Sample pre-treatment determines the clinical usefulness of acid-labile subunit immunoassays in the diagnosis of growth hormone deficiency and acromegaly

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Abstract

Objective: The usefulness of measuring the GH-dependent acid-labile subunit (ALS) in the management of GH deficiency (GHD) and acromegaly remains in question and is investigated in this study, comparing several different immunoassays for ALS.

Method: We compared the diagnostic accuracy of a commercially available polyclonal Ab-based ELISA with SDS pre-treatment (SDS-ELISA) with a monoclonal Ab-based immunofluorometric assay, using two unfolding methods (urea (UREA) and Glycine-HCl (Gly)). The corresponding molecular weight (MW) of ALS and IGFBP-3 immunoreactivity was determined. The clinical usefulness of each assay was examined in adult GH disorders.

Results: ALS was lower in GHD and higher in acromegaly using all assays. In GHD, UREA had higher sensitivity and specificity than SDS-ELISA (59 and 69% versus 41 and 51% respectively). In acromegaly, sensitivity and specificity was 94 and 87% for UREA, 81 and 36% for Gly, and 44 and 44% for SDS-ELISA. After UREA, immunoreactivity for ALS and IGFBP-3 eluted at their predicted free MW using size-exclusion chromatography, whereas ALS immunoreactivity in SDS (300–600 kDa) and Gly (250–500 kDa) was at a high apparent MW consistent with aggregation.

Conclusion: The diagnostic accuracy of ALS varies with assay choice and pre-treatment modality. UREA, which results in migration of ALS at the expected MW on a sizing column, has the highest specificity and sensitivity. Thus, if measured in an assay in which ALS is unfolded without aggregation, ALS is a clinically highly useful parameter for the assessment of GH.

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Introduction

Insulin-like growth factors (IGF) circulate in serum, for the most part, bound in a ternary complex with IGF-binding protein 3 (IGFBP-3) and acid-labile subunit (ALS). As the ALS is synthesized under direct control of GH, and primarily in hepatocytes (1–3), it may be expected to be a good indicator of GH secretion and action. ALS concentrations in adults (1, 4) and children (3, 5, 6) with GH deficiency (GHD) are lower than in normal controls; ALS increases with GH replacement therapy (3, 7, 8); and the change in ALS is highly correlated with the growth response in children (9). ALS is elevated in conditions of GH excess (4, 10) and declines with successful treatment (11). The diagnostic accuracy of ALS using the commercially available SDS-ELISA in the diagnosis and follow-up of patients with acromegaly remains unclear. It has been shown to exceed IGFBP-3 in some studies (12) but not others (13) and to be similar to (12) or less accurate than IGF-I (13).

ALS was first quantified in serum by Baxter in 1990, with a RIA using polyclonal antibodies (Ab) directed against the native ALS protein (1). As the ALS molecule contains a series of leucine-rich repeat regions commonly found in other proteins engaged in protein–protein interaction, there is the theoretical possibility that polyclonal Ab directed against native ALS may bind non-specifically to other proteins. Ab raised against specific, unique sequences of ALS represent one way to circumvent this problem. Quantitative, two-site immunoassays, using Ab raised against these specific N- and C-terminal oligopeptides, have been described by Khosravi et al. (4) and ourselves (8). These sandwich immunoassays provide the
additional advantage of measuring intact molecules rather than protein fragments. With this approach, however, pre-treatment of serum samples to unfold the ALS molecule is required for immunorecognition. The pre-treatment protocols used in the two assays described in the aforementioned articles were different from one another. It is not known precisely what effect the various pre-treatments have on ALS protein structure and whether or not these differences have clinical significance when the assay is used in the diagnosis and management of GH-related disorders.

The purpose of this work is to determine if the usefulness of ALS as a biochemical marker in the diagnosis and follow-up of GH-related diseases is related to sample pre-treatment modality and/or assay method. In addition, we sought to optimize pre-treatment in the specific monoclonal assay described by Stadler et al. (8), with respect to both immunorecognition and reproducibility, and present the methodology and validation of the new assay.

