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Differential removal of insulin-like growth factor binding proteins in rat serum by solvent extraction procedures

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Abstract. Solvent extraction of serum and other biological fluids at an acidic pH is a convenient method to remove the insulin-like growth factor binding proteins (IGFBPs); however, an incomplete removal of IGFBPs can occur and this can potentially interfere with the radioimmunoassay of insulin-like growth factors (IGFs). This study compared the removal of IGFBPs from normal adult rat serum and 5-day old neonatal rat serum by acid-gel filtration, and three solvent extraction methods, i.e., acid-ethanol (AE), acid-cryo-ethanol (ACE) and formic acid-acetone (FAA) treatments by western ligand blotting and slot-blotting analysis. In adult rat serum all three extraction methods removed nearly 75% of total IGFBPs present. For the neonatal serum, AE and FAA were very inefficient in eliminating the IGFBPs, while ACE was somewhat better, as it removed nearly 30% of IGFBPs. Ligand blots of extracted samples showed that IGFBPs of lower size range, 24 to 32 kDa (IGFBP-4, IGFBPs-1 and -2), were resistant to solvent extraction. Acid-gel filtration, in contrast, eliminated >95% of IGF-binding components in both sera. Determination of IGF-I concentrations in samples after gel filtration and extraction methods revealed lower IGF-I values in neonatal serum in acid extracted samples. These data caution against using solvent extractions for IGFBP removal in fetal/neonatal serum.
Key words. Insulin-like growth factor; insulin-like growth factor binding protein; RIA; methods; fetal serum.

Insulin-like growth factors (IGF-I and IGF-II) are mitogenic peptides with fundamental roles in growth, development and metabolism[1,2]. These growth factors in serum and other extra-cellular fluids are bound to a family of specific and high affinity IGF binding proteins (IGFBPs)[3–5]. In the rat, IGF-II and IGFB-2 show a coordinate and profound regulation with developmental age. They are high in fetal and neonatal rat serum and decrease to low adult levels by three weeks of age[6,7]; IGF-I and IGFBP-3, which are low in the fetus, increase dramatically during the postnatal period, reaching adult levels around day 40 in rats.

The dissociation of the endogenous IGF from the binding proteins and the elimination of binding proteins from the samples are essential for IGF radioligand assays[8]. Procedures involve acidification of serum or tissue sample, followed by gel-filtration[9], HPLC[10], reverse phase chromatography using C-18 silicate cartridges[11], or addition of an organic solvent (ethanol or acetone) to separate the binding proteins from the IGFs[7,12–14]. Size exclusion chromatography and HPLC procedures are laborious and time-consuming. Hence, solvent extraction methods have gained popularity because of their ease in handling several samples simultaneously. However, numerous investigators have noted that the removal of IGFBPs, especially low-size IGFBPs, is incomplete after solvent extraction[8,15–18]. In spite of this knowledge, a systematic comparison between different extraction methods is not available.

During our studies to determine serum IGFs in relation to fetal alcohol syndrome[19,20], we recognized that solvent extraction methods were inadequate in removal of IFGBPs from fetal rat serum. Therefore, in the present study, we compared the retention of IGFBPs after the extraction of the adult and neonatal rat sera with three common extraction procedures, i.e. acid-ethanol (AE)[7], acid-cryo-ethanol (ACE)[14], and formic acid-acetone (FAA)[12,13], as well as acid-gel filtration (GF). Also, IGF-1 level in each extract was quantified by radioimmunoassay (RIA).

Material and methods

The adult serum sample was pooled from six Sprague-Dawley male rats of 10–12 weeks of age which were fed regular Purina Rodent Chow, and maintained at controlled conditions of temperature, humidity and light cycle. Pooled sera from ten 5-day-old rats was designated as the neonatal serum sample.

Acid-gel filtration (GF). Samples (100 µl) of the serum pool were acidified with an equal volume of 2 M acetic acid for 30 min and subjected to gel filtration[9] on 0.9 × 30 cm Sephadex G-75 columns. The eluant was 1 M acetic acid containing 0.15 M NaCl. IGFBPs and [^{125}I]-IGF-I eluted from these columns in fractions corresponding to 0 to 50%, and 50 to 90% of the bed volume, respectively. The recovery of [^{125}I]-IGF-I was reproducibly > 85%. Column eluates were lyophilized and resuspended in RIA buffer (30 mM sodium phos-

phate, pH 7.4, containing 0.05% Tween-20, 0.02% protamine sulfate, 10 mM EDTA and 0.02% sodium azide) at 1:40 dilution.

