Journal of Chromatography, 496 (1989) 91–100
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4932

DETECTION OF MACROENZYMES IN SERUM BY HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY

JEAN-PAUL SION, MURIËL LAUREYS, ERIK GERLO and FRANS GORUS*

Department of Clinical Chemistry, Academic Hospital of the "Vrije Universiteit Brussel" (A Z - V.U.B), Laarbeeklaan 101, B-1090 Brussels (Belgium)

(First received April 4th, 1989; revised manuscript received June 28th, 1989)

SUMMARY

A rapid high-performance gel permeation chromatographic method to confirm the presence of enzymes with abnormally high relative molecular masses (macroenzymes) in serum is described. The technique requires 200 μl of serum, can be automated and has been implemented for the analysis of creatine kinase (CK), lactate dehydrogenase, amylase, and alkaline phosphatase (ALP) activities. Serum fractionation according to relative molecular mass is completed within 21 min, and 84–106% of enzyme activities are recovered in the eluted fractions. The elution patterns obtained make possible the differentiation of 40 samples containing at least 10 U/l immunoglobulin–enzyme complexes, aggregated mitochondrial CK or membrane fragments carrying ALP activity from 40 control samples without these high-mass enzyme forms.

INTRODUCTION

Various isoenzymes are currently assayed in human serum by electrophoresis or more rapid indirect tests as biological markers for the diagnosis and monitoring of a wide variety of diseases [1–7]. The introduction and, more recently, the automation of these assays in clinical chemical work has facilitated effective detection of circulating enzyme forms with a high relative molecular mass (M_r), termed macromolecular enzymes or macroenzymes, and raised questions concerning their clinical relevance [8–14]. During the past two decades it became increasingly clear that the appearance of such oversized enzyme molecules in serum is common: their proportion ranges between 0.1 and 13.8%, depending on the enzyme and the subject group studied [15–19].

Several types of macroenzyme have been identified, but so far only a few

0378-4347/89/$03.50

appear to have clinical or prognostic relevance. The presence in serum of oligomeric mitochondrial creatine kinase (CK, EC 2.7.3.2.) heralds deep cellular necrosis and poor outcome [20], whereas the appearance of membrane fragments carrying alkaline phosphatase (ALP) activity (koinozyme form of ALP, EC 3.1.3.1 [21]) reflects cholestasis [3,5]. An increasing number of serum enzymes, including CK, lactate dehydrogenase (LD, EC 1.1.1.27), ALP and amylase (AMYL, EC 3.2.1.1), have been reported to participate in circulating immune complexes. The clinical meaning of this remains uncertain [8–14,22–26], although at least in some patients their activity has paralleled autoimmune events [22–25]. In any case, their prompt identification is fully warranted in order to prevent misinterpretation of concomitantly elevated (iso)enzyme activities [27,28].

Although isoenzyme assays, e.g. based on electrophoresis or immunoinhibition, often but not invariably [29] herald the occurrence of macroenzymes in daily routine [27,28], the final proof and quantitation require molecular mass determination of the measured enzyme activity. So far, this has mainly been achieved through conventional gel permeation chromatography at atmospheric pressure, a tedious technique that is not well suited to routine applications.

This paper evaluates the potential of a recently designed high-performance gel permeation chromatographic (HPGPC) method for the fractionation of human or animal sera [30] to confirm the macromolecular nature of atypical CK, LD, ALP and AMYL activity as judged by electrophoretic screening techniques. This approach should facilitate large-scale epidemiological, clinical and biochemical studies for assessing the clinical relevance of certain types of macroenzyme.

EXPERIMENTAL

Blood samples

Blood was obtained from patients by puncture of the antecubital vein, collected in commercial plastic tubes (Sarstedt, Haasrode, Belgium), allowed to clot at room temperature and centrifuged at 1000 *g* for 15 min within 6 h after sampling. The supernatant serum was divided into aliquots and stored at −70°C until further assayed.

