MICROPLATE ASSAY FOR HYALURONAN USEFUL IN CLINICAL PRACTICE

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A microplate assay for determination of hyaluronan (HA) has been developed. In principle, HA in the sample and biotinylated HA compete for binding to hyaluronan binding protein (HABP) coated to the plate. The amount of the microplate-bound biotinylated HA is quantified with avidin peroxidase complex. This assay is useful for 10 – 500 μg/l with < 2% relative error with 50% inhibition at 80 μg/l. The assay is simple, sensitive and particularly suitable for the determination of HA in serum and other biological fluids. The assay correlated with only commercially available radioisotope kit, Pharmacia HA-50, with correlation coefficient 0.92 at $p < 0.001$. Serum HA levels of patients with osteoarthritis, rheumatoid arthritis and healthy donors determined by this assay correspond to those previously determined by different methods. These results suggest that this assay is suitable for clinical use.

Hyaluronan (HA, hyaluronic acid, hyaluronate), a high molecular weight polysaccharide, is widely distributed in connective tissue. It possess both structural and regulatory functions. Hyaluronan seems to enter the blood circulation via the lymph vessels and is then rapidly eliminated in liver[1]. Elevated HA concentration in serum is an indicator of HA turnover. It reflects both a change in an HA outflow from the tissue and an impairment in catabolism of HA in liver[2].

HA concentration in the serum of healthy adults is low[3] (20 – 100 μg/l). The concentration significantly increases in sera of patients with rheumatoid arthritis (RA) and progressive systemic sclerosis[4–6]. High HA concentrations were also found in patients with osteoarthritis of large joints[4]. The other clinical applications of serum HA determination are related to liver diseases, certain cancers and chronic renal failure[7–9].

Previously developed methods for determination of HA concentration used precipitation and chromatography followed by a colorimetric assay[10,11]. Application of these methods for serum is frequently misleading because of the large excess of other glycos-

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aminoglycans. New, sensitive methods for determination of HA concentration in biological fluids use proteins capable of binding HA by biospecific interactions. Recently published methods used hyaluronan binding protein (HABP), isolated from brain (hyaluronectin)[12], or cartilage (hyaluronan binding proteins – HABP)[13,14]. However, these methods require either use of radioactivity[15], or additional development od anti-HABP antibodies[16].

Microplate based assays without using the antibodies have also been described[17,18]. The plates were coated with HA or HABP. Biotinylated HABP was used for the detection of HA in the samples. However, in this arrangement the biotinylation can cause a decrease of HABP binding activity that makes the assay difficult to reproduce.

We present a specific, sensitive and rapid microplate method for HA determination. In our assay the HA in a sample competes with biotinylated HA for binding to HABP coated on the microplate. The amount of bound, biotinylated HA is evaluated with avidin-horse radish peroxidase (HRP) complex. The assay development is described step by step and its use for clinical practice is demonstrated.

EXPERIMENTAL

Materials

Hyaluronic acid from rooster combs, sodium salt; hyaluronidase type IX from *Streptomyces hyalurolyticus*; 1,6-diaminohexane and potassium borohydride, biotin hydrazide, biotinamidocaproyl hydrazide (CH-biotin) were purchased from Sigma (U.S.A.). *N*-hydroxysuccinimidobiotin (NHS-biotin); streptavidin–peroxidase, *o*-phenylenediamine; fluorescein isothiocyanate (FITC) and chondroitin sulfate were obtained from Serva (Germany). Trypsin was purchased from Boehringer (Germany). Radioassay test for HA determination was purchased from Pharmacia (Sweden). HA reference molecular weight standards $3.0 . 10^6$, $1.6 . 10^6$, $0.81 . 10^6$, $0.49 . 10^6$ and $0.39 . 10^6$ were kindly provided by Dr Ove Wik (Pharmacia, Sweden). Other chemicals were obtained from Lachema (The Czech Republic) and NUNC – Immuno Plates Maxisorp F96 from A/S Nunc (Denmark).