Formic acid-acetone (FAA) extraction. This was performed according to Bowsher et al.[12]. 50 µl serum aliquots were acidified with 25 µl formic acid (8 mol/l) containing Tween-20 (5 g/l), freshly prepared. After immediate vortexing, 175 µl acetone was added and the mixture was vortexed and centrifuged. The protein pellet was discarded and the supernatant diluted in RIA buffer.

Acid-ethanol (AE) extraction. As described by Daughaday et al.[2], serum samples were mixed with an acid-ethanol mixture (87% ethanol and 1.5% 2 M HCl, v/v) at a ratio of 1:4 and incubated at room temperature for 30 min. The tubes were centrifuged to obtain a firm pellet. 100 µl aliquots of the supernatants were neutralized with 0.855 M Tris base at a ratio of 5:2, i.e., 100 µl aliquot mixed with 40 µl Tris base and further diluted in RIA buffer.

Acid-ethanol cryo-precipitation. This method is identical to AE extraction except for a final step of storing the neutralized samples briefly at −20 °C[14]. Serum samples were mixed with acid-ethanol solution and centrifuged as described for AE protocol. A 100 µl aliquot of the supernatant was neutralized with 0.855 M Tris-base at a ratio of 5:2 and the mixture kept at −20 °C for 1 h. After recentrifugation, the supernatant was diluted in RIA buffer.

5 ml polypropylene tubes were used for sample preparation and centrifugation was performed at 4 °C and 5,000 *g* in a Beckman J-2HS centrifuge for all extraction procedures. The supernatants were adjusted to a final dilution of 1:40 by the addition of RIA buffer in all cases.

IGF-1 radioimmunoassay. IGF-I levels in sera extracted by different procedures and acid-gel filtration was determined by the method of Furlanetto et al.[21]. A polyclonal antiserum to IGF-I, UB3-189, obtained from Drs L. E. Underwood and J. J. Van Wyk through the National Hormone and Pituitary Program of NIH, was used. Standards (0.003 to 0.8 ng recombinant IGF-I, Amgen, Thousand Oaks, California, USA) and unknowns were incubated at 4 °C with antiserum, the final concentration being 1:10,000, in a total volume of 0.3 ml RIA buffer. After 72 h, 0.02 ng [^{125}I]-IGF-I (10,000 cpm, Amersham, Arlington Heights, Illinois, USA) was added and the incubation was terminated after an additional 16 h by the addition of normal rabbit serum and goat anti-rabbit IgG (Sigma, St. Louis, USA). The sensitivity of this assay was 3 picograms or less, and the intra- and inter-assay coefficients of variation were 5.0% and 7.8%, respectively.

Ligand blotting of IGFBPs. IGFBP depleted fractions obtained after solvent extraction procedures (equivalent in volume to 2, 8 or 16 µl of the original serum) were lyophilized. These samples were dissolved in 50 µl of SDS-gel loading buffer and co-electrophoresed along with 2 µl of original (undissociated) sera on 10% SDS-Polyacrylamide gels under non-reducing conditions. Proteins were transferred electrophoretically from the gel onto nitrocellulose membranes using Towbin buffer [25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol] at 4 °C overnight[22]. In some experiments, unextracted serum and extracted sera were immobilized in equivalent amounts on nitrocellulose using a slot blot module and vacuum filtration. The membranes were treated sequentially with 3% Nonidet-40 in PBS, 2% non-fat dry milk and 0.1% Tween-20 as described by Hossenlopp et al.[23] and incubated with [^{125}I]-IGF-I (0.03 µCi/ml) at 4 °C with and without excess unlabeled IGF-I for 15 h.

After extensive washing with PBS/0.1% Tween-20, the blots were exposed to X-Omat AR film (Eastman Kodak, Rochester, New York, USA) at −80 °C for 2–3 days. Autoradiograms were scanned using a Bio-Rad Video 620 densitometer to quantitate IGFBP content. Since the signal due to IGFBPs retained at 2 µl serum or its volume equivalents was weak for accurate comparison on autoradiograms (fig. 2), higher volume equivalents (8 or 16 µl) in two different experiments were scanned and the peak areas for the protein bands were expressed per ml serum volume.

