Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls

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RECEIVED 4 June 1990

ABSTRACT
This report describes essential requirements for the validation of a radioimmunoassay (RIA) for insulin-like growth factor-I (IGF-I) and presents solutions to some problems and pitfalls commonly observed. The preparation of IGF-I to be used as radioligand or standard has to be selected carefully since some IGF-I preparations are contaminated with variants which demonstrate different potencies for different antisera used in the RIA.

Accurate assessment of IGF-I levels in blood plasma requires an efficient extraction method for the IGF-binding proteins (IGFBPs). Extraction methods to remove the influence of IGFBPs in the RIA were compared using blood plasma of considerable differences in IGF-I/IGFBP ratios. Acidification of plasma before column chromatography on Sephadex G-75 (G75) is generally considered to be the most reliable extraction method, but it is very time-consuming. The acid–ethanol extraction (AE) of plasma is not valid in many situations. Non-parallel displacement to the IGF-I standard was observed with AE-extracted plasma samples in the RIA. In addition, a comparison of IGF-I values obtained in the RIA after AE or G75 extraction of fetal ovine plasma has shown no significant correlation.

We report an extraction technique based on a modified AE extraction followed by cryo-precipitation (AEC). AEC extraction on blood plasma reduced residual IGFBPs to a level that did not interfere in the assay. Furthermore, AEC-extracted plasma samples showed parallel displacement in the RIA to highly purified preparations of authentic IGF-I. We observed high correlations, with a slope close to unity, of IGF-I values obtained in the RIA using the AEC or G75 extraction for plasma from different species including adult and fetal sheep, rat, mouse and man. The AEC extraction provides a rapid and simple alternative to G75 extraction for blood plasma from a variety of species provided that high-affinity antisera are used for the RIA.


INTRODUCTION
Increasing interest in the physiological role of insulin-like growth factor-I (IGF-I) in vivo has led to a proliferation of immunoassay systems for the measurement of plasma IGF-I. Many of these systems, however, have not been rigorously validated, and a number of potential pitfalls are apparent which limit the value of some data obtained.

A rapidly increasing number of recombinant human IGF-I (rhIGF-I) preparations have been used for radioimmunoassays (RIAs), some of which contain variants with mismatched disulphide linkages and reveal significant heterogeneity (Buell, Schulz, Selzer et al. 1985; Meng, Burleigh & Kelly, 1988). This heterogeneity appears to be particularly common when rhIGF-I has been produced by bacterial as opposed to yeast fermentation. Two major peaks may be seen on analytical high-pressure liquid chromatography (HPLC) representing intact and misfolded IGF-I (Meng et al. 1988), which differ in biological potency and their ability to bind IGF-binding proteins (IGFBPs) (Hodgkinson, Napier, Davis et al. 1989b). The potential problems caused by such heterogeneous materials in assay systems have not been considered.

It is now appreciated that dissociation and removal of IGFBPs from circulating IGF-I is essential before plasma samples can be reliably assayed (Daughaday, Kapadia & Mariz, 1987). Acidification of plasma and separation of free IGF-I by column chromatography under acidic conditions has been generally accepted as the most reliable approach (Daughaday & Rotwein,
1989) and is regarded as the standard method. The expensive and laborious nature of this method, however, makes it unsuitable for routine assays. With the need for high-volume assays the acid–ethanol extraction (AE), as originally developed for post-natal human plasma (Daughaday, Mariz & Blethen, 1980), has been widely used during recent years. However, its use has been extended to fluids other than plasma and species other than man. Such use has often not been subject to rigorous validation. It is therefore not surprising that there is persistent uncertainty over the validity of some IGF-I RIA systems (Daughaday et al. 1987; Mesiano, Young, Browne & Thorburn, 1988).

In this paper we illustrate problems that may arise from the inappropriate selection of IGF-I preparations and the incomplete validation of an RIA for IGF-I on extracted plasma. We demonstrate the need to evaluate extraction systems across a range of samples in which the ratio of IGF-I to IGFBP can be anticipated to vary markedly. Furthermore, we present data showing that the commonly used AE extraction technique (Daughaday et al. 1980) is unsatisfactory for plasma samples obtained under extreme physiological conditions. We describe a modified AE extraction method which includes a cryo-precipitation step (AEC). This AEC extraction has validity over a wide range of different IGFBP profiles encountered in blood plasma from a variety of species including sheep, rat, mouse and man.