Preparation of Diaminohexyl HA

Hyaluronan (M. w. $1.6 . 10^6$, 1 mg/ml) was mildly oxidized with sodium periodate for 0.5 h in dark at room temperature. The reaction mixture was then dialyzed against 0.05 M sodium carbonate buffer, pH 9.5 overnight. The oxidized HA was incubated with 0.2 M 1,6-diaminohexane for 20 min at room temperature. The Schiff base formed was reduced with freshly prepared 0.1% potassium borohydride in 0.1 M sodium carbonate buffer, pH 9.5 for 20 min at room temperature. The product was then dialyzed against 0.1 M borate buffer, pH 9.0 overnight. Amino groups were determined by the trinitrobenzene sulfonic acid method[19].

Biotinylation fo Diaminohexyl HA

Solution of NHS-biotin in dimethyl sulfoxide (0.12 mol/l, 5 – 50 μl) was added to the solution of diaminohexyl HA in the 0.1 M sodium borate buffer, pH 9.0, and stirred at room temperature for 4 h. The product was then dialyzed against distilled water at 4 °C and frozen.

Biotinylation of Oxidized HA with CH-Biotin and Biotin Hydrazide

Biotinylations were performed according to the modified method of Spiegel and Wilchek[20]. Sodium periodate, 0.2 mol/l, 5 – 100 μl, was added to the 1 ml of HA in water (1 mg/ml). The mixture was incubated at room temperature in dark for 0.5 h followed by dialysis against phosphate buffered saline pH 7.4 (PBS). Then CH-biotin and biotin hydrazide solution in PBS were added. The reaction products were reduced with potassium borohydride as previously, dialyzed against the distilled water and stored frozen. The degree of biotinylation of HA was determined by the method of Der-Balian et al.[21].

Purification of HABP

HABP was isolated from bovine nasal cartilage and purified by the method of Tengblad et al.[22]. HABP was eluted from column of HA coupled to Sepharose 4B by 0.5 M sodium acetate, pH 5.8, containing 4 M guanidine hydrochloride. The effluent was concentrated and chromatographed on Sepharose 6BCL column. The second peak was collected and concentrated. Purified HABP was stored in 4 M guanidine solution at 4 °C or dialyzed against water and then lyophilized. The protein content in HABP solutions was measured by the method of Lowry et al.[23].

Depolymerization of HA

Hyaluronan from rooster combs, molecular weight of $1.6 \cdot 10^6$, 1 mg/ml was dissolved in water with gentle mixing at 4 °C overnight. The solution of HA was ultrasonicated on the Dynatech sonic dismembrator (Dynatech, Germany) at room temperature for 30 min (ref.[24]). The molecular weight of the ultrasonicated HA was determined by HPLC as $4.2 \cdot 10^4$.

Fluoresceinization of HABP

The complex of HABP with ultrasonicated HA (1 : 1 w/w, 5 mg) was dissolved in 200 μl of 0.1 M sodium carbonate buffer, pH 9.3 and 15 μl of FITC (5 mg/ml) in methanol was added. The mixture was incubated at 4 °C for 18 h and the unreacted FITC was separated on the Sephadex G-25 column (1 × 25 cm) eluted by 0.1 M phosphate buffer, pH 7.0 at 4 °C with a flow rate 10 ml/h. Further purification and characterization of FITC-labelled HABP has already been described[25].

High Performance Liquid Chromatography

The System Gold 126 Programmable Solvent Module with a 166 Programmable UV Detector Module (Beckman, U.S.A.), the RF-535 fluorescence HPLC monitor and a C-R6A Chromatograph Integrator – Chromatopac (Shimadzu, Japan) in gel permeation mode (HPGPC) were used. The ready for use stainless steel HEMA-BIO-1000 column (8 × 250 mm i.d.), 10 μm was obtained from Tessek (The Czech Republic) and the noncommercially available stainless steel column SG-10-6000-NH_2 (8 × 250 mm i.d.), 9 μm filled with aminopropyl-modified highly porous silica gel derivative was the gift of Dr I. Novák, Ph.D., Institute of Polymers, Slovak Academy of Sciences (The Slovak Republic).