Total enzyme activities

All enzyme activities were measured at 30°C on an RA-1000 random-access analyser (Technicon, Dublin, Eire) with Boehringer reagents (Mannheim, F.R.G.) for CK, LD and ALP (CBR CK-NAC Kits 475.742 and 475.769, CBR LDH-opt test combination 543.047 and 543.055 and ALP-opt test combination 415.278, respectively) and with General Diagnostics Chem-Strate Enzymatic

Amylase Reagent (Kit 33146, Organon Technica, Turnhout, Belgium) for AMYL.

Isoenzyme activities

CK and LD isoenzymes were determined electrophoretically with Paragon agarose gels and reagents (Beckman Instruments, Brea, CA, U.S.A.; Kit 655.930 for CK and 655.940 for LD).

ALP-isoenzyme fractionation was carried out with the Isopal® agarose electrophoresis kit 004 040 (Analis, Namur, Belgium). AMYL isoenzymes were separated by electrophoresis on agarose membranes (No. 470 104, Ciba Corning Diagnostics, Palo Alto, CA, U.S.A.) using 12.25 g/l β-hydroxy-4-morpholinepropanesulphonic acid (MOPSO) (Ciba Corning) as buffer. The enzymic activity of the AMYL isoenzymes was revealed by means of a slurry of substrate tablets (seven tablets in 10 ml of buffer) from the Phadebas Isoamylase Test (Pharmacia Diagnostics, Uppsala, Sweden). The substrate was allowed to react for 90 min at 56°C [31].

Isoenzyme quantitation was carried out by means of an EDC scanning densitometer (Helena Labs., Beaumont, TX, U.S.A.) used in the fluorescence mode for CK (excitation at 366 nm and emission at 400 nm) and in the absorbance mode for LD, ALP and AMYL (600 nm).

The presence of macromolecular CK was suspected on the basis of an apparent CK band with aberrant electrophoretic mobility (more cathodic than CK-MM for mitochondrial CK [20] and in between the CK-MM and CK-MB regions for macro CK-BB [15]), in some samples in conjunction with a seemingly elevated CK-B immunoinhibition test (Kit No. 418.234, Boehringer) in the absence of typical CK-MB or CK-BB fractions [28]. Macromolecular LD activity of the immunoglobulin (Ig)-LD type was suggested by an abnormal number and/or mobility of "isoenzyme" bands on the LD zymogram [23], and Ig-ALP activity by a broad electrophoretic band that migrated in between the position of adult intestinal and $liver_1$ ALP during Isopal electrophoresis and that differed from bone ALP in its physicochemical characteristics [32]. Macromolecular $liver_2$ ALP (koinozyme) and liver/bone ALP were identified on basis of their characteristic electrophoretic mobilities [32]. Finally a smeared isoamylase pattern differentiated macro-AMYL activity of the Ig-AMYL type from normal salivary (S_2) and pancreatic (P_2) fractions [31].

Fractionation by high-performance gel permeation chromatography

HPGPC of human sera was carried out at room temperature on a bio-compatible HPLC apparatus (LKB Pharmacia, Bromma, Sweden) equipped with an HPLC pump (Model 2150), a titanium injector, a GlasPac TSK 3000 SW (300 mm×8 mm I.D.) column protected by a TSK SW (40 mm×4 mm I.D.) precolumn, a UV detector with a 280-nm interference filter and an HPLC flow-

cell (Uvicord SD Model 2158), a recorder (Model 2210) and a fraction collector (Model 2211 Superrac).

Serum samples were centrifuged for 15 min at 100 000 *g* (Airfuge, Beckman) and 200-μl aliquots were subsequently injected. Alternatively, the samples could be filtered through a 0.22-μm cellulose acetate filter (Spin-X, Costar, Cambridge, MA, U.S.A.).

The elution buffer consisted of phosphate-buffered saline (PBS, 10 mmol/l phosphate buffer pH 7.4 and 135 mmol/l sodium chloride) supplemented with 5 mmol/l sodium azide. The flow-rate was set at 800 μl/min, corresponding to a working pressure of ca. 1.3 MPa. Under continuous absorbance monitoring, 320-μl fractions were collected and stored at 4 °C (although room temperature was also suitable) until being assayed for enzyme activity within 24 h.