Materials and methods

Ia. Study participants

ALS concentration was measured in 39 (16 M) normal controls (CON; students and hospital personnel), aged 18–56 (mean 30.6), 29 (23 M) patients with GHD prior to hGH replacement therapy, aged 19–61 (mean 42.3 ± 2.41 (S.E.M.)), and 16 (12 M) patients with active acromegaly (ACRO), aged 34–60 (mean 45.9 ± 2.31). Patients with GHD and acromegaly attended a tertiary care endocrine clinic (University of Munich).

GHD is defined in accordance with the Port Stephens criteria (14) of the Growth Hormone Research Society. That is, all patients had morphological evidence for hypothalamic–pituitary disease, at least one other anterior pituitary hormone deficiency (and on adequate hormone replacement other than GH at the time of sampling) and failure to increase the GH level above 3 ng/ml in either an insulin tolerance test or an arginine test. The patient charts have been reviewed in detail and 24 out of 29 contained full information. GHD was most often of adult onset (15 out of 24) and in 14 out of 15 resulted from resection of a pituitary or hypothalamic tumor. Five of fifteen also had radiation therapy. Of the 9 out of 24 with childhood onset GHD, four had a tumor resected and five had morphological evidence of pituitary abnormality and were on multiple hormone replacement. GHD had a higher body mass index (BMI) than CON (26.4 ± 1.13 versus 22.4 ± 0.39; mean ± S.E.M.; \( \text{P} < 0.01 \)).

ACRO was defined as tonically elevated GH levels and IGF-I levels exceeding the reference range for age. In this study, all had IGF-I levels > 400 ng/ml (the upper limit of normal for this age group being 320 ng/ml). Twelve out of sixteen had failure to suppress GH to <2 ng/ml during an oral glucose tolerance test (two had no OGTT recorded, one suppressed to 1.9 ng/ml and one to 1.3 ng/ml). All charts were reviewed and detailed information was available for 14 subjects. The ACRO group had a higher BMI than CON (28.5 ± 1.44; \( \text{P} < 0.01 \)). Acromegaly resulted from a macroadenoma in 13 patients. Surgical resection was done in 12 out of 13 patients (mean 11.0 ± 1.9 years; range 2.6–25.6 years) prior to blood test. Of the 13 also had radiotherapy (mean 5.0 ± 2.94 years; range 0.75–19.4 years) and 13 of 14 had received long acting somatostatin analogs (one unknown). Data collection occurred prior to the measurement of ALS by all assays, but subsequent to the patient’s identification within a patient group. ALS assays were measured on the same serum samples as the reference methods (IGF-I and IGFBP-3). All sera were obtained after written informed consent and the Ethics Committee of the University of Munich approved the studies.

Ib. Tests of diagnostic accuracy

The mean and S.E.M. for ALS concentrations of sera from each of the three groups (CON, GHD, and ACRO) were calculated. The sensitivity was defined as the percentage of affected patients with ALS <2 S.D. below the control mean (GHD), or >2 S.D. above the control mean (acromegaly). Specificity was defined as the proportion of controls with an ALS level >2 S.D. above the mean of the GHD patients in GHD and <2 S.D. below the ACRO mean in acromegaly. Positive likelihood ratio (LR+) (change in odds favoring disease, given a positive test result) and negative likelihood ratio (LR-) (change in odds favoring disease, given a negative test result) were calculated as: \( \text{LR}+ = \text{sensitivity}/(1 - \text{specificity}) \) and \( \text{LR} - = (1 - \text{specificity})/\text{specificity} \) respectively. Confidence intervals were calculated according to the method of Simel (15).

Ii.a. ALS assays monoclonal Ab-based immunoaffluorometric assay (UREA and Gly)

Reagents Oligopeptide sequences of the N-terminal (a.a. 1–34) and C-terminal (a.a. 535–563) of ALS were kindly provided by Diagnostic Systems Laboratories (DSL; Webster, TX, USA). Monoclonal Ab against these oligopeptides were established as previously described (6).