Statistical analysis. All experiments were performed at least three times and results are expressed as mean ± SEM. Differences betwen means were compared by ANOVA with Duncan's multiple range test.

Results and discussion

Considerable effort has been made to find suitable methods to remove and separate IGFs from IGFBPs. Apart from acid-gel filtration and HPLC, other methods retain some amount of binding protein, depending on species, tissue, or physico-chemical properties of proteins. Depending on the amount of IGFBP retained, its potential for interference with IGF-RIA is expected to increase. Prompted by earlier reports[15–18], including the low efficiency of C-18 reverse phase columns[24] and our own observations on the difficulty of removing IGFBPs from fetal and neonatal rat serum, we undertook a comparative study of the residual IGFBPs present in relation to different extraction methods used. Figure 1 shows the slot-blot autoradiogram depicting the amount of IGFBP retained. The acid-gel filtration fractions were devoid of any binding proteins (< 5% retention), whereas the three extraction procedures retained nearly equal amounts of IGFBPs in adult serum. The rank order of IGFBP retention for the neonatal serum was FAA > AE > ACE extractions. Acid cryo-ethanol (ACE), as reported previously[14], markedly depleted the IGFBP content in neonatal serum. Acidified whole serum in both adult and neonate pools on immo-

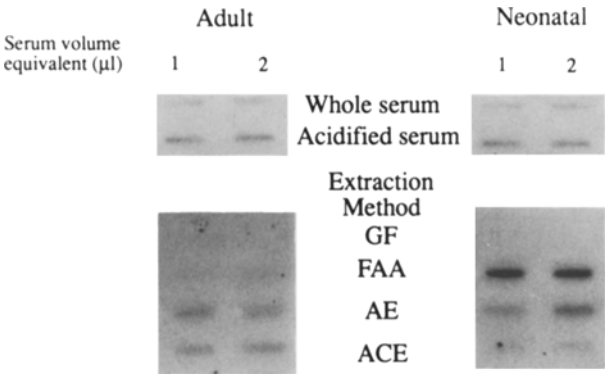

Figure 1. Autoradiographs of slot blot analysis of IGFBPs in extracted and unextracted rat sera. 1 or 2 µl of adult or neonatal sera, without or with acidification were diluted with 400 µl TBS and applied to nitrocellulose membrane (upper panel). IGF fractions derived from acid-gel filtration (GF) and various extractions were equivalent to 1 or 2 µl of the neat (whole) serum and were also slot-blotted as described in Materials and methods. The intensity of signal in the lower panel depicts comparison of extractions.

bilization, were able to bind exogenous [^{125}I] IGF-I, demonstrating the dissociation of the complex. The signal seen with the whole serum (without acidification) may represent the unsaturated binding proteins, as suggested by others[5].

The slot blotting data provides an overall comparison of the total binding proteins present. It does not, however, furnish information on the molecular size of IGF-binding proteins retained in the extraction procedures.

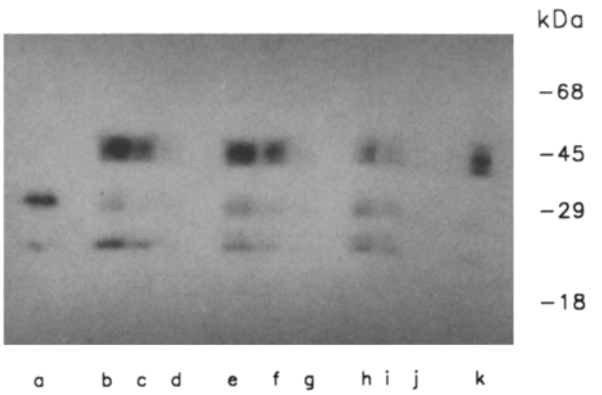

Figure 2. Ligand blot analysis to determine IGFBP retention in adult rat serum after solvent extraction procedures. IGF fractions from ACE (lanes b, c, d) AE (lanes e, f, g) and FAA (lanes h, i, j) extracted adult serum (equivalent to 2, 8 and 16 µl of the whole serum) were lyophilized and subjected to SDS-PAGE along with 2 µl of adult serum (lane k) for comparison of IGFBP abundance before and after extraction. Lane a represents 2 µl of unextracted neonatal serum showing different molecular species of IGFBPs. Lane designation for rest of the autoradiogram is as follows: lanes d, g and j correspond to 2 µl equivalents, lanes c, f, and i correspond to 8 µl equivalents; and lanes b, e, and h refer to 16 µl equivalents. Extracted serum of 8 and 16 µl volume were included to ensure adequate signal on the autoradiogram for quantitative purposes. Compare lane k (2 µl whole serum) with lanes d, g, and j to assess the quantitative retention of IGFBP in 2 µl adult serum after ACE, AE, and FAA extractions. Molecular weight markers are shown on right.

