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Reliability of the Western ligand blot method for the simultaneous relative estimations of serum insulin-like growth factor binding proteins (IGFBPs)

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SUMMARY. It is well established that nutrition is an important regulator of both serum insulin-like growth factor-I (IGF-1) and its binding proteins (IGFBPs). The Western ligand blot method (WLB) for simultaneous determinations of IGFBPs in serum or plasma samples was evaluated and validated with emphasis on its reproducible capabilities. After electrophoretic separation and transfer, the membranes were incubated with a mixture of recombinant labeled human (GF-I/IGF-II (rhIGF-I/rhIGF-II) and band intensities measured by autoradiography. The typical electrophoretic profile for pig serum, as determined with molecular weight markers, showed four main bands of approximately 42-39, 32, 30-28 and 24 kDa which seemed to correpond to IGFBP-3, IGFBP-2, IGFBP-1 and IGFBP-4 respectively. Likewise, a triplet of approximately 42-39 kDa (IGFBP-3), a broad area called IGFBP-30 region (most probably IGFBP-1, -2 and -3 variants) and a third band of ~ 24 kDa (IGFBP-4) were seen in rat samples. Determination of IGFBP-2 and -1 in rat serum samples, as two separate bands on 12% gels was difficult due to their close electrophoretic migration and possibly to the reported lower levels of IGFBP-2 in adult rat serum. Dilutions tested on 0.2 µm nitrocellulose membranes with samples volumes between 0.25 to 1.5 µl (1:10-1:60 dilutions), showed IGFBPs curves with good linearity which suggest first, that there exist a quantitative relation between the amount of each protein and the densitometric response and second, that the transfer of the proteins was linear across the range of 0.25 to 1.5 µl (1:10-1:60 dilutions). Moreover, the results also suggest that losses were equally spread and that the proteins retained their binding properties after the transfer process. Reproducibility showed intra-assay coefficients of variation (CVs) of 15% or lower using either a transfer device without cooling system or a combination of a transfer device with cooling system and manually defined band boundaries. In summary, it was shown that the optimized experimental conditions here described for the WLB method, allow realiable simultaneous measurements of the main pig and rat serum IGFBPs and therefore, could be utilised to detect changes in the serum profile after dietary manipulations.
Key words: Western ligand blot, insulin, binding proteins.

RESUMEN. Confiabilidad del método de «Western ligand blot» para la determinación simultánea de las proteínas de unión de los factores de crecimiento similares a la insulina (IGFBPs) en suero. La nutrición es un regulador importante de los niveles séricos de IGF-1 y de sus proteínas de unión (IGFBPs). En este trabajo, el método de Western Ligand Blot (WLB) fue evaluado y validado con énfasis en la reproductibilidad de la técnica, con el fin de realizar determinaciones simultáneas de las IGFBPs en suero o plasma. Una vez realizada la separación electroforética de las proteínas y sus transferencia a membranas de nitro-celulosa, las membranas se incubaron con una mezcla de IGF-1/IGF-II recombinantes humanos marcados radiactivamente y se determinaron las intensidades de las bandas por autorradiografía. El perfil electroforético típico del suero de cerdo mostró cuatro bandas principales de aproximadamente 42-39, 32, 30-28 y 24 kDa que corresponden probablemente a IGFBP-3, IGFBP-2, IGFBP-1 e IGFBP-4, respectivamente. De manera similar, el suero de rata mostró un triplete de aproximadamente 42-39 kDa (IGFBP-3) y una banda amplia de aproximadamente 30 kD que se designó como región IGFBP-30 (probablemente IGFBP-1 y -2 junto con variantes de IGFBP-3). Debido a la movilidaad electroforética muy semejante de las proteínas IGFBP-2 y -1 de rata en poliacrilamida al 12% no fue posible realizar su determinación en forma individual. Otro factor que dificulta el análisis de IGFBP-2 de rata son los niveles circulantes tan bajos que se han reportado para esta especie.
La evaluación de la linearidad del método se realizó aplicando volúmenes de suero comprendidos entre 0.25-1,5 µl (diluciones 1:10-1:60), sobre membranas de nitrocelulosa de 0.2 µm de tamaño de poro. Los resultados mostraron linearidad para cada IGFBP, lo cual sugiere que existe una relación cuantitativa entre las concentraciones de cada una de las proteínas y la respuesta densitométrica y que la transferencia de las proteínas fue lineal en el rango de 0.25-1.5 µl de suero (diluciones 1:10-1:60). Además, los resultados indican que las proteínas transferidas conservan sus propiedades de unión al ligando y que las posibles pérdidas de proteína durante el proceso de transferencia se distribuyeron uniformemente. La determinación de la reproductibilidad del método mostró coeficientes de variación (CV) intraensayo del 15% o menores, para transferencias en cámaras refrigeradas o sin refrigeración y mediante definición manual de las bandas. En resumen, en este trabajo se muestra que el método de WLB en las condiciones experimentales aquí descritas, permiten en forma confiable la determinación simultánea de las principales IGFBPs en suero de cerdo y de rata. Por lo tanto, este método podría ser útil para detectar cambios en los perfiles séricos de dichas proteínas como consecuencia de manipulaciones nutricionales.
Palabras clave: Método "Western ligand blot", insulina, proteínas de unión.