Binding of Modified Hyaluronans to HABP–FITC

HABP–FITC in 0.5 M sodium acetate buffer, pH 5.8 containing 4 M guanidine hydrochloride was diluted 11 times and mixed with HA or biotinylated HA solutions (0.2 mg/ml) at the ratio 1 : 1 (v/v).

The mixture was incubated for 1 h at 8 °C and injected into the HPGPC system. The elution time and peak areas of the HA–HABP complex were determined as described previously[25,26].

HA Standards

The high molecular weight HA standards and the ultrasonicated HA were prepared by dilution of the stock solution (1 mg/ml water) with PBS, pH 7.4, containing 0.1% Tween 20, 3% (w/v) bovine serum albumin and 0.02% merthiolate, to the concentrations 15, 30, 60, 120, 240 and 480 μg/l. The standard solutions were stored in the refrigerator at 4 °C. The HA concentration was determined by the carbazol method[27].

HA Digestion

Standard HA solution, 100 μl, and the test serum were incubated with 20 μl of *Streptomyces hyaluronolyticus* hyaluronidase (0.5 U) and 100 μl of 0.1 M phosphate buffer, pH 6.2, for 18 h at 37 °C. Control samples were incubated under the same conditions without hyaluronidase. Each sample was assayed immediately after incubation.

Assay Procedure

The microplate was coated with HABP (0.8 μg/well) in a 0.1 M sodium carbonate buffer, pH 9.4 containing 0.2 g/l merthiolate for 1 h at 4 °C. Plates were washed 3 times with washing solution (0.14 M NaCl, containing 0.05% Tween 20). Standards and serum (100 μl) were applied to the wells and incubated for 2 h at 8 °C. All assays were done in duplicates. Negative control wells contained 100 μl PBS/Tween with 3% bovine serum albumin. After subsequent washing, different preparations of biotinylated HA (0.1 – 0.3 μg/ml) in 100 μl solution containing 3% of BSA was added and incubated for 1 h at room temperature. The plate was washed 3 times and streptavidin–peroxidase (diluted 1 : 3 000) in the washing solution containing 3% BSA was added. The microplate was then incubated for 45 min at room temperature, washed 3 times with washing solution and finally incubated with 100 μl of substrate solution (0.5 mg/ml *o*-phenylenediamine, 0.033% hydrogen peroxide in 20 mM citric acid, 50 mM sodium hydrogen phosphate). The reaction was stopped with 100 μl of 0.1 M sulfuric acid and absorbance at 492 nm was measured on Biomec microplate reader (Beckman, U.S.A.).

Subjects

Sera were obtained from 42 patients with rheumatoid arthritis (RA) and 22 with osteoarthritis (OA) treated in the Research Institute of Rheumatic Diseases, Piestany. Twenty three patients met the American Rheumatism Association (ARA) criteria for a diagnosis of definite or classic RA (ref.[28]), and 19 had seronegative RA. All patients were treated with various nonsteroidal anti-inflammatory drugs and 14 patients with RA were treated with prednisone < 10 mg/day. Altman's[29] classification was used to diagnose of OA. Fifteen patients with OA took nonsteroidal anti-inflammatory drugs. Blood from patients was collected between noon and 2.00 p.m. Control sera were obtained from 12 healthy volunteers and stored at −20 °C until use.

RESULTS

Binding of HABP to the Microplate

A crucial step for further development of the HA assay was binding of HABP to the microplate. Binding was measured using biotinylated HA at 4 °C, 24 °C and 37 °C for 1, 2 and 4 h. The optimal temperature was found to be 4 °C. HABP exhibited a high affinity to the microplate surface which reduced coating time to 1 h (Table I). Better coating efficiency showed 0.1 M sodium bicarbonate buffer, pH 9 over 0.1 M phosphate, pH 7.2. Efficient HABP concentrations for optimal coating were in the range 4 – 24 μg/ml. Optimal conditions selected for routine coating were 8 μg/ml HABP in 0.1 M bicarbonate buffer, pH 9 at 4 °C for 1 h. Under those conditions the background was measured less than 0.05 absorbance unit. Longer incubation of the HABP coated microplate with BSA did not show any effect on the background.