MATERIALS AND METHODS

Materials

Recombinant hIGF-I produced by bacterial fermentation (rh-met-IGF-I) batch 742.44 and HPLC-purified misfolded rh-met-IGF-I (misfolded IGF-I) (Meng et al. 1988) were provided by Dr B. D. Burleigh, IMC, Terre Haute, IN, U.S.A. The international reference standard rhIGF-I preparation 87/518 (IRR IGF-I) was obtained from the National Institute for Biological Standards and Control, Potters Bar, Herts, U.K. Recombinant hIGF-I produced by yeast fermentation (y-rhIGF-I) batch CGP 35'126 was provided by Dr K. Mueller and Dr W. Maerky, Ciba-Geigy Ltd, Basel, Switzerland. Ovine IGF-I (oIGF-I), purified from sheep serum, was a gift from Dr L. Moore, Wallaceville Agricultural Research Centre, Wellington, New Zealand. Human amniotic fluid IGFBP (IGFBP-1) was a gift from Professor K. Hall, Karolinska Hospital, Stockholm, Sweden.

Plasma samples

Fetal and adult sheep plasma were used for detailed demonstration of potential pitfalls of the RIA for IGF-I since samples covering considerably differing IGF-I/IGFBP ratios (Butler & Gluckman, 1986) were available from the same species. In addition, sheep plasma appears to have caused problems in the IGF-I RIA in some laboratories (Mesiano et al. 1988). Adult sheep plasma was obtained from Romney ewes at varying nutritional planes ranging from chronic undernutrition to feeding at very high levels and also from ewes at late gestation. Fetal sheep plasma was obtained from chronically catheterized fetuses of Romney ewes mated to Suffolk rams. The gestational ages ranged from 100 to 144 days and samples were obtained from fetuses of markedly different nutritional states. Rat plasma was obtained from adult male and female Charles River Wistar rats and male dwarf rats (Charlton, Clark, Robinson et al. 1988) ranging from 50 to 180 days of age. Mouse plasma was collected from male and female Charles River CD-I mice and mice selected for high and low plasma IGF-I (Blair, McCutcheon, Mackenzie et al. 1988); the age range was 20–110 days. Human plasma was obtained from normal volunteers, patients with either active acromegaly or hypopituitarism (with the approval of the Auckland Hospital Research Ethical Committee). All blood samples were collected in heparinized tubes (15 units heparin/ml) and immediately placed on ice. The plasma was separated within 30 min of collection and frozen at −20 °C.

Extraction techniques for plasma samples

Standard acid–ethanol extraction

AE extractions were carried out according to the method of Daughaday et al. (1980). Plasma samples were thoroughly mixed with an acid–ethanol mixture (87·5% ethanol and 12·5% 2 mol HCl/l, v/v) at a ratio of 1 : 4 and incubated at room temperature for 30 min. The tubes were then centrifuged at 1800 g for 30 min at 4 °C. An aliquot of the supernatant was then transferred into fresh test tubes and neutralized with 0·855 mol Tris base/l at a ratio of 5 : 2. Recoveries of 125I-labelled y-rhIGF-I added to plasma were 89·3 ± 2·6% (n = 7) for adult and 96·3 ± 1·6% (n = 7) for fetal sheep plasma.

Acid–ethanol cryo-precipitation method

Plasma samples were thoroughly mixed with an acid–ethanol mixture (87·5% ethanol and 12·5% 2 mol HCl/l, v/v) at a ratio of 1 : 4 and incubated at room temperature for 30 min. The tubes were then centrifuged at 3000 g for 30 min at 4 °C to obtain a firm pellet of precipitated protein. The supernatant was decanted into fresh test tubes and neutralized with 0·855 mol Tris base/l at a ratio of 5 : 2. The samples were then stored at −20 °C for 1 h and immediately centrifuged at 3000 g for 30 min at 4 °C. A second precipitate was observed which was particularly marked.
in fetal sheep and rat samples. The supernatant was
decanted into fresh test tubes. Preliminary studies had
shown that maximal precipitation was achieved under
these conditions. Recoveries of $^{125}$I-labelled y-rhIGF-I
added to plasma were 91.6 $\pm$ 2.7% ($n$ = 7) for adult
and 93.2 $\pm$ 6.3% ($n$ = 7) for fetal sheep plasma. All AE
or AEc extractions of plasma were performed on the
same day of their use in the RIA or Superose 12
chromatography.

