. Chromatogr., 431 (1988) 471.
11 H Mueller, Z. Anal. Chem., 332 (1988) 464.
12 L. Siekmann, J. Clin Chem. Clin. Biochem., 23 (1985) 137.
13 D. Stamm, J. Clin. Chem. Clin. Biochem., 20 (1982) 817.