This is important, as specific binding proteins of different sizes and properties are present in adult and neonate rat sera. Therefore, samples extracted were subjected to ligand blotting at equivalent amounts corresponding to 2, 8 and 16 µl of neat (untreated) serum to quantitate the BPs retained. Figure 2 presents the ligand blot results of adult serum IGFBPs. At 2 µl adult serum volume (lanes d, g and j) all three extraction procedures (AE, ACE, and FAA) almost depleted the samples of IGFBPs; with 8 µl and 16 µl sera, however, AE and ACE appear to retain higher BP content than FAA, consistent with the slot blot data shown in figure 1. Figure 3 depicts the extent of individual IGFBPs retained in adult serum as quantitated by densitometry. Similar data for net IGFBP retention in adult serum is presented in the upper panel of figure 6. Nearly 75% of the total IGFBP was removed from adult serum by the extraction procedures. FAA extraction appeared to

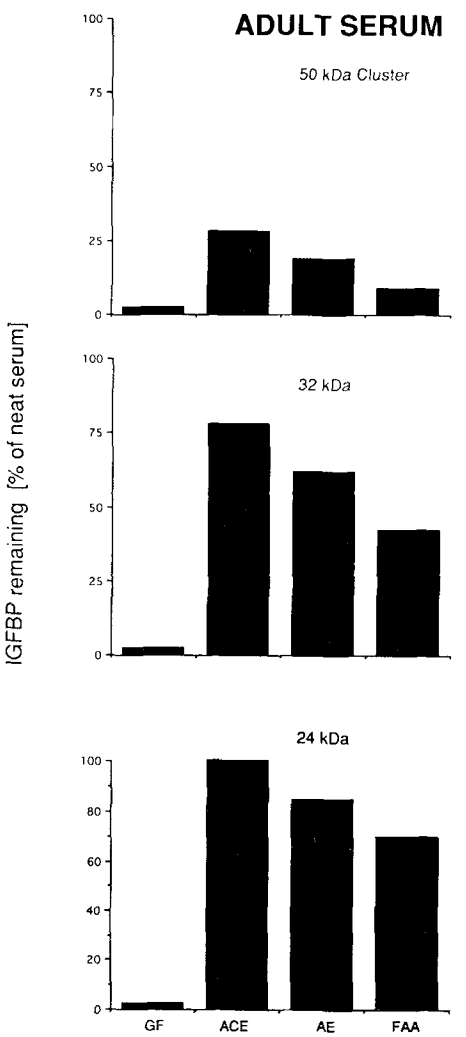

Figure 3. Individual IGFBPs remaining in adult rat serum after extraction procedures. IGFBPs remaining in relation to the BPs in neat (whole) serum were quantitated by densitometric scanning of slot blot autoradiograms as described in Materials and methods.

