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A Candidate Reference Method for the Determination of Uric Acid in Serum Based on High Performance Liquid Chromatography, Compared with an Isotope Dilution-Gas Chromatography-Mass Spectrometer Method

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Summary: A method based on isocratic high performance liquid chromatography (HPLC) with UV detection at 292 nm is proposed as a candidate reference method for the determination of uric acid. Data obtained by this method are compared with those from an isotope dilution-gas chromatography-mass spectrometric method (ID-GC-MS), using [1,3-$^{15}N_2$]uric acid as internal standard and selected mass detection at m/z = 456 and m/z = 458.

The inaccuracy of the ID-GC-MS method is maximally 0.4% for NBS-SRM-909 control sera with a concentration of 483 µmol/l. The coefficient of variation between days is 0.26% – 0.80% and 0.37 – 0.90% for 14 control sera from other suppliers.

The maximum bias of the HPLC method is 0.6%, and the coefficient of variation between days is 0.31% – 0.65% for NBS-SRM-909 control sera. The coefficient of variation between days for the other 14 control sera tested is 0.35% – 0.66%.

Comparison of the HPLC method with the reference ID-GC-MS method resulted in a coefficient of correlation of r = 0.9998 (n = 14). The concentration of uric acid in the tested control sera ranged from 160 to 624 µmol/l.

Introduction

During the last ten years several procedures for the determination of uric acid in different body fluids have been published. They employ the method of reversed-phase HPLC with electrochemical or spectrophotometric detection, for samples of serum, urine (1 – 19), amniotic fluid (20) as well as cerebrospinal fluid (21). The described methods suffer mainly from lack of the precision required for a reference method, and the fact that no internationally accepted standard material was used for the control of accuracy. The first HPLC-based candidate reference method was based on isotope dilution-high performance liquid chromatography with radioactive uric acid as internal standard (22).

The aim of this study was to establish an HPLC-method with high precision and accuracy to serve as candidate reference method for the determination of uric acid. The use of guard columns eliminated the requirement for complex sample preparation techniques, which reduce both the simplicity of the method and reliability of the analytical results.

To test the accuracy of the presented method, its analytical results were compared with those obtained by an isotope dilution-gas chromatographic-mass spectrometric (ID-GC-MS) method. From the well established methods (23 – 24), the method of *Siekmann* was chosen, which uses [1,3-$^{15}N_2$]uric acid as internal standard.

Materials and Methods

Chemicals

Uric acid (purity 99.7% ± 0.1%) used for preparation of the standard solutions was a Standard Reference Material (SRM 913, National Bureau of Standards, Washington, D. C. 20234); [1,3-$^{15}N_2$]uric acid (99% 1,3-$^{15}N_2$- isotopic enrichment) was obtained from Amersham International (Amersham, UK); uricase (EC 1.7.3.3) was purchased from Boehringer Mannheim (Mannheim, FRG); N-methyl-N-trimethylsilyl trifluoroacetic amide was supplied by Macherey & Nagel (Düren, FRG). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, FRG), SIGMA (Munich, FRG) and Aldrich (Steinheim, FRG). The pyridine used was stored over molecular sieve 4 µm, purchased from Merck (Darmstadt, FRG). Demineralized water was prepared with a Milli-Q apparatus from Millipore GmbH (Eschborn, FRG). The anion exchange resin AG1x2, acetate form, was supplied by BioRad (Munich, FRG).

Samples

Human serum for determination of accuray of the methods was a Standard Reference Material (SRM 909, National Bureau of Standards, Washington, D. C. 20234). All other control sera were given by Prof. Dr. Dr. *D. Stamm* (Deutsche Gesellschaft für Klinische Chemie e. V., Zentrale Referenzinstitution, Max-Planck-Institut für Psychiatrie, Abteilung Klinische Chemie, Kraepelinstr. 10, D-8000 München 40).

Instruments and settings

Isotope dilution — gas chromatography — mass spectroscopy

A Finnigan MAT 1020 combined gas-chromatograph quadrupole-mass-spectrometer with Perkin Elmer Sigma-3 gas-chromatograph, equipped with an FS-SE 30/CB 25 m × 0.32 mm capillary column (Macherey & Nagel, Düren, FRG) was used.

GC-conditions: The carrier gas was Helium at 100 kPa inlet pressure, the split-exit was set at 10 ml/min, the split-ratio was set to a value about 1 : 10, the injector temperature was 230 °C, the oven temperature was isothermal 210 °C, the transfer line was heated to 230 °C.