**Sephadex G-75 extraction using 1 mol formic acid/l**

Acid gel chromatography was performed according to
the method of Hintz & Liu (1977). Plasma samples of
1 ml were acidified with 100% formic acid to a final
concentration of 1 mol/l and incubated for 15 min to
dissociate IGF-I from its IGFBPs. The acidified serum
was then chromatographed on Sephadex G-75 (G75)
(Pharmacia, Uppsala, Sweden) using a glass column
(10 $\times$ 600 mm). The column was pre-equilibrated with
2 ml 1% (w/v) bovine serum albumin (BSA) acidified
with 100% formic acid to a final concentration of
1 mol/l. The samples were eluted with 0.25 mol formic
acid/l at a flow rate of 0.5 ml/min and fractions of 1 ml
were collected into silicone (Coatasil; Ajax Chemicals
PTY, Auburn, Australia)-coated glass tubes containing
50 $\mu$1 1% (w/v) BSA in distilled H$_2$O. The column was
calibrated with Dextran Blue, $^{125}$I-labelled y-rhIGF-I and
$^{125}$I, and the volume of eluate containing the
native IGF-I (Darcy’s constant (KD) = 0.28–0.75) was
measured and lyophilized. These lyophilized ‘free’ IGF-I
fractions were reconstituted in assay buffer before
further analysis. Recoveries of $^{125}$I-labelled y-rhIGF-I
added to plasma were 95.2 $\pm$ 4.5% ($n$ = 7) for adult
and 92.4 $\pm$ 3.8% ($n$ = 7) for fetal sheep plasma. To
investigate the interference of IGFBPs in the RIA, IGFBP-
containing fractions (KD = 0–23) of G75-extracted
sheep plasma were lyophilized, reconstituted in assay
buffer and the insoluble denatured protein was removed
by centrifugation at 3000 g for 30 min at 4 °C. Only the
soluble IGFBP fractions were used for further analysis.

**Sep-Pak extraction**

This procedure for extraction of IGF-I from plasma
involves adsorption on C-18 silicate minicolumns
(SepPak; Waters Associates, Milford, MA, U.S.A.)
(Daughaday et al. 1987; Wallis, Daniels, Ray et al.
1987). The column was prepared with rapid sequential
washes of 5 ml isopropanol, 5 ml methanol and 10 ml
4% (v/v) acetic acid. Fetal or adult sheep plasma
(250 $\mu$l) was acidified with 1 ml 0.5 mol HCl/l and then
incubated at room temperature for 30 min. The acidifi-
ced sample was loaded onto the column over 2 min
and incubated for 1 min. The column was then
washed with 10 ml 4% (v/v) acetic acid to remove the
IGFBP. The IGF-I was eluted with 4 ml methanol
over 3 min. The eluate was dried at room temperature
in a stream of air, dissolved in RIA buffer and incu-
bated at room temperature for 15 min to improve
solution of the sample. Recoveries of $^{125}$I-labelled
y-rhIGF-I added to plasma were 65.5 $\pm$ 18.6% ($n$ = 15)
for fetal and 71.8 $\pm$ 19.1% ($n$ = 8) for adult sheep
plasma. We observed that the timing of the loading of
the acidified sample and the elution of the IGF-I frac-
tion were critical. Very small changes in timing (in the
range of 10–15 s) caused large variation. In addition
we observed that the extracted samples did not
uniformly redissolve in H$_2$O or assay buffer after they
had been evaporated. The Sep-Pak extraction method
was for these reasons abandoned and not investigated
in detail in the RIA.