HA Modification

Another important factor for the assay was quality of biotinylated HA. Both, the size and degree of modification affect competition of biotinylated HA with the sample HA for microplate bound HABP. Periodate concentration 1 – 20 μmol/mg HA was tested for further diaminohexyl and biotinyl derivatization of HA. Molecular weight of HA $1.6 \cdot 10^6$ decreased exponentially with concentration of periodate (Fig. 1). The subsequent reactions of oxidized HA with 1,6-diaminohexane did not change substantially the HA molecular weight (not shown). It was found that 2 – 6 biotin groups per 5 disaccharide units of HA provided absorbance 1.0 – 2.0 at 492 nm per 30 min of incubation with the HRP substrate.

TABLE I
Comparison of HABP binding (in %) to the microplate under different conditions. Maximal binding for 1 h at 4 °C was taken as 100%

Temperature, °C	Incubation time, h		
	1	2	4
4	100.0	100.0	92.3
24	74.4	65.4	50.0
37	66.0	55.1	46.1

HA–HABP Interaction

Hyaluronan is a very complex macromolecule with a variety of extraordinary properties. Therefore, a detailed analysis of HA–HABP interaction was important to understand which kind of HA needs to be used in the assay and what is the relationship between the degree of HA modification and its applicability as the standard for the practical assay purposes. On the other hand, this study would suggest what size of naturally occurring HAs our assay matches best and what is the relationship between the molecular weight of HA standards and actually measured HA size in a biological sample.

The complex formation of HABP–FITC with unmodified high molecular weight HA ($1.6 . 10^6$), low molecular weight ultrasonicated HA ($4.2 . 10^4$) (Fig. 2*a*) was comparable with modified biotinylated HAs (Fig. 2*b*). One can also see that hydrazide derivatives of biotin further decreased the molecular weight of oxidized HA. HPGPC chromatograms detected with fluorescence show that about 65 – 83% of HABP–FITC was bound to different HAs. However, amount of denaturated HABP–FITC unable to bound HA was not possible to estimate.

Standardization

Standard curves of HAs in binding assay with different biotinylated HAs are shown on Fig. 3. There are no significant differences between binding of biotinylated HA prepared with 1,6-diaminohexane or aminocaproic acid spacers and with biotin hydrazide without spacer, respectively. Observed differences are likely due to various degrees of biotinylation.

An effect of high and low molecular weight HA on the assay was tested with M. w. of HA of $1.6 . 10^6$ and $4.2 . 10^4$ (Fig. 4). The low molecular weight HA competed with

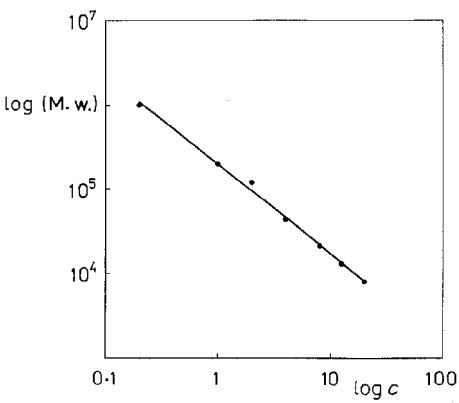

FIG. 1
Effect of periodate oxidation on the molecular weight of HA. HA was oxidized by different concentrations (*c*, μmol) of sodium periodate and the molecular weight of HA was measured as described

biotinylated HA significantly better than high molecular weight HA. The values of 50% inhibition were 80 μg/l and 320 μg/l for low and high molecular weight HA, respectively. Therefore, the low molecular weight standard was selected for serum HA deter-