Protein molecular mass calibrators (gel permeation calibration kit, Pharmacia) were dissolved in elution buffer (final concentration 5 mg/ml, except ferritin, 1 mg/ml) and injected to establish molecular mass reference curves. Thyroglobulin (M_r = 669 000, Pharmacia, 5 mg/ml, in elution buffer) was used to determine the void volume (V_0) whereas sodium azide (2.5 mol/l in elution buffer) was used to assess the total column volume (V_t). For each eluted peak, the elution volume (V_e) was measured and the average value (K_{av}) was calculated according to $K_{av} = (V_e - V_0)/(V_t - V_0)$. The position where serum IgM, IgG and albumin were eluted was assessed by nephelometric analysis of the column fractions (BNA nephelometer, Behring, Marburg, F.R.G.) using Behring reagents (antisera Nos. OSAT 15, OSAS 15 and OSAL 15, respectively), according to the manufacturers specifications.

Occasionally, an increase in pressure was noted under normal working conditions. When the recommended maximum pressure for the column (2.0 MPa) was reached, 200 μl of a 1 g/l trypsin solution (EC 3.4.21.4, Boehringer) was injected and the flow-rate was lowered to 0.01 ml/min for overnight elution. This procedure normalized the pressure and permitted 500–1000 injections per column. The precolumn was replaced after 100 injections. After completion of the M_r analysis of the patient samples described under Results, the HPLC system was further automated as follows: a WISP 710B automated injector (Millipore/Waters, Milford, MA, U.S.A.) was incorporated, and the flow-rate was adjusted to 0.3 ml/min for overnight operation, yielding run-times of 56 min.

Immunoprecipitation

In a few selected sera, immunoprecipitation of possibly present IgG–CK-BB complexes with protein A-Sepharose CL-4B (Pharmacia) was attempted according to the procedure of Bauer et al. [33].

Statistical analysis

The statistical significance of differences between means of experimental groups was assessed by a two-tailed Student's *t*-test.

RESULTS

Following injection of a mixture of four M_r standards (Pharmacia), namely ribonuclease A (M_r 13 700), ovalbumin (dimer; M_r 86 000), aldolase (M_r 158 000) and ferritin (M_r 440 000), a linear M_r calibration curve was obtained (Fig. 1; inset). Also displayed in Fig. 1 is the typical absorbance pattern, observed after injection of 200 µl of serum and obtained from Uvicord monitoring (280 nm) at the column outlet. The first peak corresponds roughly to the IgM-