**Assessment of residual IGFBPs**

The presence of IGFBPs was investigated in neutralized
AE and AEc extracts, Sep-Pak extracts, Sephadex
G-75 extracts of plasma samples and also in plasma
diluted with either 0.01 mol phosphate-buffered saline
(PBS)/l, pH 6.2, containing 0.37% (w/v) EDTA, 0.02% (w/v)
sodium azide, 0.5% (w/v) BSA (assay buffer) or 0.01 mol PBS/l, acid–ethanol mixture and 0.855 mol Tris base/l at a ratio of 1 : 4 : 2 : 1, pH 7.8
(PTA buffer) for both fetal and adult ovine plasma.
IGF-I binding was determined by in-vitro binding studies
using $^{125}$I-labelled y-rhIGF-I (Hodgkinson, Moore,
Napier et al. 1989a). All samples were used at a 1 : 7
dilution of plasma equivalents to allow a direct com-
parison between the different extraction techniques.
The samples (1 ml) were incubated with approximately
500 000 c.p.m. $^{125}$I-labelled y-rhIGF-I for 90 min at
room temperature. Preliminary studies demonstrated
that maximal binding was achieved after incubation
for 30 min at room temperature and binding remained
constant for 24 h. Aliquots of 500 $\mu$l were subjected to
high-performance gel chromatography on a Superose
12 column (10 mm $\times$ 300 mm, Pharmacia) fitted to a
Pharmacia fast-protein liquid chromatography sys-
tem. The column was equilibrated with 0.1 mol PBS/l
buffer containing 0.15 mol NaCl/l and 0.02% NaN$_3$
(pH 7.2) and the samples were eluted with the same
buffer at a flow rate of 0.5 ml/min. Fractions of
0.25 ml were collected after an 8-min delay and the
radioactivity in the eluate was determined in a gamma
counter. The Superose 12 column was calibrated
before use with a range of $M$, markers.

**RIA of IGF-I**

The y-rhIGF-I was iodinated by a modified chloramine
T method (Gluckman, Johnson-Barrett, Butler et al.
1983). The $^{125}$I-labelled y-rhIGF-I was purified by
exclusion chromatography on a prealbuminized
Sephadex G-50 (Pharmacia) column (10 mm $\times$
600 mm). Fractions of 40 drops were collected using

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4°C. The preprecipitated normal PBS/1,1 containing neutralized assay properties activity and is distributed provided antisera glutaraldehyde immunized in our laboratory in New Zealand White rabbits immunized with rh-met-IGF-I (batch 742.44) by a glutaraldehyde conjugation to keyhole limpet haemocyanin emulsified in complete Freund's adjuvant. These antisera are of high affinity for IGF-I and low cross-reactivity with rhIGF-II (batch 2/1, Dr D. Burleigh). We also used a polyclonal antiserum (batch C549/804) provided by Drs J. Van Wyk and L. Underwood and distributed by the Hormone Distribution Program of the NIADDK for comparison. Purified oIGF-I, which is very similar to hIGF-I in structure and biological activity (Francis, McNeil, Wallace et al. 1989), demonstrated parallel displacement to y-rhIGF-I, IRR IGF-I and rh-met-IGF-I for all four antibodies. Details of the properties of the four antisera are shown in Table 1.

Assay procedure
The incubation mixture consisted of 100 μl diluted neutralized acid–ethanol solution containing the standard or test plasma and 200 μl assay buffer containing IGF-I antiserum. After preincubation for 60 min at room temperature, 125I-labelled y-rhIGF-I (20 000 c.p.m.) was added in 200 μl assay buffer and the incubation was continued for 18–24 h at 4°C. Bound and free IGF-I were separated by addition of 1 ml of a preprecipitated second antibody containing 0.01 mol PBS/1, 1% (v/v) sheep antirabbit γ-globulin, 0.1% (v/v) normal rabbit serum and 5% polyethylene glycol 6000. The test tubes were then incubated for 1 h at room temperature and centrifuged at 3000 g for 30 min at 4°C. The supernatant was decanted and the radioactivity in the remaining pellet was determined. The standard curve for the measurement of plasma samples was derived from AEC-extracted pooled adult sheep plasma or various preparations of IGF-I described above in multiple dilutions using PTA buffer.