MS-conditions: selected mass detection at m/z = 456 ± 0.25 and m/z = 458 ± 0.25, 0.28 seconds per scan, the resolution was set to approximately 10% valley by manual tuning.

High performance liquid chromatography

HPLC was performed on a Bruker Model 31 Chromatograph with Rheodyne injection valve (20 µl injection-loop), 20 × 4 mm RP-18 guard column, 250 × 4 mm RP-18 analytical column, both filled with LiChrosorb (Merck; Darmstadt, FRG) RP-18, 5 µm material. The guard-column has to be replaced every 40—60 injections due to contamination with serum components. The analytical column must be replaced every month for the same reason.

For UV-detection a Bruker-Knauer filter photometer No. 97.00, equipped with a 292 nm interference filter, set to 0.08 absorbance units full scale, was used. Integration of area was carried out on a Hewlett-Packard Integrator.

The multiwavelength chromatograms were obtained with a Waters 990 Photo-Diode-Array-UV-Vis Detector with the following settings: wavelength range 210 nm—330 nm, resolution 4 nm, 1 scan per second, 23 ms sampling time; 10 sampling times were averaged per scan; sensitivity was set to high (7 points).

The eluent was 0.05 mol/l potassium dihydrogenphosphate buffer, pH 4.90. Flow rate was set to 1 ml/min, corresponding to a pressure of 120 bar.

Glassware

Volumetric flasks (10 ml and 100 ml) were calibrated by filling with water at 20 °C and weighing the contents. The 25, 50 and 100 µl gas tight syringes, equipped with repeating adapter (Hamilton Bonaduz AG; Bonaduz, Switzerland) were calibrated by weighing procedures before use.

Samples for HPLC- and GC-MS measurements were prepared in plastic reaction vials (3 ml) (Sarstedt; Nümbrecht, FRG). For the GC-MS procedure, the final evaporation of methanolic ammonia and the derivatisation procedure were performed in Micro-Vials (Wheaton; Millville, NJ, USA).

A 5 µl syringe was used for injection into the GC. A 25 µl syringe with a Rheodyne-tip was used for injection into the HPLC.

Procedures and measurements

Weighing procedures

The uric acid certified reference material and the isotopically labelled uric acid were weighed on a micro balance (Mikrowaage 708501, Fa. Sartorius, Göttingen, FRG). All other weighing procedures, including the calibration of volumetric flasks and the Hamilton syringes, were done on a semimicro balance (Halbmikrowaage 2004 MP, Fa. Sartorius, Göttingen, FRG).

Serum reconstitution

Serum samples (Standard Reference Material) and control sera were reconstituted by the following procedure. The label was torn off completely, and residual paper and adhesive were removed by wiping the vials with an acetone-moistened tissue; a number was scratched on each vial for specimen identification. After removal of the metal closure, serum particles adherent to the stopper were dislodged by repeatedly tapping the bottom of the vial. The stopper was then dislodged to equalize air pressure, and the vial plus stopper was weighed with a precision of 0.1 mg. The amount of water (19—22 °C) specified for the reconstitution of the serum specimen, was pipetted into the vial.

The control sera were pooled by pipetting 5 ml from each of 5 reconstituted vials, using an officially calibrated pipette and mixing carefully.

After reconstitution, the empty vials were cleaned and dried, then weighed to determine the previous content of lyophilisate of each vial.

Four pools were prepared and measured in the same manner to obtain the mean concentration for each given control serum.

Preparation of standards

For ID-GC-MS, the internal standard was prepared by dissolving 2 mg of isotopically enriched [1,3-$^{15}N_2$]uric acid in 10 ml 0.013 mol/l ammonia under sonification. The internal standard was frozen in portions of 1 ml at −71 °C. The uric acid standard solution was prepared by dissolving 2 mg uric acid (SRM 913) in 100 ml 0.013 mol/l ammonia under sonification.

For HPLC analysis, the standard solution was prepared by dissolving 6 mg uric acid (SRM 913) in 100 ml 0.013 mol/l ammonia under sonification.

For ID-GC-MS measurements as well as for HPLC measurements, two standards were prepared freshly each day.