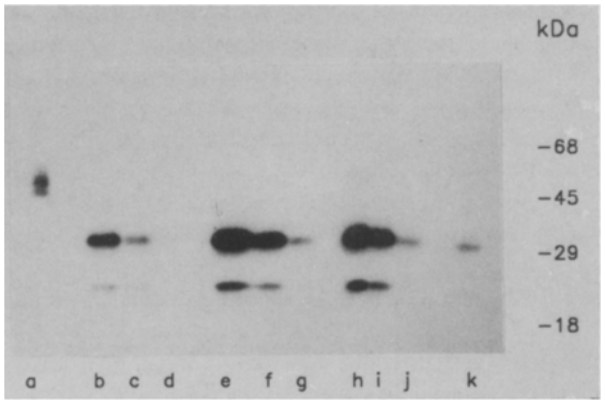

Figure 4. Ligand blot of neonatal rat serum IGFBPs after extraction procedures. IGF fractions obtained after ACE, (lanes b, c, d) AE, (lanes e, f, g) and FAA (lanes h, i, j) extractions of the neonatal serum were adjusted in volume to 2, 8 and 16 µl of the whole serum. These samples were lyophilized and electrophoresed on 10% SDS-polyacrylamide gels along with 2 µl of neat neonatal serum (lane k) and 2 µl of adult rat serum (lane a). The gels were further processed for ligand blot analysis as described in Materials and methods. For assessment of IGFBP removal in 2 µl neonatal serum, compare lane k with lanes d, g, and j. Sample volumes in other lanes were: lanes c, f, and i – 8 µl serum equivalents; lanes b, e, and h – 16 µl serum equivalents.

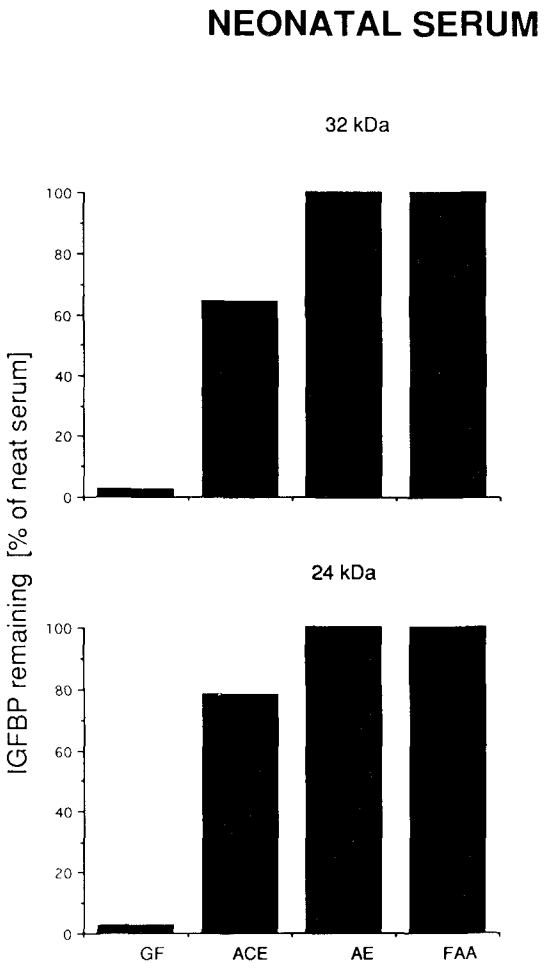

Figure 5. Densitometric quantitation of individual IGFBPs remaining in rat neonatal serum after solvent extractions. The abundance of 32 kDa and 24 kDa IGFBP bands on ligand blots of extracted sera were compared with those in neat serum to calculate the amount of binding proteins remaining.

eliminate BPs to a greater extent than the other extraction techniques.

IGFBP removal in neonatal serum. Figure 4 represents a ligand blot analysis performed after the extraction procedures applied to the neonatal serum. In contrast to the removal of IGFBPs of the adult serum, the 32 and 24 kDa binding proteins were largely retained in the neonatal serum (compare lanes g and j with k). ACE extraction reduced the neonatal IGFBPs appreciably (lanes d and k), in agreement with the results of figure 1. Densitometric quantitation of the ligand blots of neonatal serum revealed that 24 and 32 kDa were retained at 100% by AE and FAA, and 70% in ACE extract (fig. 5). Nearly, 30% of the total BPs was removed by ACE, but not by AE and FAA methods (fig. 6, lower panel).

Radioimmunoassay of IGF-I. Figure 7 shows mean ± SEM values of IGF-I concentration in pooled fetal serum extracted by various methods. IGF-I values were 73%, 31% and 29% respectively in FAA, ACE and AE compared to acid-gel filtration method ($N = 4\text{-}7$, $p < 0.05$ for all comparisons). Unextracted fetal rat serum yielded the lowest IGF-1 values. For the adult rat serum, FAA and ACE yielded slightly higher values (462 ± 45 and 484 ± 20 ng/ml) than acid-gel filtration (361 ± 35 ng/ml). IGF-I levels in AE and in whole serum were 295 ± 25 and 173 ± 19 mg/ml respectively ($N = 4\text{-}9$).