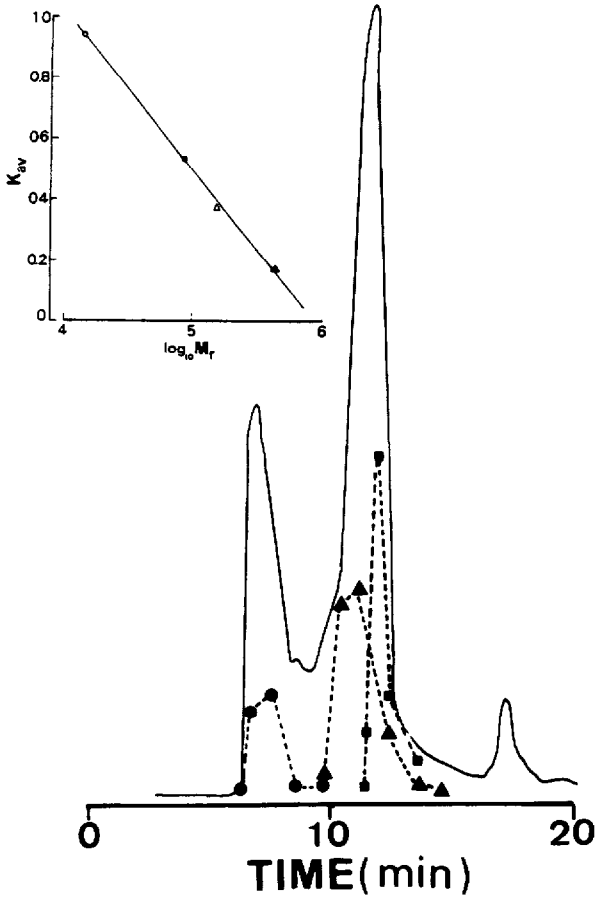

Fig. 1. High-performance gel permeation chromatography of human serum on a GlasPac® TSK 3000 SW column. The solid line represents the absorbance profile at 280 nm as monitored at the column outlet, while the dotted line denotes the elution position of IgM (circles), IgG (triangles) and albumin (squares) as determined by nephelometric analysis. Inset: M_r calibration curve obtained by plotting the K_{av} values observed with the GlasPac column against the logarithm of the assigned M_r for the following protein standards: (○) ribonuclease A (13 700); (●) ovalbumin (dimer; 86 000); (△) aldolase (158 000); (▲) ferritin (440 000).

containing void, the second composite peak contains mainly IgG and albumin and the third peak approximately coincides with the salt peak [30].

In Fig. 2 the distribution of enzyme activity throughout the eluted fractions in the case of macromolecular enzyme activity is contrasted with the distribution for a normal control serum for CK (Fig. 2a), LD (Fig. 2b), AMYL (Fig. 2c) and ALP (Fig. 2d). The recovery of the enzyme activities was 84–106% after HPGPC and a decrease in activity of less than 5% was noted when the serum fractions were incubated for 24 h at 4 °C. Comparable calibration curves, absorbance patterns and enzyme recoveries were obtained when 0.1 *M* ammonium acetate was used as elution buffer.

As illustrated in Table I, HPGPC confirmed the macromolecular nature of at least part of the CK activity in 25 out of 29 suspected cases of Ig–CK-BB

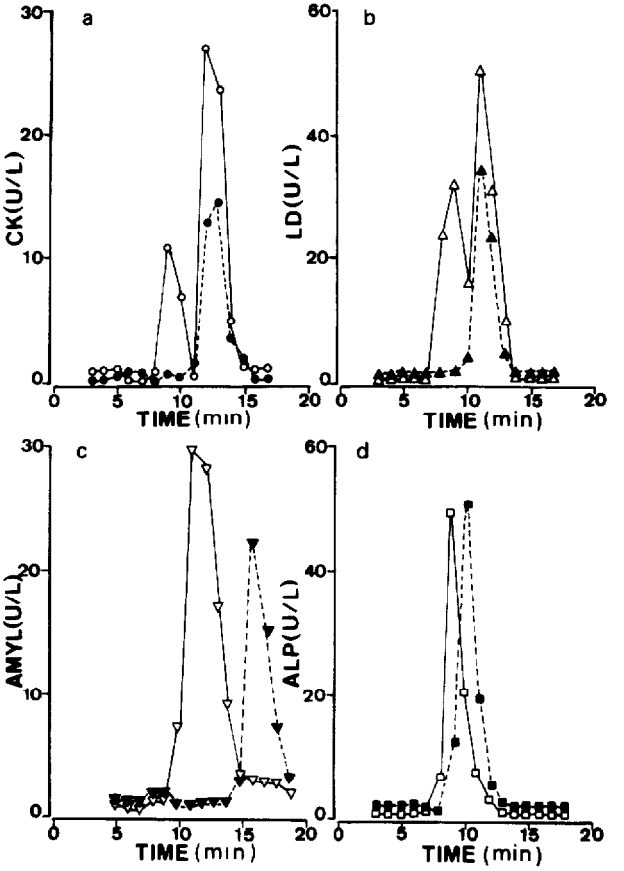

Fig. 2. Elution profile of enzyme activity during HPGPC of sera with (solid line; open symbols) or without (broken line; closed symbols) macromolecular enzyme forms: (a) CK; (b) LD; (c) AMYL; (d) ALP. For clarity, not all the fractions are included.