HPLC measurements

Standards and serum samples were diluted with HPLC eluent buffer to give concentrations of 20 to 40 µmol/l. The required amount of diluent was weighed and the amount of standard or serum pipetted with a 50 µl calibrated syringe. Then the diluted samples were filtered through a membrane filtration device with a pore diameter of 0.45 µm (Sartorius GmbH; Göttingen, FRG) and 20 µl were injected for HPLC analysis.

Low standard, serum sample and high standard were consecutively injected in triplicate. The concentrations of uric acid in the serum samples were obtained by integration of peak areas using linear interpolation between the bracketing standards.

GC-MS measurements

Standard samples were prepared by pipetting 20 µl of internal standard into 150 µl, 200 µl or 250 µl of standard solution in Micro-Vials. The solvent was evaporated at 60 °C under a nitrogen-stream and the residue was finally incubated with 50 µl derivatisation mixture (N-methyl-N-trimethylsilyl trifluoracetic amide/pyridine 1 + 2) at 60 °C overnight.

Serum samples were prepared by pipetting 20 µl internal standard, 50 µl serum and 500 µl demineralized water into a vial. The solution was gently mixed up and equilibrated for 30 min. Then the samples were transferred to Pasteur pipettes (2.5 cm length, 0.5 cm inner diameter) filled with 1 ml anion exchange resin. After washing with 2 ml demineralized water and 500 µl 12 mol/l acetic acid, the samples were evaporated under a nitrogen-stream at 80 °C. The residue was dissolved in 500 µl ammonia in methanol (50 ml 25% NH_3 solution + 950 ml methanol). After centrifugation in an Eppendorf centrifuge 5414 for 5 min, the supernatant was transferred into Micro-Vials and treated as described above.

For GC-MS measurements 1.5 µl were injected, and areas were calculated with the Finnigan-MAT integration software. Subsequent calculations were performed, using the computational procedure described by *Siekmann* (25).

Results

Specificity

The chromatograms (fig. 1) of standard mixtures of uric acid, creatinine, hypoxanthine, xanthine, uridine, thymine, inosine-5′-phosphate, orotic acid, allopurinol and oxipurinol, each at a concentration of 45 µmol/l clearly demonstrate that all substances tested are well separated from uric acid. Relative retention times with respect to uric acid are given in table 1.

Nevertheless, uricase degradations of the control sera were performed prior to quantitative analysis, to ensure the homogeneity of the uric acid peak. Interfering substances were never observed at the retention time of uric acid; the same was true for pooled patient sera.

Precision

Precision between days was tested with appropriate pool sera; aliquots were stored seperately at −70 °C and were thawed and equilibrated at room temperature prior to sample processing.

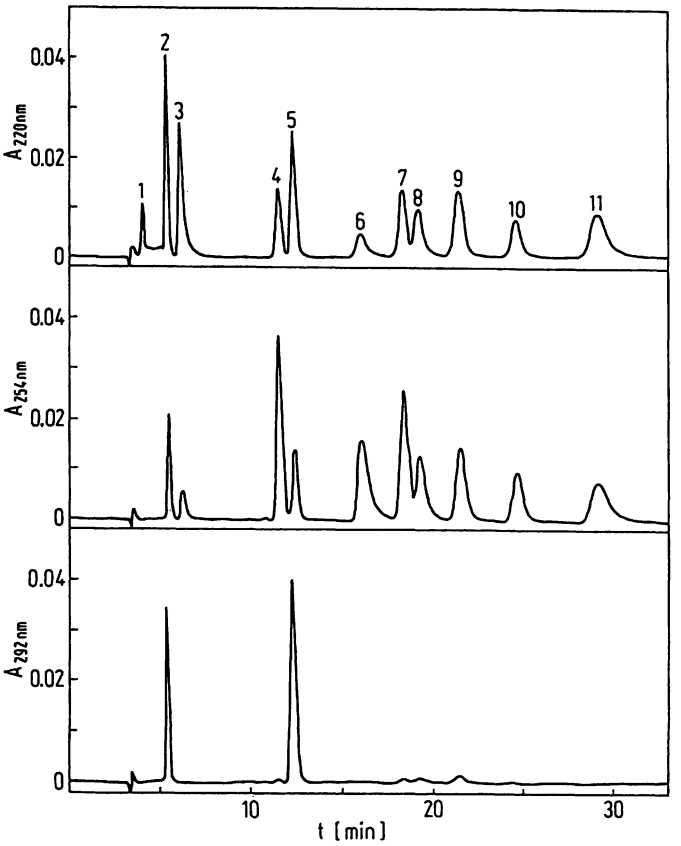

Fig. 1. Multiwavelength chromatograms performed at 220 nm, 254 nm and 292 nm with concentrations of 45 µmol/l of each substance; peak identification:
1. allantoin,
2. orotic acid,
3. creatinine,
4. inosine-5′-phosphate,
5. uric acid,
6. hypoxanthine,
7. uridine,
8. xanthine,
9. thymine,
10. oxipurinol,
11. allopurinol