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INTRODUCTION

Most of IGFs in body fluids, do not exist as free polypeptides but are bound to a familiy of IGF binding proteins (IGFBPs). So far, six IGFBPs have been identified and their primary structures determined by molecular cloning from human and rat tissue libraries (1). Although each of the proteins consist of 200-300 aminoacids and the alignment of cysteines in the amino and carboxy-terminal ends has been well preserved, these proteins represent different gene products with little homology in the midregion. Each of the binding proteins has an unique pattern of expression and hormonal regulation (1). IGFBP-3 is present in the circulation in concentrations that are 10 to 100 fold higher than IGFBP-2 and -1, respectively, whereas serum concentrations of IGFBP-4, -5 and -6 are low (2). The knowledge about the functional roles of the IGFBPs remains incomplete but the largest differences between the *in vivo* insulin and IGF-I effects are related to their presence.

It is established that nutrition regulates the IGFBPs levels and that they respond to the energy and protein content of the diet in several species. In the rat both fasting and protein deficiency decreases IGFBP-2 (3) whereas protein restriction may increment the hepatic IGFBP-2 mRNA (4) although the effect on the serum levels of the protein are less clear. In addition it has been demonstrated that the hepatic IGFBP-1 mRNA is markedly increased by protein restriction and fasting (5).

At present one of the most commoly used methods for the analysis of serum IGFBPs is the Western Ligand Blot method (WLB), first described by Hossenlopp et al (6) for studies of the proteins using labeled IGF-I as ligand. The quantitative potential of the method for measurements of the hIGFBP-1 biological activity was later assessed by Grissom et al (7) using labeled IGF-II and it was suggested that IGFBP-1 and possibly other IGFBPs, can be adequately measured by the technique since similar concentration and nearly identical sensitivities were found among quantitation of IGFBP-1 by specific hIGFBP-1 radioimmunossay (RIA) and by WLB.

The present study was performed to further describe the intra-assay variation when the WLB method is employed for determinations of IGFBPs and suggest ways in which these variations could be reduced to enable its utilization for the simultaneous analysis of the main pig and rat serum IGFBPs using a mixture of iodinated IGF-I and IGF-II tracers.

MATERIAL AND METHODS

Plasma samples: Normal human and rat blood samples (≈500 µl) were taken in EDTA coated tubes from normal volunteers and normal rats (Sprague-Dawley, 3-4 months fed with a standard chow). Pig blood samples were obtained from catheterised animals (40 kg). Samples were stored in the refrigerator during 30 min and centrifuged. Plasma was kept at-20 °C until use.