Pre-treatment of sample sera Urea. Ten microlitres of serum were pipetted into a 5 ml polystereene tube, 15 µl of urea solution were added (5 M urea, 0.625 M NaCl in 25 mM phosphate buffer, pH 8.8) and the mixture was incubated for 30 min at ambient temperature on a horizontal shaker (3 M urea/0.375 M NaCl final concentration). Assay buffer (0.05 M Tris–HCl, 150 mM NaCl, 0.02% Tween 40, 0.05% NaN₃, 0.5% BSA, 0.05% bovine
γ-globulin, 20 mM DTPA) was then added (2475 µl), resulting in a serum dilution factor of 250. This solution was then vortexed to assure homogeneity and was used immediately for assay measurement.

**Acid-neutralization (Gly).** Twenty microlitres of serum were pipetted into a 5 ml polystyrol tube, 80 µl Gly solution (0.2 M, pH 2.7) was added and samples were incubated for 30 min at ambient temperature. Neutralizing solution (100 µl of 0.5 M Na3PO4, pH 12.0) was added, followed immediately by 1800 µl PBS with 0.65% BSA to bring the final serum dilution to 1:100. Samples were then measured in duplicate in the assay as described below.

**Assay standards** A serum pool from seven healthy adult males (age 24–37 years) was ascribed a value of 1000 mU/ml. The serum pool was utilized in the generation of the calibration curve, and as one of the longitudinal assay control samples.

**Calibration curve for urea pre-treatment** Pre-treatment of the standard curve was as above, except that after the pre-treatment, the standard pool sample was diluted to a serum concentration of 1:50 with assay buffer (resulting serum concentration is fivefold that of the sample). This solution (5000 mU/ml standard) was then serially diluted in the assay buffer, with resulting dilutions of 1:100, 1:250, 1:500, 1:1000, 1:2500, and 1:5000 (2500, 1000, 500, 250, 100, and 50 mU/ml) for standard curve generation.

**Calibration curve for acid-neutralization pre-treatment** As noted above, the unknown serum samples, after Gly pre-treatment, were only diluted to 1:100. For calibration purposes, the initial dilution after neutralization of the serum pool assigned 1000 mU/ml had a serum dilution factor of 1:10. This was utilized as the 10 000 mU/ml standard, and was then serially diluted with assay buffer 1:20, 1:50, 1:100, 1:200, 1:500, and 1:1000 corresponding to standard concentrations of 5000, 2000, 1000, 500, 200, and 100 mU/ml respectively.

**Biotinylation of mAb clone 7H3** Biotin-ε-amidocaproyl-γ-butyryl-N-hydroxysuccinimide ester (Sigma) was dissolved in DMF at a concentration of 1 mg/100 µl. Protein A purified anti-C terminal ALS mAb 7H3, 1 mg/ml, was mixed with 25 µl 1 M phosphate buffer (pH 9.0) and a 12-fold molar excess of biotin and incubated at ambient temperature for 2–4 h. Labeled protein was separated from uncoupled biotin by application to a Superdex 30 fast protein liquid chromatography (FPLC) column. The biotinylated protein was stored in 50 mM Tris–HCl buffer, pH 7.8, containing 0.1% BSA and 0.02% NaN₃ at 4 °C.

**Assay procedure** Anti-ALS N-terminal mAb (clone 5C9) was adsorbed to a 96-well flat bottom polystyrene microtite plate (NUNC, Wiesbaden, Germany), in a concentration of 1000 ng mAb in 200 µl 50 mM sodium phosphate buffer, pH 9.6. The plates were sealed with a self-adhesive film and were stored at 4 °C for at least 12 h and up to 1 month. Prior to assay, the coating solution was aspirated, and the plate was washed three times (Wallac Delfia plate washer; Wallac, Turku, Finland) with washing solution (25 mM phosphate buffer, 0.05% Tween 20, 0.005% NaN₃). After being tapped dry, 175 µl of assay buffer containing 100 ng of biotinylated mAb 7H3 was added to each well. Twenty-five microlitres of standard or sample was then pipetted into each well, and the re-sealed plate was incubated either for 4 h at ambient temperature on a horizontal shaker or overnight at 4 °C. After a threefold wash step, the plates were incubated with 10 ng/well streptavidin–europium in assay buffer for 30 min. The plates were then washed sixfold, 200 µl enhancement solution was added and the plates were incubated 15 min at ambient temperature on a horizontal shaker. Time-resolved fluorescence was then measured utilizing a Delfia 1232 fluorometer (Wallac). All samples are measured in duplicate. Following glycine pre-treatment, 50 µl sample or standard was added to each well, and 100 ng biotinylated mAb 5C9 was added in 150 µl assay buffer.