Tab. 1. Relative retention times of probably interfering substances

Substance	Relative retention time
Allantoin	0.330
Orotic acid	0.439
Creatinine	0.498
Inosine-5′-phosphate	0.932
Uric acid	1.000
Hypoxanthine	1.297
Uridine	1.485
Xanthine	1.556
Thymine	1.732
Oxipurinol	1.991
Allopurinol	2.362

The coefficient of variation (CV) within run (n = 20) is less than 0.5% for analysis of sera (data shown in table 2); the CV between days (n = 20) is less then 0.8% for analysis of sera (data shown in table 3).

Tab. 2. Within-run precision of the HPLC method for the determination of uric acid in serum (n = 20)

Mean µmol/l	Standard deviation µmol/l	CV %
266	1.3	0.5
322	1.6	0.5
513	2.5	0.5
552	2.2	0.4

Tab. 3. Day to day precision of the HPLC method for the determination of uric acid in serum (n = 20)

Mean µmol/l	Standard deviation µmol/l	CV %
245	1.7	0.7
357	2.9	0.8
511	2.5	0.5
592	4.7	0.8

Accuracy

For SRM 909 the maximum deviation from the certified value was 0.6% in HPLC, which is only slightly higher than the bias obtained from the ID-GC-MS measurements (see table 4).

The results for the concentration of uric acid in 14 control sera, as obtained with both HPLC and ID-GC-MS, are shown in table 5 and figure 2.

The standardized main component analysis yielded the following relationship:

$$c_{\text{uric acid (HPLC)}} = 1.00595 \times c_{\text{uric acid (GC-MS)}} - 3.834\ \mu\text{mol/l}$$

The coefficient of correlation was $r = 0.9998$ ($p < 0.0005$).

Practicability

The number of analyses per day is dependent on the required precision. The weighing procedure for the diluent buffer is the most time consuming step in sample preparation. The time required for preparation of the standards is about 15 min. Precluding the analysis of 5 serum pools, the complete dilution procedure takes about 1 h. Since the absence of interfering substances was shown for all tested control sera by uricase decomposition studies, one HPLC run with 6 standard- and 3 serum injections takes about 20 min. Therefore, if two threefold-determinations of each serum pool are performed, the HPLC measurements will take about 5.5 h, if one run with an appropriate dilution of SRM 909 is intercalated in every 2 runs.

Tab. 4. Concentration of uric acid in the Standard Reference Material SRM-909 (human serum), obtained by ID-GC-MS and HPLC
CV = coefficient of variation between days (n = 10)
%Bias = 100 × ($c_{\text{obtained}} - c_{\text{certified}}$) / $c_{\text{certified}}$
$c_{\text{certified}}$ = 483 µmol/l

ID-GC-MS			HPLC		
Mean µmol/l	CV %	Bias %	Mean µmol/l	CV %	Bias %
483	0.5	0.0	483	0.3	0.0
481	0.8	−0.4	481	0.5	−0.4
482	0.7	−0.2	480	0.7	−0.6
482	0.3	−0.2	482	0.5	−0.2
482	0.7	−0.2	480	0.6	−0.6
482	0.2	−0.2	483	0.4	0.0

Tab. 5. Concentration of uric acid in the 14 control sera, obtained by ID-GC-MS and HPLC
%Deviation = 100 × ($c_{\text{IC-GC-MS}} - c_{\text{HPLC}}$) / $c_{\text{ID-GC-MS}}$

ID-GC-MS		HPLC		Deviation %
Mean µmol/l	CV %	Mean µmol/l	CV %	
328	0.56	328	0.52	0.00
510	0.53	510	0.38	0.00
500	0.74	502	0.64	−0.40
296	0.46	290	0.50	2.03
320	0.57	320	0.50	0.00
624	0.61	622	0.52	0.32*
160	0.78	162	0.66	−1.25*
485	0.31	486	0.50	−0.21
286	0.37	282	0.44	1.40
306	0.49	299	0.35	2.29
313	0.90	310	0.77	0.96
306	0.49	302	0.56	1.31
321	0.62	320	0.49	0.31
403	0.51	402	0.48	0.25