Sample preparation: Samples were mixed with buffer Laemmli (8) containing SDS boiled for 3 min and kept on ice water short before electrophoresis. For analyses of reproductibility 1:10 serum dilutions were processed mixing 10 µl of the sample buffer with 10 µl of sample and adding 80 µl of a 1:1 dilution of sample buffer in water (Lalou, pers: comm. 1994). Then, 15 µl volume equivalent to 1.5 µl of the original sample were loaded onto the gels (10 wells). Pig and rat standard curves were run either by mixing 0.5, 1.0, 1.5 and 2.0 µl samples directly with a mixture of sample buffer in water (1:1) to a final volume of 15 µl or in 1:10, 1:15, 1:30, 1:60 and 1:150 dilutions respectively. In the later case, 15 µl of the mixture were loaded onto the gel and were equivalent to 1.5, 1.0, 0.5, 0.25 and 0.1 µl of the original sample, respectively.

IGF preparation and labeled IGFs. Recombinant human IGF-1 (rhIGF-I, batch DSQ-23) and rhIGF-II (batch HJDF01) were from Pharmacia (Stockholm, Sweden) and GroPep (Adelaide S.A., Australia) respectively. Iodination of each IGF was carried out by the Iodogen method (9). Briefly, 20 µl (2 µg) of IGF were added to an iodogen coated tube containing 10 µl of 0.5 M phosphate buffer (pH 7.4). The reaction was let to proceed for 4 min at room temperature under gentle agitation and was stopped by transferring the mixture to a siliconized tube containing 0.05 M phosphate buffer (pH 7.4). The labelled IGFs were purified by gel filtration on Sephadex G-25 PD 10 columns (Pharmacia Biosystems AB) equilibrated in 0.05 M phosphate buffer (pH 7.4). Specific activities achieved by this method ranged between 80-100 µCi/µg. In addition, aliquots of ^{125}I-IGF-II were further purified before the assays through Sephadex G-50 (1x17 cm) equilibrated in 0.1 M ammonium acetate (pH 7.4) containing 0.2% bovine serum albumin (BSA Sigma RIA grade). The iodinated products were stored at -20 °C until use.

Gel electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) was performed by the Laemmli method (8) under non reducing conditions running usually two gels on the Novex, c-cell apparatus (Mini-cell. Sorrento Valley Blvd, CA. USA). Samples dilutions or directly prepared (15 µl) were loaded onto 12% Tris-glycine pre-cast polyacrylamide gels (1 mm; Novex, Sorrento Valley Blvd, CA), electrophoresed stepwise at 30-40 mA (100-150 V) per gel during 2 h until the dye front reached the gel bottom.

Electrotransfer: The process was carried out as described by Hosssenlopp (6). Before transfer, each gel slab was soaked in transfer buffer (0.015 M Tris base, 0.12 M glycine, pH 8.3 containing 15% of methanol). The equilibrated gels were placed on buffer saturated transfer nitro-cellulose membranes either 0.45 or 0.2 µm (Sorrento, Valley Blvd CA, and Bio Rad Transfer Medium, Richmond, CA, USA) and packaged between two buffer saturated Whatman N° 3 filter papers pads and two Bio Rad Schotch Bride pads. The electrotransfer was next performed in transfer buffer at 300 mA (constant current) for 3 h using in initial experiment the Novex Blot Module (San

Diego, CA, USA) whitout cooling system which besides required less volumen of buffer to fill the tank. In the following experiments, the Hoefer apparatus (Hoefer Scientific Instruments, CA, USA) equipped with a cooling device was employed. The need to use a second transfer device obeyed to the higher intra-assay coefficients of variation obtained when the transfer was carried out in the x-cell module without cooling (see below). Evaluation of the retention capacity of 0.2 μm nitro-cellulose membranes was carried out using molecular weight standards (Rainbow™, Amersham) and by the analysis of the linearity of standar curves.