**IIb. ALS assay–polyclonal antibody-based ELISA (SDS ELISA)**

ALS was also measured utilizing the total ALS ELISA kit kindly provided by DSL (Webster). Measurements were carried out according to kit insert. The signal was measured with an SLT-Tecan Spectra ELISA reader (Crailsheim, Germany). This assay utilizes polyclonal antisera directed against the specific N- and C-terminal ALS sequences. Sample pre-treatment is with SDS (0.05% final concentration). The pre-treatment for this assay is referred to as SDS and the assay as SDS-ELISA. The freeze–thaw cycle stability, reproducibility and linearity of this assay have been previously reported (4).

**III. Insulin-like growth factor I (IGF-I) and IGF-binding protein 3 (IGFBP-3) assays**

Serum IGF-I concentrations were measured using the IGF-I RIA from BioMerieux (Nurtingen, Germany). This assay is based on a previously described method (16) involving acidification and blocking of IGFBP’s by excess IGF-II. Intra- and inter-assay coefficients of variation were determined at 4.0 and 7.0% respectively. IGFBP-3
concentrations were measured by the active IGFBP-3 ELISA from DSL (Webster). Intra- and inter-assay coefficients of variation were 6.5 and 9.2% respectively. Age- and sex-adjusted normative data for IGF-I and IGFBP-3 were provided by the manufacturers. All tests were performed by an experienced researcher blinded to the patients’ clinical status.

IV. Size-exclusion chromatography

Untreated and pre-treated serum from the assay standard pool described above was fractionated with a calibrated Superdex 200 HiLoad (60 cm × 16 mm) FPLC column (Pharmacia) at neutral pH using PBS (pH 7.4). The size-exclusion chromatography column was calibrated with low molecular weight and high molecular weight gel filtration kits (Pharmacia), as previously described (17). For the chromatography of untreated serum, 500 µl serum pool was run over the column. After 1:1 dilution of fractions with assay buffer, each aliquot was UREA pre-treated as above. After pre-treatment, the samples were diluted only 1:15 (instead of 1:250) in order to obtain a measurable signal. ALS concentration was determined as under UREA, outlined above.

Pre-treated serum (pre-treated either with UREA or Gly by the methods described above, except that the final dilution was to 1:15 instead of 1:250) was also separated over the column. In addition, one aliquot of serum was SDS pre-treated by 15-fold dilution with DSL ALS sample buffer (0.05% SDS) for 30 min prior to application to column. The aliquots were diluted 1:1 with assay buffer and were then measured directly in the assay. IGFBP-3 levels were also measured in the column fractions from untreated and pre-treated serum using the two-site assay employing polyclonal goat anti-IGFBP-3 Abs, as provided by DSL (Webster).

Results

The mAbs had been raised against peptides corresponding to specific N- and C-terminal sequences of the ALS molecule. These oligopeptide sequences used for immunization are undetectable by the mAbs in the ALS protein’s natural conformation, i.e. no signal detected in untreated serum samples. Pre-treatment of the serum is therefore required to facilitate immunorecognition of ALS by these mAbs. No influence on the measured ALS concentrations was seen when serum samples were pre-incubated with increasing concentrations of IGFBP-3 (up to 2500 ng/ml; DSL, Webster).

Sensitivity and assay range

A typical dose–response curve obtained using samples pre-treated with UREA and Gly is shown in Fig. 1. The theoretical lower limit of quantification of the assay, as defined by the mean + 3 s.d. of a 30-fold zero standard (non-serum blank measured in 30 replicates), was 8.5 mU/ml.

Freeze–thaw cycle stability

To determine stability with repeated freeze–thaw cycles, serum obtained before and after pre-treatment was exposed to one to five freeze–thaw cycles (freezing for 12 h at −20 °C and thawing for at least 2 h at room temperature (21 °C)/cycle). Measurement of ALS in five serum samples over the five cycles ranged from 92 to 116% of the expected concentration (mean of 103.4%), suggesting that ALS in serum is stable to freeze–thaw.