* Analysis was performed from single vials, not from pools, as described in Materials and Methods

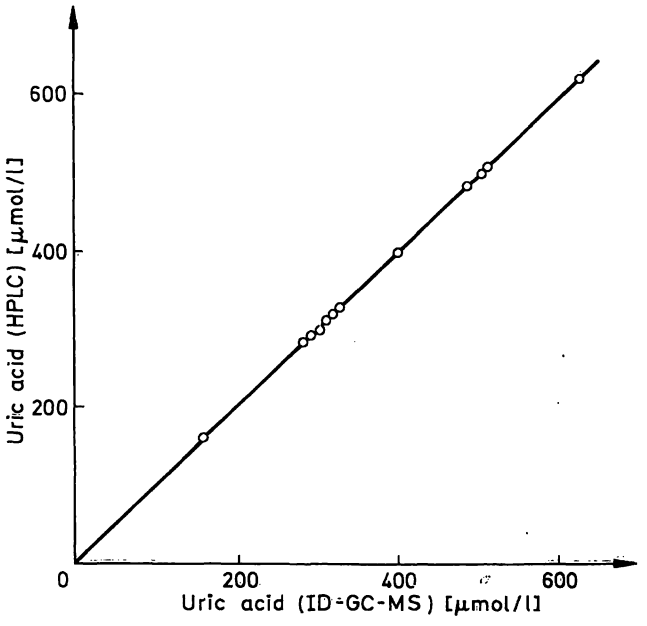

Fig. 2. Correlation of the analytical results for uric acid determination in 14 control sera with HPLC and ID-GC-MS

Conclusions

The HPLC method for determination of uric acid presented in this paper has high precision and accuracy. The coefficient of variation within run does not exceed 0.5%, and the day to day variation does not exceed 0.8%. For Standard Reference Material SRM 909, the deviation from the certified value was no larger than 0.4%. Thus, the inaccuracy of the method presented is not worse than the imprecision.

The comparison of the analytical results for the 14 control sera obtained by the established ID-GC-MS method with those obtained by the described HPLC method yielded a coefficient of correlation r = 0.9998. As can be seen in table 5; the largest difference between the methods is 2.29%; for 8 of the 14 control sera the difference is less than or equal to 0.40%. Large differences were observed in the case of two control sera, where the reference method value determined by ID-GC-MS was 2.03% or 2.29% higher than the the value determined by HPLC. This may result from specific serum matrix effects, which affect only the accuracy of one or of both used methods. Since HPLC- and ID-GC-MS measurements were done with samples from the same serum pools, other sources of error could be excluded.

To our knowledge, this is the first report of the comparison of an HPLC- with an ID-GC-MS method for the determination of uric acid. Moreover, no previously published HPLC method for this analyte has been evaluated using Standard Reference Material for standards as well as control sera; and in all previously reported studies, accuracy was determined by standard-recovery experiments only.

Another advantage of our HPLC method is the simplification of the sample preparation by the use of guard columns, which also contributes to the high reproducibility. As shown by *Kojima* et al. (26) for the Amicon MPS-1 ultrafiltration system and by *Sakuma* et al. (27) for several other deproteinizing methods, such as zinc hydroxide, sodium tungstate, trichloroacetic acid, perchloric acid and acetonitrile, all these procedures reduce the accuracy to such an extent, that they are disqualified as sample preparation techniques for a reference method.

Although the method is used in our laboratory only for the determination of reference method values, it may be used for the determination of uric acid in patient sera, if it is suspected that the routinely used methods are affected by interfering substances, as reported for hyperxanthinaemia (28).

This method is the first HPLC method to be evaluated for application as candidate reference method for the determination of uric acid in serum according to the recommendations of the International Federation of Clinical Chemistry (29–32).

An unsolved problem is the stability of uric acid in aqueous solution. Uric acid can be autoxidized by oxygen-derived radicals (33–34). This reaction is temperature-dependent, therefore the preparation of the uric acid standard solutions has to be carried out as carefully as possible. Temperatures above 20 °C should be avoided. This is also necessary for the reconstitution of the control sera.