Quenching and detection of the IGFBPs: After transfer, the membranes were dried at room temperature and soaked at 4 °C for 1 h in 10 mM Tris, 0.15 M NaCl, 0.5% BSA, 0.2% Tween 20 with gentle agitation. Ligand incubation was performed in 1.5 ml of 10 mM Tris, 0.15 M NaCl, 0.5% BSA (1 mg/ml) containing a mixture of ^{125}I-IGF-I and ^{125}I-IGF-II (200.000 cpm each) in plastic bags at room temperature in flat position. Initial incubation with the ligand was carried out for 48 h at 4 °C but later, overnight incubation at room temperature was adopted (Lindgren, pers. comm). Optimised incubations were performed by adding 1 mM EDTA to the quenching and ligand mixtures. After the washing steps, the membranes were dried at room temperature and exposed to Hyperfilm (ECL Amersham, Sweden). Because the IGFBPs are present in plasma in varying concentrations, the membranes were sometimes exposed to the film for short periods (1-2 days) and then re-exposed to a new film for longer periods (2-7 days). This approach allowed us to perform relative determinations of the different IGFBPs over a wide range of concentrations.

Evaluation of results: Film spots were scanned in one dimension (Millipore Corporation Imaging System, Eisenhower Parkway, Suite Arbor, MI, USA) defining band boundaries either automatically or manually. The integrated optical density (IODs) values of each band in their respective track were then compared and intra-assay variation calculated (% CV). Attempts to compare directly the IODs among gels (inter-assay variation) were not possible due to the inherent variation of the IOD values on gels run the same day. It also depended on the exposure times needed to obtain good signals.

RESULTS

Sample preparation: The main interest of this work was to evaluate the reliability of the WLB method with the aim to make simultaneous relative estimations of the IGFBPs content in serum or plasma. The study of the reproductibility capabilities of the technique was performed emphasising on the principal sources of error, particularly those derived from employing very small sample volumes. Commonly 1:10 - 1:15 dilutions (1.5 - 1.0 μl of serum) were optimal to detect the 39-42, 32, 28-30 and 24 kDa proteins and dilutions above 1:60 (0.25 μl) were usually undetectable, while below 1:10 tended to give a plateau on the standard curves, probably due to saturation of the membranes at high protein concentrations (i.e 39 to 42 kDa protein, IGFBP-3).

Separation pattern: In agreement with the known molecular weight of each IGFBP (10,11), the observed bands (Figure 1) after WLB were: 39 to 42, 32, 28 to 30 and 24 kDa which most probably correspond to IGFBP-3, IGFBP-2/IGFBP-1 and IGFBP-4. Each band exhibited slight different patterns among the species. The ~39-42 kDa (IGFBP-3) was the most abundant protein in all samples tested. In rat, it consisted of a triplet of bands, whereas both pig and human samples exhibited doublet. In rat samples, the second spot between ~28-30 kDa termed IGFBP-30 region most likely contain IGFBP-2 and -1 together with IGFBP-3 variants and not completely separated on 12% gels due to their similar molecular weights. Meanwhile, both pig and human samples, showed both IGFBP-2 and -1 as two separated bands with molecular masses of ~32 and ~28-30 kDa, respectively. Finally, the third band migrated with a molecular weight of ~24 kDa and most probably corresponds to IGFBP-4. Each band was specific for the ligands as both, cold IGF-II were able to displace them (data not shown). In addition their concentrations seemed to change in each specie as could be seen from the band intensities.

Electrophoresis: The autoradiographic pattern of rat, pig and human serum IGFBPs (Figure 1) showed that a good protein separation is obtained on 12%, 1 mm gels (see Materials and Methods), whereas with 1.5 mm gels, no further improvement in separation was obtained.