As UREA pre-treated samples were not stable to freeze–thaw with levels of only 61–85% of the expected concentration, a freshly prepared calibration curve was utilized for each assay.

<table>
<thead>
<tr>
<th>CONCENTRATION (mU/ml)</th>
<th>PRE-TREATMENT</th>
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<tbody>
<tr>
<td></td>
<td>UREA</td>
</tr>
<tr>
<td>01</td>
<td>1544</td>
</tr>
<tr>
<td>50</td>
<td>3989</td>
</tr>
<tr>
<td>250</td>
<td>13974</td>
</tr>
<tr>
<td>1000</td>
<td>44069</td>
</tr>
<tr>
<td>2500</td>
<td>85798</td>
</tr>
<tr>
<td>5000</td>
<td>142942</td>
</tr>
</tbody>
</table>

Figure 1  Representative dose–response curves. Urea pre-treatment was performed as described in the Materials and methods section.
To achieve equivalent amounts of calibrator serum pool with Gly pre-treatment, the dilution in PBS/0.65% BSA was to 1:50 (5000 mU/ml). Twenty-five microliters of diluted sample were added to each well and samples were measured in duplicate.
Assay reproducibility

The intra-assay coefficient of variation was determined by the measurement of ten duplicates of three serum samples on a single plate. At concentrations of 310, 1192 and 2688 mU/ml, the intra-assay coefficients of variation were 5.8, 3.1 and 2.9% respectively. To assess the inter-assay coefficient of variation, the same three sera were assessed, in duplicate, on 12 separate days. The respective inter-assay coefficients of variation were 11.0, 5.9 and 8.8% respectively.

Linearity

The linearity of the assay was determined by the dilution of four pre-treated samples in assay buffer and results were compared with the calculated expected results. The measured results were, on average, 98.8% of expected with a range for the four samples of 95.8–102.4%.

The freeze–thaw cycle stability, reproducibility, and linearity of Gly assay are previously reported (8).

Size-exclusion chromatography

When non-pre-treated serum is separated with a neutral size-exclusion column (Superdex 200 FPLC column), the highest concentrations of both ALS and IGFBP-3 are measured in a peak at approximately 140 kDa, consistent with the molecular weight of the ternary complex. A smaller shoulder on the ALS peak is found at 85–90 kDa, corresponding to the MW of free, glycosylated ALS. The smaller peak at approximately 40 kDa in the IGFBP-3 assay is consistent with free IGFBP-3 (Fig. 2).

After pre-treatment of the serum with UREA the primary ALS peak is found at 85–90 kDa, no peak is found at 140 kDa and a second, flat but broad peak corresponding to a molecular weight of 250–500 kDa is apparent (Fig. 2). When SDS is used for pre-treatment no 85–90 kDa peak is seen and a single broad peak at 300–600 kDa is found (Fig. 2). Gly also results in the absence of immunoreactivity corresponding to 85–90 kDa, but a single broad peak from 250 to 500 is apparent (Fig. 2). IGFBP-3, on the other hand, is measured after all pre-treatments in a single peak at the expected MW of 40–50 kDa. Only after UREA pre-treatment is the majority of the immunoreactivity of ALS detected at 85–90 kDa, representing uncomplexed, ‘free ALS’.

Diagnostic discriminatory capacity of ALS using the three assays

Growth hormone deficiency

ALS concentrations are lower in GHD than in CON when measured with all three assays (P<0.0001 for GHD versus CON, in all ALS assays; Table 1). Overlap between GHD and a normal control is apparent with all assays (Fig. 3). Serum ALS concentrations in the groups CON, GHD, and ACRO are shown in Table 1 and Fig. 3. Agreement between the assays, as judged by the correlation analysis, is greatest in GHD (R² for UREA versus SDS-ELISA 0.90 and UREA versus Gly 0.76) and is shown in Fig. 4.

The sensitivity and specificity of serum ALS concentration as a test for GHD are highest for UREA (59 and 69% respectively) and lowest for the SDS-ELISA (41 and 51% respectively; Table 2). The likelihood ratio of having GHD based on a single low ALS measurement is 1.9, suggesting some usefulness using UREA compared with Gly or SDS-ELISA.