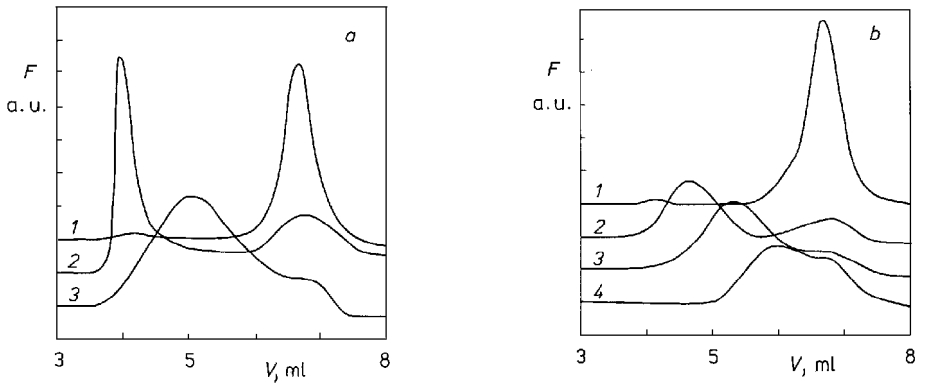

FIG. 2

HPGPC fluorescence chromatograms of HABP–FITC alone and in the mixture with different HAs (*a*) and different biotinylated HAs (*b*). Concentration of HABP–FITC 500 μg/ml, HAs 1 mg/ml, and biotinylated HAs 0.2 mg/ml. Sample volume was 20 μl and fluorescence (*F*) was detected at 495 nm E_x and 520 nm E_m. *V* is elution volume. Chromatograms were shifted on *y*-axis for better demonstration; in *a*: *1* by two, *2* by one a.u.; in *b*: *1* by three, *2* by two, and *3* by one a.u. For *a*: *1* HABP–FITC alone; *2* HABP–FITC and high molecular weight HA ($1.6 \cdot 10^6$); *3* HABP–FITC and low molecular weight ultrasonicated HA ($4.2 \cdot 10^4$). For *b*: *1* HABP–FITC alone; *2* HABP–FITC and biotinylated HA prepared from 1,6-diaminohexyl HA and NHS-biotin; *3* HABP–FITC and biotinylated HA prepared from HA and CH-biotin; *4* HABP–FITC and biotinylated HA prepared from HA and biotin hydrazide

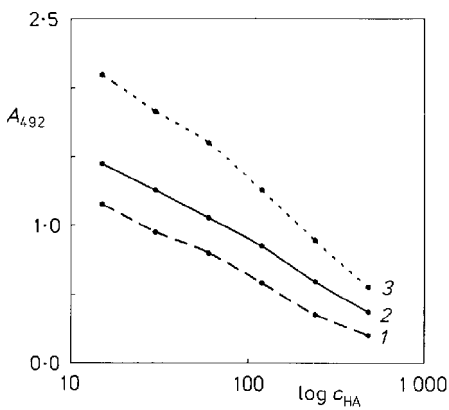

FIG. 3

Standard curves of HA determination with different biotinylated HAs at 492 nm. The microplates were coated with 8 μg/ml HABP for 1 h at 4 °C. Concentration of biotinylated HA was 1.0 μg/ml and concentration of HA (c_{HA}, μg/l) varied from 15 to 480 μg/ml. Biotinylated-HAs were prepared by periodate oxidation followed with biotinylation as described in Experimental: *1* biotin hydrazide, *2* 1,6-diaminohexane and NHS-biotin, *3* CH-biotin; degree of biotinylation was 2 – 8 biotin groups per 5 disaccharide units of HA

mination. A standard solution of ultrasonicated HA was prepared in PBS and the different concentrations of Tween 20 (0.01, 0.1, 1.0%) and BSA (0, 3, 6%) were compared. The maximum reproducibility and the highest slope of the standard curve was observed at concentration 0.1% of Tween 20 and 3% BSA.