Transfer membranes: The evaluation of retention capacity of 0.2 and 0.45 μm nitro-cellulose membranes suggested that 0.2 μm membranes better retained the proteins as judged by the higher IOD values obtained. High linearity was achieved as deduced from the coefficients of correlation r^2 ~0.939 to 0.993 (for pig) IGFBPs and ~0.99 (for rat) IGFBPs (Figure 2) after 2-4 days of exposure. This suggests a quantitative relationsh between the amount of each protein on the nitro-cellulose and the densitometric response. Notwithstanding, 0.2 μm membranes possesses a retention capacity limit which tend to deviate the IOD response when exceeded.

Quenching conditions and incubation with tracer: Quenching conditions including pre-treatment of dry membranes with 0.5% BSA, and 0.2% Tween 20 during 1 h at 4 °C (Lalou, pers. comm.) resulted in good signal with low background, which were improved when 1mM EDTA was included in both the quenching and the ligand solutions. Improved results may probably be associated with inhibition of proteolysis either of the IGFBPs or the ligand itself. An additional improvement in the spots was further achieved when freshly purified ^{125}I-IGF-II was utilized before incubation. This allowed shorter exposure times with sharper bands and lower backgrounds after periods of exposure as long as 3 to 7 days. Shorter exposition times were remarkable useful with the 39 to 42 kDa area which could be detected and scanned after periods not longer that 24 h. Other IGFBPs had to be exposed for longer times due to their lower signal intensities (lower serum concentration).

Evaluation of results: The reproducibility of the WLB method was tested running pig and rat serum samples (Tables 1 and 2). Table 1 shows that when running pig serum the intra-assay variation in the doublet (42 and 39 kDa) was not notoriusly influenced by either automatic or manual procedures during band definition (from 12% to 8% for the 42 kDa band or from 12% to 14% for the 39 kDa respectively). However, the variation of both 32 and 28-30 kDa proteins (probably IGFBP-2 and -1) could be reduced from 31% to 21% and from 21% to 15% respectively, using the manual procedure. Since the variation still appeared high, the possibility of protein damage due to excessive heat generation during the transfer process could not be discarded. To exclude this possibility, the transfer was carried out in an apparatus which included a cooling system. The results (Table 2) indicated that the transfer under these conditions together with a manual procedures of band boundaries definition, reduced the variation from 16 to 12% for the 39 to 42 kDa area and from 25 to 13% for the IGFBP-30 region in rat serum samples. We had previously found these variations to be around 26% for both regions using a chamber without cooling system and the variations were reduced at some degree (to 16% for 39 to 42 kDa) by the manual procedure (data not shown).

TABLE 1

Reproducibility of the WLB method. Pig plasma samples in dilutions 1:15 were separated and transferred to 0.2 µm nitro-cellulose membranes in a device without cooling system. The coefficient of variation (CV) of pig 42 and 39 kDa, 32 kDa and 28-30 kDa were calculated from band areas defined (a) automatically or (b) manually (see Materials and Methods). Autoradiography was carried out for 4-5 days. (n, number of wells taken into account)

Protein	CV[a]	n	CV[b]	n
IGFBP-3	13	9	11	9
(~42 kDa)	7	6	2	6
	16	9	11	9
mean	12		8	
IGFBP-3	9	9	18	9
(~39 kDa)	9	6	10	6
	19	9	14	9
mean	12		14	
IGFBP-2	23	9	17	9
((~32 kDa)	21	6	11	6
	48	9	32	9
mean	31		20	
IGFBP-1	9	9	17	9
(~30 kDa)	35	6	12	6
	18	9	15	9
mean	21		15	

TABLE 2

Reproducibility of the WLB method. Rat plasma samples in dilutions 1:10 were separated and transferred to 0.2 µm nitro-cellulose membranes in a device with cooling system (a) or without it (b). The coefficients of variation (CV) of rat 39-42 kDa and IGFBP-30 region were calculated from band areas manually defined. Autoradiography was carried out for 5-6 days. (n, number of wells taken into account)