The number of patients with GHD whose ALS falls within 2 s.d. of the mean for CON is 12 out of 29 with UREA, compared with 18 out of 29 with Gly and 17 out of 29 with SDS-ELISA. Thus, sample pre-treatment influences diagnostic usefulness of ALS in GHD, with UREA providing somewhat better diagnostic
discrimination. However, the overlap between GHD and CON does not allow for an accurate diagnosis of GHD on the basis of a single ALS measurement using any method. Only 4 out of 29 of the same GHD patients had an IGF-I level within 2 S.D. of the control group, and the sensitivity, specificity and likelihood ratios suggest that IGF-I is more useful in the diagnosis of GHD. This may, however, reflect a selection bias, as subnormal IGF-I was used as one of the criteria to define GHD in our center.

**Acromegaly** ALS concentrations are higher in the patients with acromegaly than in controls (P < 0.0001) using all ALS assays. The assays correlate less closely with each other in ACRO than in GHD sera (R² for UREA versus SDS-ELISA 0.26 and R² for UREA versus Gly 0.52), as illustrated in Fig. 4. Although there is almost complete separation of CON and ACRO by the use of the UREA assay, considerable overlap exists using the other assays (Fig. 3).

The sensitivity of UREA and Gly for the diagnosis of acromegaly is 81–94%, compared with only 44% with SDS-ELISA. The specificity of the ALS concentration in diagnosing acromegaly is 46% with SDS-ELISA, similar to Gly (49%), but much lower than 87% found by UREA. The likelihood ratio of a positive or negative (normal) test using ALS measurement after UREA is much higher than Gly, SDS-ELISA, or IGFBP-3. Furthermore, using UREA and Gly, 1 out of 16 and 3 out of 16 patients with acromegaly respectively had an ALS concentration within 2 S.D. of the control group, in contrast to 9 out of 16 when ALS is measured by the SDS-ELISA.

The sensitivity and specificity of IGF-I were 100 and 97% respectively and the likelihood ratios also suggest excellent differentiation of acromegaly from normal using IGF-I. However, the sera from patients with ACRO were selected on the basis of an IGF-I > 400 ng/ml.

**Discussion**

In this series of adult patients with GHD and at least one additional anterior pituitary hormone deficiency, ALS levels were lower than in the control population using all assays. However, a significant overlap between GHD and controls was noted. The sensitivity and specificity calculated for this adult population ranged from 40 to 60% and 51 to 69% respectively, depending on the ALS assay used. UREA pre-treatment of sera before ALS measurement led to the highest sensitivity and specificity. These values are slightly lower than those reported by Juul et al. (18), who found in a re-test of young adults treated with GH as children, the sensitivity and specificity of ALS in confirming the result from GH provocative testing (cut-off 7.5 ng/ml) to be 72 and 75.8% respectively. The sensitivity and specificity of IGF-I in our cohort of GHD patients (86 and 90% respectively) is considerably higher than was seen in Juul’s study (76 and 72%). This likely results from the stringent selection criteria applied to our patients (all with organic pituitary pathology, at least one other pituitary hormone insufficiency and a subnormal IGF-I).

There is conflicting evidence in the literature regarding the age dependency of ALS levels. In our relatively small cohort of control individuals, we found no significant effect of age on ALS levels. Although one study had shown an effect of age in males (18), more recent studies have not confirmed this (12). If age does impact ALS levels, the older age of our patient group would tend to overestimate the sensitivity and specificity in GHD and underestimate the sensitivity and specificity in acromegaly. However, this would apply equally to all ALS assays compared in this study and would therefore not explain differences between the methods of serum pre-treatment for ALS unfolding.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>IGF-I and ALS concentrations in each of the groups as measured by each assay.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GHD (n= 29)</td>
</tr>
<tr>
<td>ALS–UREA (mU/ml)</td>
<td>432.6±261.7 (42%)</td>
</tr>
<tr>
<td>ALS–Gly (mU/ml)</td>
<td>350.1±244.3 (32%)</td>
</tr>
<tr>
<td>ALS–SDS-ELISA (mg/l)</td>
<td>21.2±6.6</td>
</tr>
<tr>
<td>IGF-I (ng/ml)</td>
<td>58.1±37.3 (30%)</td>
</tr>
<tr>
<td>IGFBP-3 (ng/ml)</td>
<td>2310±1064 (45%)</td>
</tr>
</tbody>
</table>

Values are mean ± s.d. (% mean is of control group).