TABLE I

K_{av} VALUES AND APPARENT M_r VALUES AS DETERMINED BY HPGPC IN SAMPLES WITH ELECTROPHORETIC PROFILES SUGGESTING THE PRESENCE OF NORMAL OR MACROMOLECULAR ENZYME FORMS

Enzyme	Electrophoresis	HPGPC elution pattern			Reported M_r[a] (kDa)
		K_{av} (mean ± S.D.)	n	Apparent mean M_r (kDa)	
CK	Ig-CK-BB	0.23 ± 0.06[b]	25	295	
	Mitochondrial	0.24 ± 0.05[b]	2	280	
	MM ± MB	0.56 ± 0.03	18	78	81
LD	Ig-LD	0.12 ± 0.04[b]	4	550	
	LD_{1-5}	0.44 ± 0.05	9	134	134
AMYL	Ig-AMYL	0.36 ± 0.12[b]	3	185	
	S_2+P_2	1.09 ± 0.01	6	< 10	40–50
ALP	Ig-ALP	0.18 ± 0.01[c]	3	420	
	Koinozyme ($liver_2$)	0.06 ± 0.03[b]	3	> 700	
	$Liver_1$/bone	0.31 ± 0.02	7	235	240

[a]See ref. 34.
[b]$p < 0.001$ versus control with normal M_r (Student's t-test).
[c]$p < 0.02$ versus koinozyme (Student's t-test).

complexes (macro CK-BB; range 10–150 U/l by electrophoresis), selected because of indicative agarose electrophoresis patterns. Only one of the four samples that possibly contained small amounts of macro CK-BB (4–10 U/l) could be analysed for CK activity after incubation with protein A-Sepharose [33]. This procedure removed 11 U/l CK (30% of initial activity) which is higher than the −4 to +4 U/l precipitated in six normal controls with comparable total CK activities. There was no sample left for CK-B immunoinhibition, as was also the case for the three other sera with suspected low levels of macro CK-BB activity. For two sera that apparently contained macromolecular mitochondrial CK forms (70–140 U/l) on the ground of their typical electrophoretic migration, a peak of CK activity with abnormally high M_r was detected in both cases. By contrast, no such peak was detected for eighteen electrophoretically normal control sera. In 20 out of the 25 macro CK positive samples, part of the CK activity (10–90%) eluted at the same K_{av} as control samples.

Suspected cases of macromolecular Ig–LD complexes (as judged by agarose electrophoresis) were also confirmed by HPGPC (Table I) on four occasions and, as for CK, several elution patterns (three out of four) displayed both a high M_r and a normal M_r peak of LD activity. Only the latter peak was observed in all nine control sera (Table I). Reinjection of pooled high M_r fractions did

yield only one peak with an identical elution volume, indicating that no significant dissociation of macromolecular complexes had occurred during fractionation.

For ALP and AMYL, all electrophoretically suspected cases of Ig–enzyme complexes were confirmed as macromolecular entities by gel permeation (three for each enzyme). In all six cases, 100% of the enzyme activity was in the macromolecular form, with a K_{av} value that differed significantly from that of the controls ($n=6$ for AMYL and $n=7$ for ALP), as well as from the koinozyme form (also termed "liver$_2$ band" or "fast-liver" ALP) in the case of ALP that was eluted near the void (Table I). Using M_r calibration curves similar to that shown in Fig. 1, apparent mean M_r values for both normomolecular and macromolecular CK, LD, AMYL and ALP fractions were computed (Table I). Except for AMYL, a good agreement with previously reported M_r values for enzymes in serum was noted for the control samples [34]. For all enzymes tested, the amount of macroenzyme activity determined by HPGPC correlated well with the size of the atypical electrophoretic band (e.g. $r=0.971$ for CK by linear regression analysis).

After completion of the M_r analysis in patient's sera, the HPGPC method was further refined by incorporating an automatic injector and lowering the flow-rate. This modification allowed the system to operate overnight while improving the lifetime and performance of the column.