	Intra-assay variation		Intra-assay variation	
Protein	CVa	n	CVb	n
IGFBP-3	6	8		
(~39-42 kDa)	11	8	17	9
	16	10	13	10
	6	10	17	8
	22	10		
mean	12		16	
IGFBP-30	11	8		
REGION	16	8		
(~28-30 kDa)	20	10	18	9
	8	10	30	10
	7	10	16	8
mean	12		21	

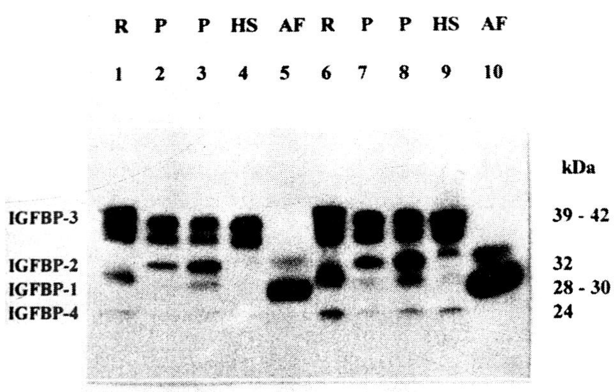
DISCUSSION

The aim of this work was to further evaluate the WLB method, originally described by Hossenlopp et al (10) for the simultaneous analysis of the IGFBPs in serum. We were particulary interested in finding ways to reduce the sourcess of variation in order to have a reliable technique.

To improve the reliability, some modifications of the method were introduced and validated. Examples of such modifications included sample volume and sample preparation, and the evaluation of both the pore size of nitro-cellulose membranes and their retention capacity. In addition, ligand binding specificity was studied by the use of both IGF-I and IGF-II tracers. Moreover, two transfer chambers, and two ways of definition of band boundaries during the scanning process were studied.

Sample preparation was designed in order to reduce the inherent errors of taking very low sample volumes since those could by themselves introduce variation into the results. The optimal sample dilution (1:10) (Figure 1) employed for the reproductibility assays, shows that both the 39 to 42 kDa area as well as, the IGFBP-30 region (rat) and the 32 and 28 to 30 kDa proteins (IGFBP-2 and -1 pig) were possible to analyse as early as 1-3 days after autoradiography when fresh and pure tracer was employed. In addition, after those days of exposure, the membranes did not appear saturated with the IGFBPs, not even the 39 to 42 kDa proteins which are present in the highest concentrations. Therefore any determination carried out at this sample dilution will fall into the linear part of the curve.

FIGURE 1
Autoradiographic pattern of plasma IGFBPs by the WLB method. Seven microliters (lanes 1-5) or 1.5 µl (lanes 6-10) of rat (R), pig (P), human serum (HS) and amniotic fluid (AF) were mixed with sample buffer in water (1:1) to a final volume of 15 µl and separated onto 12% polyacrylamide gels as described under Materials and Methods. Transfer was perfonned on a device without cooling system to 0.2 µm nitro-cellulose membranes. The molecular weight position of 39 to 42, 32, 30 and 24 kDa are marked. Autoradiography was carried out for 4 days.