The mechanisms by which IGFBPs interfere with the IGF-RIA to provide higher or lower IGF concentrations appear to be complex. These may include a simple competition for the ligand binding to the antibody and of the IGFBP. Thus interference with the calculation of bound versus free IGFs would falsely elevate IGF concentrations[15]. Further, if the IGF-antibodies used are directed against an epitope partially blocked by any of the IGFBPs, only a portion of IGFs will be measured[25]. Three extraction procedures appear to be applicable to quantitate IGFs in adult rat serum. A significant finding of the present study is that, in fetal or neonatal rat sera where IGFBP-2 is prominent, acid-solvent extraction procedures fail to remove the IGFBPs to a large extent and, therefore, are not appropriate to determine IGFs. Although IGF-II predominates in fetal life[5–7], IGF-I, which is present in small amounts, appears to be more susceptible to alteration under different pathological conditions[26,27]. Thus, acid gel filtration, which achieves a complete removal of IGFBPs appears to ensure an accurate determination of rat IGFs during prenatal and neonatal periods.

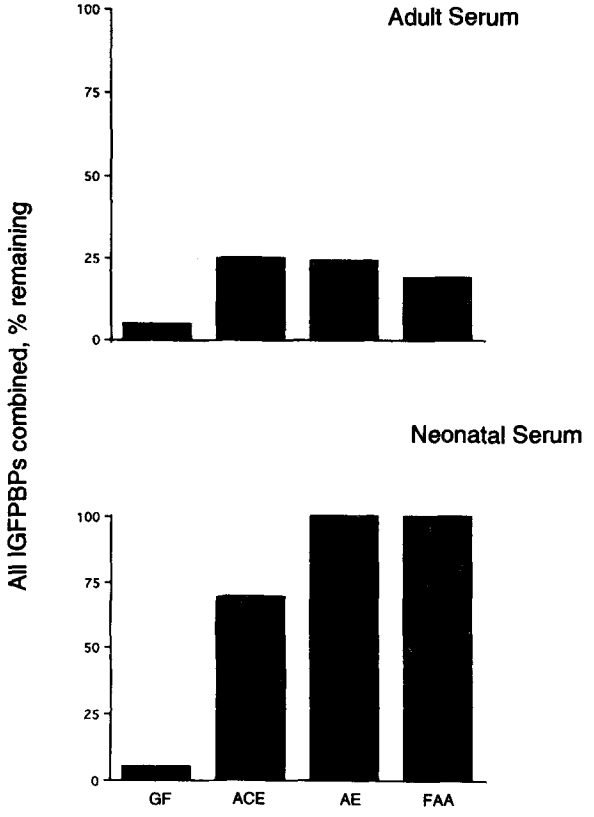

Figure 6. Amount of IGFBP (all molecular species, combined) retained in adult and neonatal rat sera as calculated from densitometry of ligand blots. Total IGFBP remaining in IGF fractions after acid-gel filtration (GF) and extraction procedures is expressed as a percentage of the IGFBPs in neat serum. Densitometric scanning 17 of slot-blots (see fig. 1) was performed to acquire the data for GF. The results are mean of three independent experiments.

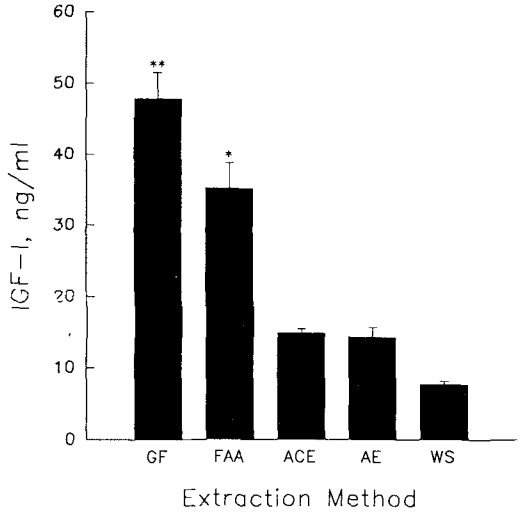

Figure 7. IGF-I concentration in neonatal rat serum after separation of IGFBPs by different methods. WS = whole serum. **$p < 0.001$ vs. WS, AE, ACE and $p < 0.05$ vs. FAA. *$p < 0.01$ vs WS, AE, ACE and $p < 0.05$ vs. GF.

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