DISCUSSION

The HPGPC method described for fractionation of serum enzyme activity requires 200 μl of sample, can readily be automated and is performed within 21 min. The recoveries of CK, LD, AMYL and ALP activities were more than 84% and the enzyme activities remained stable for at least 24 h. Different types of macroenzyme, including Ig–enzyme complexes, oligomeric mitochondrial CK and koinozyme ALP, could be recognized on basis of their characteristic elution pattern, down to ca. 10 U/l in the case of macro CK, which is near the detection limit attainable with other techniques, such as immunoprecipitation [33] or densitometric zymogram scanning [28], that have been used to screen for the presence of macroenzymes.

Except for AMYL, HPGPC generated fairly accurate estimates of apparent M_r values for the enzymes tested [34]. It is conceivable that a modest degree of solute–gel interaction [35] might have occurred in the case of AMYL, resulting in a slight retardation on the column and hence a lower apparent M_r. Likewise, non-covalent interactions are probably also responsible for the fact that, by analogy with previous reports [36], bovine albumin and ovalbumin (Pharmacia) eluted as dimers in our HPLC system. Although such interactions render the present HPGPC method unreliable for accurate M_r estimation, they do not compromise its ability to discriminate between normal and

macromolecular enzyme forms. Indeed, using this technique we were able to demonstrate the macromolecular nature of CK, LD, AMYL or ALP activity in 40 out of 44 sera, selected on basis of indicative electrophoretic isoenzyme patterns. In four sera the suspected low-level (less than 10 U/l) macro CK-BB activity could not be recovered by HPGPC. Only in one sample was indirect evidence obtained by immunoprecipitation that favoured the presence of small amounts of such complex, whereas the three remaining sera were not available for further testing.

In contrast to the macro AMYL and Ig-ALP-like macro ALP elution profiles, the vast majority of the macro CK or macro LD patterns also displayed a normal enzyme peak of variable relative importance that was also noted on electrophoresis. The reason for this difference in elution patterns should be further investigated by studies of patients, as it is unlikely that the presence of the second normal peak reflects dissociation of enzyme complexes during chromatography. In addition, the ratios of the activities of macroenzymes and normal enzymes observed with HPGPC and electrophoresis appeared similar.

Although in this study electrophoresis appeared a good predictor of the presence of macroenzymes, there have been reports about macroamylases not evidenced by a broad band [29], as well as about macro CK-BB bands mimicking [37] or adsorbing [27] CK-MB activity. Molecular mass estimation therefore remains the method of choice for confirmation of the presence of macroenzymes, suspected on basis of electrophoretic characteristics, decreased enzyme clearance or raised (iso)enzyme activity in serum as judged by various assays [9–11, 28]. HPGPC fractionation is furthermore likely to provide additional information concerning the stoichiometry of enzyme–antibody interactions within enzyme complexes, the possible Ig class involved (IgM versus IgG/IgA) or the presence of enzyme fragments as described in transient hyperphosphatasemia [38]. The present method should be useful in supporting systematic epidemiological, clinical and biochemical approaches that further explore the significance of macromolecular enzyme species.

ACKNOWLEDGEMENTS

We thank Prof. Dr. D. Pipeleers, Dr. A. Foriers (both Department of Metabolism and Endocrinology, V.U.B.) and Dr. R. Zeeuws (Department of Clinical Chemistry, Academic Hospital of the V.U.B.) for their advice and support. The technical assistance of L. De Pree, P. Goubert and G. Stangé and the secretarial work of A. De Swaef, A. De Nil and M. Van Eeckhoutte are gratefully acknowledged. This work was supported by the Belgian "Fonds voor Geneeskundig Wetenschappelijk Onderzoek" (Grant No. 3.0088.86).