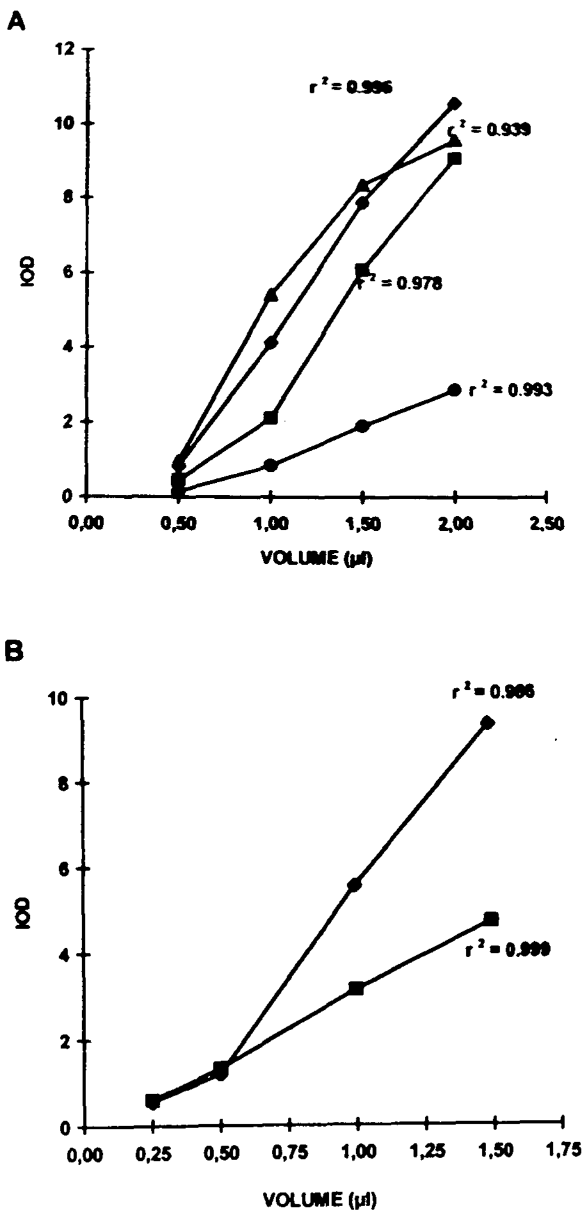
The retention capacity of the two nitro-celluylose membranes (0.2 or 0.45 µm) held together revealed that proteins present at higher concentrations (IGFBP-3) tended to saturate the inner membrane and IGFBPs were also detected on the outer membrane. Moreover, samples signals which seemed to be caused by the sandwich tightness around this area and were more evident when the transfer was performed on a device without cooling system. Therefore, it seemed as if at tight contact within the transfer sandwich mediated and facilitated the transfer of proteins. Despite that all these facts could be interpreted as losses, the standard curves for pig and rat serum IGFBPs showed linearity for each IGFBP tested (Figure 1). Hence it can be assumed that the losses were equally spread over the whole range of molecular weight of the tested proteins and proportional to the original protein amount. In this context, Grisson et al (7) have found by quantitative measurements by radioimmunoassay, linear losses of pure hIGFBP-1 across both 0.45 and 0.2 µm membranes. In this study, only 5% of the total IGFBP-1 was found over a second outer 0.2 µm membrane increasing to 21% when 0.45 µm membranes were used. Both pore sizes trapped 94.7% of the proteins, respectively. In our work, 0.2 µm membranes exhibited higher IODs with stronger and sharper bands in agreement with the notion (7) that, both the capacity as well as the retention of the proteins are improved by using this pore size.

In contrast to other authors (6,7) who have used an iodine-labeled IGF-I, a mixture of 125 I-labeled IGF-I and IGFF-II ligands was utilized in this work enhancing the final signal manifested as stronger and sharper bands. We consider that enhanced signals are achieved in response to the similar opportunity for each IGFBP to bind the ligand for which its affinity is higher (12-14). Thus, it is possible to obtain complexes with large stability and hence less probability of band density losses during the wash period.

Since the measurements of IGFBPs levels rely on their ability to bind IGFs, to test the specificity of the binding is of importance for the technique. Several data may support the IGFBPs integrity. First, both cold IGF-I and IGF-II were able to displace laveled ligands from the proteins when the protein specifity was tested. The ability to bind and/or displace either cold or labeled IGFs reveals that the IGFBPs binding capacity was retained after transfer and attachment to nitro-cellulose membranes. Increasing serum volumes showed the capacity to bind growing amounts of ligands. In addition, the results demonstrated that SDS treatment lead to the releasement of endogenous ligand. We however , do not know the extent of it. In this respect, Bisack (15) has claimed that SDS treatment is insufficient for the dissociation of large IGFBP molecular weight complexes therefore leading to underestimation of the total amount of IGFBP levels.