![Figure 3 ALS levels in GHD (x), acromegaly (o), and controls (+). Measured with each immunoassay (mU/L for UREA and Gly and mg/L for SDS-ELISA).](https://www.eje-online.org)
In this study, we confirm the observation of other authors (1, 4, 10) that ALS levels are elevated in acromegaly. In contrast to Baxter’s findings, we found that the usefulness of ALS quantification in the diagnosis of GH excess is dependent on the assay used for testing. The sensitivity using UREA exceeded 90% compared with only 45% for the polyclonal SDS-ELISA. Specificity in the SDS-ELISA was 46% and substantially higher at 87% after UREA pre-treatment. In other studies, 89% of patients with acromegaly had an ALS level greater than the 97th percentile of healthy controls and ALS had a sensitivity of 90% and a specificity of 71% in assessing clinical activity in acromegaly based on questionnaire and using the SDS-ELISA assay (4, 19).

Figure 4 Scatter plot of ALS levels comparing (A) UREA versus SDS-ELISA and (B) Gly versus SDS-ELISA. Groups are GHD (●), control (□), and acromegaly (▲).

Table 2 Diagnostic accuracy of ALS concentration measured with various assays, IGFBP-3 and IGF-I in the diagnosis of GHD and acromegaly (95% confidence interval in brackets).

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>LR_{pos}</th>
<th>LR_{neg}</th>
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<tr>
<td><strong>GHD</strong></td>
<td></td>
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<tr>
<td>UREA</td>
<td>59 (41–77)</td>
<td>69 (54–84)</td>
<td>1.9 (1.1–3.3)</td>
<td>0.59 (0.37–0.96)</td>
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<tr>
<td>Gly</td>
<td>38 (20–56)</td>
<td>67 (52–82)</td>
<td>1.15 (0.60–2.19)</td>
<td>0.93 (0.65–1.33)</td>
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<tr>
<td>SDS-ELISA</td>
<td>41 (23–59)</td>
<td>51 (35%–67)</td>
<td>0.84 (0.49–1.44)</td>
<td>1.16 (0.75–1.78)</td>
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<td>IGFBP-3</td>
<td>86 (73–99)</td>
<td>77 (64–90)</td>
<td>3.74 (2.07–7.76)</td>
<td>0.18 (0.06–0.39)</td>
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<td>IGF-I</td>
<td>86 (73–99)</td>
<td>90 (81–99)</td>
<td>8.6 (3.36–22.0)</td>
<td>0.16 (0.06–0.39)</td>
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<tr>
<td><strong>Acromegaly</strong></td>
<td></td>
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<tr>
<td>UREA</td>
<td>94 (82–100)</td>
<td>87 (76–98)</td>
<td>7.23 (3.16–16.5)</td>
<td>0.07 (0.01–0.46)</td>
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<tr>
<td>Gly</td>
<td>81 (62–100)</td>
<td>49 (33–65)</td>
<td>1.56 (1.08–2.34)</td>
<td>0.39 (0.13–1.13)</td>
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<tr>
<td>SDS-ELISA</td>
<td>44 (20–68)</td>
<td>46 (30–62)</td>
<td>0.82 (0.46–1.44)</td>
<td>1.22 (0.67–2.21)</td>
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<tr>
<td>IGFBP-3</td>
<td>63 (40–86)</td>
<td>13 (2–24)</td>
<td>0.72 (0.49–1.08)</td>
<td>2.85 (1.01–8.02)</td>
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<tr>
<td>IGF-I</td>
<td>100</td>
<td>97 (92–100)</td>
<td>33.3 (4.84–229.7)</td>
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</tbody>
</table>