References

1. Pachla, L. A. & Kissinger, P. T. (1975) Clin. Chim. Acta *59*, 309–312.
2. Kiser, E. J., Johnson, G. F. & Witte, D. L. (1978) Clin. Chem. *24*, 536–540.
3. Milner, J. A. & Perkins, E. G. (1978) Anal. Biochem. *88*, 560–565.
4. Brown, N. D., Kintzios, J. A. & Koetitz, S. E. (1979) J. Chromatogr. *177*, 170–173.
5. Pachla, L. A. & Kissinger, P. T. (1979) Clin. Chem. *25*, 1847–1852.
6. Putterman, G. J., Skaikh, B., Hallmark, M. R., Sawyer, C. G., Hixson, C. V. & Perini, F. (1979) Anal. Biochem. *98*, 18–26.
7. Wung, W. E. & Howell, S. B. (1980) Clin. Chem. *26*, 1704–1708.
8. Hausen, A., Fuchs, D. Koenig, K. & Wachter, H. (1981) Clin. Chem. *27*, 1455–1456.
9. Ingebretsen, O. C., Borgen, J. & Farstad, M. (1982) Clin. Chem. *28*, 496–498.
10. Nissen, P. (1982) J. Chromatogr. *228*, 382–386.
11. Miyazaki, H., Matsunaga, Y., Yoshida, K., Arawaka, S. & Hashimoto, M. (1983) J. Chromatogr. *274*, 75–85.
12. Palmisano, F., Desimoni, E. & Zambonin, P. G. (1984) J. Chromatogr. *306*, 205–214.
13. Hatch, L. L. & Sevanian, A. (1984) Anal. Biochem. *138*, 324–328.
14. Iriyama, K., Yoshiura, M., Iwamoto, T. & Ozaki, Y. (1984) Anal. Biochem. *141*, 238–243.
15. Zhiri, A., Houot, O., Wellman-Bednawska, M. & Siest, G. (1985) Clin. Chem. *31*, 109–112.
16. Green, D. J. & Perlman, R. L. (1980) Clin. Chem. *26*, 796–797.
17. Bennett, M. J., Patchett, B. P. & Worthy, E. (1984) Med. Lab. Sci. *41*, 108–111.
18. Topp, H., Sander, G., Heller-Schoech, G. & Schoech, G. (1985) Anal. Biochem. *150*, 353–358.
19. Soto-Otero, R., Méndez-Alvarez, E. & Sierra-Marcuño, G. (1986) Anal. Letters *19*, 1107–1119.
20. Krstulovic, A. M., Bertani-Dziedzic, L. A., Gitlow, S. E. & Lohse, K. (1979) J. Chromatogr. *164*, 363–372.
21. Niklasson, F. (1983) Clin. Chem. *29*, 1543–1546.
22. Siekmann, L., Siekmann, A., Mackrodt, D. & Johnen, C. (1986) Fresenius Z. Anal. Chem. *324*, 279–280.
23. Siekmann, L. (1985) J. Clin. Chem. Clin. Biochem. *23*, 129–135.

24. Ziegler, J. M., Houot, O., Hernot, M. & Siest, G. (1985) Clin. Chim. Acta *147*, 135–143.
25. Siekmann, L. & Breuer, H. (1982) J. Clin. Chem. Clin. Biochem. *20*, 883–892.
26. Kojima, T., Nishina, T., Kitamura, M., Kamatani, N. & Nishioka, K. (1986) Clin. Chem. *32*, 287–290.
27. Sakuma, R., Nishina, T. & Kitamura, M. (1987) Clin. Chem. *33*, 1427–1430.
28. Hande, K. R., Perini, F., Putterman, G. & Elin, R. (1979) Clin. Chem. *25*, 1492–1494.
29. Stamm, D. (1982) J. Clin. Chem. Clin. Biochem. *20*, 817–824.
30. Stamm, D. (1979) J. Clin. Chem. Clin. Biochem. *17*, 283–297.
31. Büttner, J., Borth, R., Boutwell, J. H., Broughton, P. M. G. & Bowyer, R. C. (1979) Clin. Chim. Acta *98*, 129F–143F; (1980) J. Clin. Chem. Clin. Biochem. *18*, 69–77; 78–88.
32. Büttner, H. (1976) Pure Appl. Chem. *45*, 69–73.
33. Grootveld, M. & Halliwell, B. (1987) Biochem. J. *243*, 803–808.
34. Maples, K. M. & Mason, R. P. (1988) J. Biol. Chem. *263*, 1709–1712.

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