Statistical analysis
Statistical testing for parallelism of displacement for extracted plasma samples was performed by paired comparison of the IGF-I values obtained from the same extracts assayed at a twofold difference in concentration (Snedecor & Cochran, 1989). Linear regression analysis was used to correlate the IGF-I values obtained after AE or AEC extraction with G75 extraction on the same plasma samples. The IGF-I values obtained after G75 extraction were used as dependent variables for regression analysis in all comparisons. Bonferroni confidence limits were used for the t-test where multiple comparisons were performed (Snedecor & Cochran, 1989). All data are reported as means ± s.d. unless otherwise stated.

RESULTS

Selection of reference standard
Detailed competitive binding studies were performed with three of our own antisera and antiserum C549/804 using y-rhIGF-I as radioligand and rh-met-IGF-I, misfolded IGF-I, y-rhIGF-I or IRR IGF-I as the unlabelled ligand in the RIA. An aliquot of the same sheep plasma pool was also analysed with each antiserum after AEC extraction. The apparent IGF-I values for the AEC-extracted sheep plasma pool were calculated separately using each of the four different IGF-I preparations as reference standard (Table 2). Considerable differences in apparent IGF-I values were observed for AEC-extracted sheep plasma when either rh-met-IGF-I or misfolded IGF-I were used as standards in the RIAs.

Table 1. Characteristics of insulin-like growth factor-I (IGF-I) antisera. Maximum binding (corrected for non-specific binding) ranged between 25% and 45% of total tracer added for different antisera. Recombinant human IGF-I produced by yeast fermentation was used as radioligand and unlabelled ligand for the assessment of the minimal detectable dose (MDD) and the half-maximal displacement (ED50); values are means ± s.d. Recombinant human IGF-II was used to assess the cross-reactivity with IGF-II.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Final dilution</th>
<th>MDD (ng/tube)</th>
<th>ED50 (ng/tube)</th>
<th>Cross-reactivity with IGF-II (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>863/5 (n=5)</td>
<td>1:75 000</td>
<td>0.06 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0-5</td>
</tr>
<tr>
<td>861/5 (n=4)</td>
<td>1:50 000</td>
<td>0.08 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0-5</td>
</tr>
<tr>
<td>878/4 (n=5)</td>
<td>1:15 000</td>
<td>0.10 ± 0.03</td>
<td>0.31 ± 0.04</td>
<td>0-05</td>
</tr>
<tr>
<td>C549/804 (n=4)</td>
<td>1:5000</td>
<td>0.06 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>2-0</td>
</tr>
</tbody>
</table>

0.01 mol PBS/1 (pH 6.2) containing 0.1% (v/v) gelatin as eluent. The specific activity of 125I-labelled y-rhIGF-I was 120–160 μCi/μg. Only the eluates corresponding to the IGF-I monomer were pooled and stored in aliquots at 4°C. This material was used within 2 weeks of iodination.
TABLE 2. Apparent IGF-I values (means ± S.D.) of an acid–ethanol cryo precipitation (AEC)-extracted sheep plasma pool expressed in terms of recombinant hIGF-I produced by bacterial fermentation (rh-met-IGF-I) or misfolded IGF-I or recombinant hIGF-I produced by yeast fermentation (y-rhIGF-I) or international reference IGF-I (IRR IGF-I)

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>rh-met-IGF-I</th>
<th>Misfolded IGF-I</th>
<th>y-rhIGF-I</th>
<th>IRR IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>861/5 (n = 3)</td>
<td>287 ± 8.4*</td>
<td>110 ± 8.4*</td>
<td>201 ± 19.6</td>
<td>208 ± 12.6</td>
</tr>
<tr>
<td>863/5 (n = 3)</td>
<td>228 ± 13.8</td>
<td>112 ± 7.7*</td>
<td>217 ± 21.0</td>
<td>227 ± 19.6</td>
</tr>
<tr>
<td>878/4 (n = 3)</td>
<td>389 ± 37.1*</td>
<td>2765 ± 152**††</td>
<td>206 ± 32.2</td>
<td>245 ± 32.9</td>
</tr>
<tr>
<td>C549/804 (n = 3)</td>
<td>479 ± 13.3††</td>
<td>3493 ± 452**††</td>
<td>290 ± 28.7†</td>
<td>344 ± 9.8†</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 compared with IRR IGF-I for the same antiserum; †P < 0.05, ††P < 0.01 compared with antiserum 861/5 (t-test using Bonferroni confidence limits for multiple comparison).