REFERENCES

1 F. Kanemitsu and T. Okigaki, J. Chromatogr., 429 (1988) 399.

2 M. Maekawa, J. Chromatogr., 429 (1988) 373.
3 W H. Fishman, Am. J. Med., 56 (1974) 617.
4 J.A. Lott and J.M. Stang, Clin. Chem., 26 (1980) 1241.
5 D.W. Moss, Clin. Chem., 28 (1982) 2007.
6 J.J. Zakowski and D.E. Bruns, Crit. Rev. Clin. Lab. Sci., 21 (1985) 283.
7 A. Burlina, Clin. Biochem., 12 (1979) 71.
8 H. Lang and V. Würzburg, Clin. Chem., 28 (1982) 1439.
9 P.M. Crofton, Crit. Rev. Clin. Lab. Sci., 16 (1982) 161.
10 I. Maire, Y. Arthur and G.J. Sanderink, Ann. Biol. Clin., 45 (1987) 269.
11 Y. Arthur, G.J. Sanderink and I. Maire, Ann. Biol. Clin., 45 (1987) 277.
12 W. Stein, J. Bohner and M. Bahlinger, J. Clin. Chem. Clin. Biochem., 25 (1987) 837.
13 M. Nagamine and K. Okochi, Clin. Chem., 29 (1983) 379.
14 M.E. De Broe, T.E. Mets, G.G. Leroux-Roels and R.J. Wieme, Ann. Int. Med., 90 (1979) 30.
15 P. Urdal and S. Landaas, Clin. Chem., 25 (1979) 461.
16 R.W. Forsman, Clin. Biochem., 19 (1986) 250.
17 J. Bohner, W. Stein, R. Steinhart, U Würzburg and M. Eggstein, Clin. Chem., 28 (1982) 618.
18 J. Delanghe, I. De Scheerder, M. De Buysere, L. Algoed and J. Robbrecht, Atherosclerosis, 60 (1986) 215.
19 D. Barrows, J.E. Berk and L. Fridhandler, N. Engl. J. Med., 280 (1972) 1352.
20 C.J. Berk, J. Am. Med. Assoc., 243 (1975) 2058.
21 M.E. De Broe, M. Borgers and R.J. Wieme, Clin. Chim. Acta, 59 (1975) 369.
22 J. Biewenga and T.E.W. Feltkamp, Clin. Chim. Acta, 64 (1975) 101.
23 F. Gorus, W. Aelbrecht and B. Van Camp, Clin. Chem., 28 (1982) 236.
24 O. Peters, F. Gorus and B. Van Camp, Clin. Chem., 28 (1981) 1826.
25 S.J. Podlasek, R.A. McPherson and G.A. Threatte, Clin. Chem., 31 (1985) 527.
26 M.A Virji, Clin. Chim Acta, 151 (1985) 223.
27 W. Stein and J. Bohner, Clin. Chem., 31 (1985) 1189.
28 F. Gorus, V. Claessens, P. Goubert and M. Laureys, Clin. Chem., 34 (1988) 1174.
29 C. Kammeraet and J.A. de Jong, Clin. Chem., 31 (1985) 1078.
30 M. Vercammen, F. Gorus, A. Foriers, O. Segers, G. Somers, M. Van De Winkel and D. Pipeleers, Diabetologia, 32 (1989) 611.
31 P. Leclerc and J.C. Forest, Clin. Chem., 28 (1982) 37.
32 V.O. Van Hoof, L.G. Lepoutre, M.F. Hoylaerts, R. Chevigné and M.E. De Broe, Clin. Chem., 34 (1988) 1857.
33 K. Bauer, P.M. Bayer, E. Deutsch and F. Gabl, Clin. Chem., 26 (1980) 297.
34 D.W. Moss, A.R. Henderson and J.F. Kachmar, in N.W. Tietz (Editor), Textbook of Clinical Chemistry, W.B. Saunders, Philadelphia, PA, 1st ed., 1986, Ch. 5, p. 619
35 R.A. Jenik and J.W. Porter, Anal. Biochem., 111 (1981) 184.
36 C. Lazure, M. Dennis, J. Rochemont, N.G. Seidah and M. Chrétien, Anal. Biochem., 125 (1982) 406.
37 J. Medeiros, F. Greco, D. Walsh and B. Gerson, Clin. Chem., 31 (1985) 1393.
38 E. Schoenau, K.H. Herzog and H.J. Boehles, Clin. Chem., 32 (1986) 2211.