Discrimination of patients with GH excess from normal is not only relevant for the diagnosis of acromegaly, but is also important for monitoring treatment efficacy in this disorder. Furthermore, in the follow-up of GH replacement therapy, it must be ensured that iatrogenic GH excess is avoided. IGF-I has been considered the best single test in acromegaly, but as some patients with adequately suppressible GH after oral glucose load have elevated IGF-I levels, further biochemical indicators of disease activity are required (20). Our study is not suitable to compare the usefulness of IGF-I and ALS in acromegaly, because IGF-I > 400 ng/ml was a patient selection criterion. Rather, a similar study examining the usefulness of IGF-I to classify patients with acromegaly would need to assign participants to groups based on another standard (e.g. on the basis of an elevated ALS level) to adequately address this question. ALS has been shown to have better discriminatory capability than IGFBP-3, -1, and -2 (20). An elevated IGF-I level was a selection criterion for the diagnosis of acromegaly also in that study, making it difficult to interpret the usefulness of IGF-I in the diagnosis of acromegaly. In a study comparing numerous assays of GH-dependent proteins with clinical symptoms in acromegaly, IGF-I, IGFBP-3 and ALS (using SDS-ELISA) were equally related to disease activity (12). Although the direct comparison of IGF-I and ALS is difficult within the confines of the currently available studies, our study suggests that ALS is a parameter which shows good discriminatory ability in the monitoring of acromegaly. Its diagnostic usefulness is, however, dependent on the serum pre-treatment modalities and the immunoassay applied.

The data presented here suggest that differences exist in the diagnostic usefulness of assays for ALS, particularly at high ALS concentration such as in acromegaly. Although ALS is known to form complexes with IGFBP-3 and -5, it is very unlikely that the differences between assays result from interference of IGFBP-3 or -5 as pre-treatment renders the molecule unable to associate with IGF-binding proteins.
Furthermore, increasing concentrations of IGFBP-3 had no influence on the signal measured.

When proteins are denatured, as achieved during the various pre-treatment regimes outlined in this paper, the resulting protein structure is dependent not only on the protein but also on the denaturing reagents. The denatured state can be compact (i.e. significant secondary structure is retained) or can be denatured unfolded (21). The reaction of many proteins in urea, which is thought to function by weakening hydrophobic interaction, is to a relatively compact denatured state. Acidification, on the other hand, results in the acquisition of large net charge by the molecule, which may lead to a more highly unfolded state (21). The mechanism and state of unfolding resulting from SDS is not completely understood, although considerable secondary structure can be retained (22). These effects are, however, protein specific and do not permit the exact prediction of how ALS reacts to the various denaturants. ALS and IGFBP-3 after pre-treatment are no longer measured in the ternary complex form, evident from the size-exclusion studies. Only UREA pre-treatment results in the majority of the immuno-reactivity being measured at the molecular weight corresponding to free ALS (approximately 64% as judged from the area under the curve compared with none with SDS and Gly). The reasons for the shift to higher molecular weight of ALS after pre-treatment with SDS and Gly are unclear. Potentially, the denatured ALS protein forms larger aggregates. This ‘shift’ was also observed by Khoosravi using SDS pre-treatment (4). Apparently, after SDS or Gly pre-treatments, no free ALS exists. It is conceivable that aggregation occurs to a greater extent with SDS and acidification pre-treatments, because the denatured monomeric structure that these procedures result in is more likely to aggregate than the structure resulting from urea denaturation.

Our study findings are novel, as they suggest that the diagnostic accuracy of ALS in human pathology varies with the assay used and indicate that UREA resulted in elution after pre-treatment at the expected molecular weight in contrast to what is found when using the commercially available assay. The findings that UREA pre-treatment resulted in the measurement of the majority of ALS at the MW consistent with free ALS, and had the highest sensitivity and specificity in GHD and acromegaly, support the conclusion that higher diagnostic usefulness may result from a greater proportion of uncomplexed monomeric ALS after pre-treatment.

In summary, ALS measurement using specific monoclonal Ab requires pre-treatment and the diagnostic accuracy of ALS varies with pre-treatment modality. ALS, when exposed to pre-treatment that does not induce aggregation, is a useful indicator of GH status in acromegaly and, to a lesser extent, in GHD.

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