These differences could be explained by the observation that the rh-met-IGF-I and the misfolded IGF-I preparations contain approximately 35% and 90% IGF-I variants with mismatched disulphide bonds respectively (Hodgkinson et al. 1989b).

The IGF-I values for AEC-extracted sheep plasma samples were comparable when expressed in terms of y-rhIGF-I or IRR IGF-I and the same antiserum was used. The similar immunopotency of these two IGF-I preparations appears to be related to their high degree of purity. Both contain more than 90% correctly folded material as assessed by HPLC (Hodgkinson et al. 1989b; information documents from National Institute for Biological Standards and Control). However, small but significantly (P < 0.05) elevated apparent IGF-I values were observed with antiserum C549/804 in comparison with antisera 865/5, 861/5 and 870/4 even when the correctly folded IGF-I preparations were used as reference standards. This difference cannot be explained by the relatively high cross-reactivity of IGF-II (about 2%) with antiserum C549/804 (Daugaday & Rotwein, 1989) but may relate to the different immunogen used to raise this antiserum.

Comparison of extraction methods

Efficacy of removal of IGFBPs

We examined the elution profiles of plasma diluted in assay buffer and PTA buffer as well as AE-, AEC-, Sep-Pak- and G75-extracted plasma samples after incubation with 125I-labelled y-rhIGF-I followed by high performance gel chromatography on a Superose 12 column (Fig. 1). The recovery of added tracer in the eluates was approximately 100% for plasma diluted with assay buffer, 70% for PTA buffer-diluted plasma, 85% for AE- and 98% for AEC-extracted plasma. The reduced recoveries for PTA buffer-diluted and AE-extracted plasma were related to adsorption of aggregated protein to the column supports. All extraction procedures as well as plasma diluted with PTA buffer (its main effect leads to denaturing of plasma proteins) abolished tracer binding in the 150 kDa region. In addition the amount of tracer binding in the 35–65 kDa region was markedly reduced. The efficiency of the various extraction techniques calculated as percentage radioactivity eluted in the 35–65 kDa region was G75 > Sep-Pak > AEC > AE > PTA buffer dilution for both adult and fetal ovine plasma.

Assessment of interference of IGFBPs in the RIA

Because both IGFBP and IGF-I concentrations may be regulated independently and all extraction methods leave some residual IGFBP, it is important to validate assay systems against samples of high or low IGF-I/ IGFBP ratios. We used adult and fetal sheep plasma to represent these two extremes (Butler & Gluckman, 1986; Hodgkinson et al. 1989a). To demonstrate the nature of possible interference of IGFBPs and acid–ethanol-denatured protein in the IGF-I RIA a series of competitive binding assays were performed using fetal and adult sheep plasma samples subject to dilution in assay buffer or different extraction techniques. All experiments in this series were performed using antibody 878/4 and 125I-labelled y-rhIGF-I tracer (Fig. 2). The data illustrated in Fig. 2 describe a general phenomenon observed with plasma samples from various species. Similar observations were also made with other antisera used in the present investigation.

The G75-extracted plasma samples displaced in parallel with the rh-met-IGF-I, oIGF-I and the y rhIGF-I standards. The AEC-extracted plasma samples showed identical displacement to G75-extracted plasma samples and also demonstrated complete parallelism to both IGF-I standards. While AE-extracted plasma samples showed near parallelism to the IGF-I standards at highly diluted concentrations, at lower dilutions AE-extracted plasma samples diverged from the G75 extracts. A similar behaviour was observed with samples diluted with PTA buffer which contained a large amount of denatured protein and IGFBP. The non-parallelism was particularly marked.
in fetal samples and could be explained by high concentrations of IGFBP and incomplete precipitation of acid–ethanol-denatured proteins in AE extracts. Plasma samples diluted in assay buffer did not show parallelism with the rh-IGF-I standards and demonstrated considerably less potency in comparison with the respective G75 extracts.