Specificity and Reproducibility

Specificity of the assay was demonstrated by digestion of serum samples with *Streptomyces hyalurolyticus* hyaluronidase. Pretreatment of the serum samples with hyaluronidase decreased about 96% of the detected HA. On the other hand, the closest structural

TABLE II
Within-run and between-run precision of assay

Serum	No. of assay	HA, μg/l	*Cv*, %
	Within-run precision of the assay		
1	10	68.0 ± 6.4	9.7
2	10	187.3 ± 3.3	1.7
3	10	377.0 ± 46.2	12.2
	Between-run precision of the assay		
1	8	56.0 ± 4.9	8.7
2	8	146.0 ± 11.9	8.2
3	8	218.0 ± 17.9	8.2

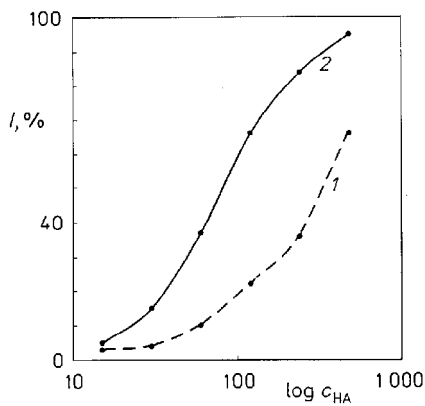

FIG. 4
Inhibition of biotinylated HA binding to the HABP coated microplate by high and low molecular weight HA. *1* High ($1.6 \cdot 10^6$) molecular weight HA, *2* low ($4.2 \cdot 10^4$) molecular weight HA. Plate was coated with 8 μg/ml HABP at 8 °C for 1 h. Concentration of biotinylated HA was 1 μg/ml

relative of HA – chondroitin sulfate did not interfered the assay at the concentrations < 100 mg/l (data not shown). The reproducibility of the assay was tested on 54 determinations of different pooled sera. The results of within-run and between-run precision of the assay are on Table II. Coeficient of variation (*Cv*) for within-run and between-run determinations varied in the range 1.73 – 12.25%. Recovery of HA added to serum was 96.7 – 120% (Table III). The detection limit was 10 μg/l. The concentrations of HA in serum determined with different biotinylated HA were closely comparable. The results of linear regression of serum HA concentrations determined with different biotinylated HA are on Table IV. All correlations were statistically significant at the level $p < 0.001$.

TABLE III
Recovery of HA added to serum

Serum μl	HA added ng	Amount of HA ng/ml		Measured/Expected . 100, %
		measured	expected	
50	30	60	62	96.7
50	60	108	90	120.0
50	30	155	150	103.0
50	60	210	180	116.0

TABLE IV
Comparison of serum HA concentrations determined with different biotinylated HA evaluated by linear regression

HA-biotins compared	r^a	Slope ± SE	Intercept ± SE
I and II	0.989	1.034 ± 0.06	–10.9 ± 6.6
I and III	0.995	1.143 ± 0.04	17.4 ± 4.4
II and III	0.994	1.092 ± 0.04	5.2 ± 4.7

I Biotinylated HA prepared with 1,6-diaminohexane and NHS-biotin; II biotinylated HA prepared with CH-biotin; III biotinylated HA prepared with biotin hydrazide; 8 different samples were measured. [a] All correlations were significant ($p < 0.001$).

Clinical Studies

Individual sera from 12 healthy donors were assayed and the mean content of HA was found to be 65.2 ± 7.2 μg/l (mean ±SD). Sera from OA (n = 22) and RA (n = 42) patients show significantly ($p < 0.001$) higher content of HA than those of healthy controls. The mean HA content for OA sera was 97.4 ± 28.0 μg/l and for RA sera 187.3 ± 111.4 μg/l (Fig. 5). Our assay correlated with the commercial Pharmacia HA-50 kit for serum HA determination of RA (n = 14) patients with correlation coefficient 0.92 and the significance $p < 0.001$.

DISCUSSION

Coating of HABP and its specific activity are important from point of view of background and the range of HA concentrations detectable in the assay. We optimalized routine coating conditions for HABP as 8 μg/ml of HABP in 0.1 M sodium bicarbonate buffer, pH 9 at 4 °C for 1 h.