The IGFBPs separation pattern (Figure 2) showed the typical three/four main proteins present in serum (16,17). Our first approach was to measure each band within the 39 to 42 kDa rata triplet but it was a complex task due to their very similar electrophoretic mobility on 12% gels. Therefore determination of those bands was performed by determining the whole area (see below). Regarding the 32 and 28 to 30 kDa proteins (IGFBP-2 and -1) in rat samples, an additional complexity was found. The proteins could not be sufficiently separated on 12% gels or the concentrations were too low to be detected by WLB. Due to this pattern, the area was denominated IGFBP-30 region, where the two proteins could be present. Several aspects have to be kept in mind: first, some authors claim that IGFBP-2 could be absent in adult rat serum (18), however, in our lab it has been detected by immunoblot and moreover both IGFBP-2 and -1, exhibited a doublet of bands (data nor shown). Second, in the rat the IGFBP-30 region contains IGFBP-2 and -1, and in addition it has been reported to exhibit 30 kDa fragments of IGFBP-3 (19) which still are able to bind IGFs although with lower affinity (20). These proteins could be present in different amounts (21), furthermore, multiple IGFBPs variants differing in isoelectric points, have recently been reported by two-dimensional gel electrophoresis (22), which altogether give more complexity to this region.

FIGURE 2
Standar curves for WLB analysis of pig IGFBPs (A: ◊ 42 kDa;□ 39 kDa; Δ 32 kDa and O 28-30 kDa) and rat (B: ◊ 39-42 kDa (triplet) and□ IGFBP-30 region. Plasma samples ranging from 0.5 to 2.0 µl I(pig) were applied to 12% SDS-PAGE gels diluted in sample buffer to a final volume of 15 µl (A) or in dilutions from 1:10 to 1:150 (B) as describe under Materials and Methods. A typical analysis using a mixture of labeled IGF-I and IGF-II is shown. The autoradiography was performed for 3-4 days. Coefficients of correlation (r^2) are indicated for each protein.



The reproductibility of the WLB method showed that the highest variability was found with samples applied to the gel edges. Moreover two gels which were run on the same day under equal conditions, frequently tended to show different IOD values, which largely depended on the transfer process. Intra-assay variation indicated that the transfer as well as the way the bands are defined and/or integrated may have a large impact on the results. Thus, we reached CVs values for rat 39 to 42 kDA (IGFBP-3) and IGFBP-30, of 12 and 13% respectively when these two parameters were optimezed. Assay reproducibility has continuously been checked in our lab running rat samples and CVs of 5.8% for 39 to 42 kDa (IGFBP-3) and 9.6% for IGFBP-30 were found. It means that CVs below 10% could be attainable when all the previously discussed parameters are carefully controlled.

Although the quantitative capabilities of the WLB method have previously been studied by Grisson et al (7) using hIGFBP-1, our approach will allow the simultaneous measurements of the main IGFBPs in serum samples. This objective is attainable by runing and comparing fixed samples volumes with a control sample that has been run on a gel as many times as possible. Running a sample several times on the gel will give a measurement of the assay reproducibility which is the key to make the assay valid.

In summary, this work has shown that with carefully controlled parameters like sample preparation, protein transfer and integration of areas, the Western Ligand Blot method may be a good and precise analytical technique for the simultaneous relative estimations of plasma 39 to 42, 32 and 28 to 30 kDa proteins (e.i IGFBP-3, -2 and -1) and therefore, changes in the serum pattern could be analysed in a direct and reliable way.

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