HABP requires at least ten carbohydrate residues in HA chain for the efficient recognition[30]. Periodate oxidation of HA chain is one of the chemical modifications that substantially impairs HA binding to HABP. We found the optimal concentration of periodate 2 – 5 μmol per microequivalent of HA which was still sufficient for further derivatization. Beside the decrease of molecular weight and amount of intact carbohydrate subunits in the HA chain, the chemical modification can also alter the structure of HA to the more rigid and extended one, increasing the hydrodynamic volume of the macromolecules[31]. This can explain the increased capability of the assay to detect low

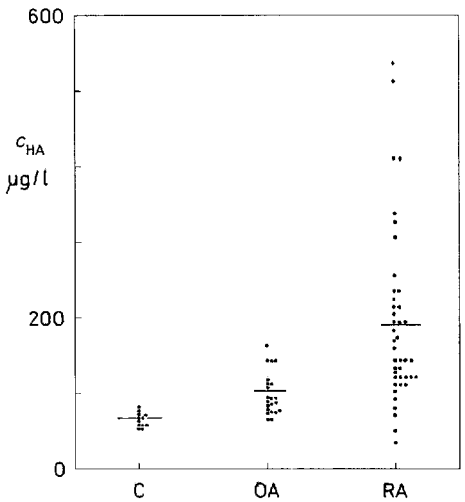

FIG. 5
Concentration of HA (c_{HA}, μg/l) in serum of healthy controls and patients suffering with OA and RA. Horizontal lines indicate the mean value. The difference between the healthy controls and patients was statistically significant at $p < 0.001$

molecular weight HA compared to those of high molecular weight. In the assay where HABP is fixed to the microplate, the rigid low molecular weight biotinylated HA showed better accessibility to it then the high molecular weight unmodified globular HA.

The study of HA–HABP interaction allowed us to estimate the size of HA in the biological samples. If we used high molecular weight HA standards in experiments with sera, it was evident that serum HA binds to HABP better. Therefore, low molecular weight HA standards were selected for the determination of serum HA. It also suggested that HA in the sera of healthy donors and in patients with RA is of low molecular weight. This is in agreement with HPLC of HABP complexes with the sample/standard HAs on the size exclusion columns. The chromatography led to estimate of molecular weight of RA patients HA in the range from 10^3 to 10^5 (ref.[32]). Fosang et al.[17] also described the higher competitiveness with low molecular weight HA-derived oligosaccharides (M. w. 4 000 – 12 000) in the ELISA microplate based assay where the plates were coated with HA. In the non-competitive sandwich technique with coated chondrosarcoma proteoglycan, a similar dependence of sensitivity on the molecular weight of sample/standard HAs was also found[33].

Our results of within-run and between-run precision of the assay are comparable to those obtained by other techniques of HA determination[34]. Correlation coefficient of serum HA determination for our assay and for the Pharmacia HA-50 commercialy available kit for RA patients ($n = 14$) was 0.92 at the statistical significance $p < 0.001$. This matches the range 0.89 – 0.98 ($p < 0.001$) of correlation coefficients calculated for several different methods of serum HA determination published by Lindquist et al.[34]. The detection limit of our assay reaches the minimum of the serum HA concentrations of healthy controls (10 – 100 μg/l depending on the age)[7].

Our assay for determination of serum HA is versatile and sensitive. The working time, reproducibility and accuracy of our assay are comparable with those of the other authors or commercial Pharmacia HA-50 kit. However, our approach avoids the use of unstable and hazardeous radiolabelled materials or preparation of chemically modified HABP which is difficult to reproduce in routine use. Moreover, we describe all important steps for the assay development in detail with a special emphasize on the molecular weight of assayed HA and its role in the standardization and an outcome of the sample HA determination. Our microplate arrangement also offers efficient automatic or semiautomatic processing. Therefore, we find our assay to be a supervising alternative for the determination of HA in serum and other body fluids in clinical practice.

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