**Significance of residual IGFBPs in the RIA**

Since all extraction methodologies leave some low molecular weight IGFBPs in the extract we elucidated whether residual IGFBPs interfere in the RIA. When purified human IGFBP-1 was used in increasing concentrations instead of unlabelled ligand in the IGF-I RIA it demonstrated apparent displacement of $^{125}$I-labelled y-rhIGF-I in a dose-dependent manner (Fig. 3). This phenomenon may be due to interference in the RIA by binding of IGF-I to soluble IGFBP-1 which is not precipitable under the assay conditions and thus decreases the availability of ligand to the antibody. More importantly, up to 2 ng IGFBP-1 per tube added to the standard curve did not interfere in the RIA. Assuming that the G75 and AEC extraction systems remove at least 80% of IGFBP from blood plasma (authors’ unpublished results) this means that plasma concentrations of IGFBP-1 up to 3.5 µg/ml, which are well in excess of plasma concentrations commonly observed (Povoa, Roovete & Hall, 1984; Cotterill, Cowell, Baxter et al. 1988), do not interfere in the assay.

For further investigation of the possible interference of IGFBPs we added soluble IGFBP fractions of G75-extracted fetal plasma to the IGF-I RIA (Fig. 4). The fractions containing soluble IGFBP interfered in the IGF-I RIA in a dose-dependent fashion when used instead of unlabelled ligand. In addition, complex competition occurred when soluble IGFBP fractions were added to the y-rhIGF-I standard curve in concentrations equivalent to or greater than those used for the analysis of fetal plasma samples. In contrast, similar fractions of AEC-extracted adult and fetal sheep plasma did not interfere with the standard curve.

**Comparison of G75, AE and AEC extractions for the radioimmunological determination of IGF-I**

The validity of AEC and its advantage over the traditional AE extraction was demonstrated by correlating the IGF-I values obtained after either AE or AEC extraction methods against G75 extraction. In addition, all extracted samples were routinely analysed at a two-fold difference in concentration to allow testing for parallelism by paired comparison. The within-assay
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variation was ≤6% and the between-assay variation was ≤12% for all antisera used.

A representative example of the inadequacy of AE is shown in Fig. 5 using antiserum 878/4 in the RIA. Although the correlation between values obtained with either extraction technique was very high for adult sheep samples, the slope of the regression line deviated from unity (adult sheep plasma analysed at 100 μl extract: \( r = 0.97 \), \( P < 0.01 \), \( y = -68 + 1.48x \) and analysed at 50 μl extract: see Fig. 5a). There was no significant correlation for fetal sheep samples when 50 μl of extract was used (Fig. 5b) and the correlation only approached significance (\( P < 0.05 \)) for samples analysed at 100 μl extract (\( r = 0.71 \), \( y = 105 + 0.67x \)). Testing for parallelism by assaying samples at a twofold difference in dilution of AE extracts revealed...
non-parallelism which was particularly marked in fetal samples (paired comparison of AE-extracted plasma samples; adult, \( P<0.01 \); fetus, \( P<0.001 \) and of G75-extracted plasma samples; adult, \( P=0.70 \); fetus, \( P=0.89 \)). This suggests some interfering factors remain in the AE-extracted plasma samples.

![Figure 5](image1)

**Figure 5.** Relationship between values of IGF-I obtained in the radioimmunoassay of samples extracted by Sephadex G-75 (G75) or acid–ethanol (AE) extraction. (a) Adult or (b) fetal sheep blood plasma samples were extracted by G75 or AE. The linear regression for adult plasma samples was \( r = 0.96, P<0.001, y = -81.2 + 1.35x \). There was no significant correlation \( (P>0.05) \) for fetal sheep plasma samples. The antiserum was 878/4 and values are expressed in terms of the international reference preparation of IGF-I (batch 87/518).

In contrast, IGF-I values obtained after AEC extraction showed a highly significant correlation with IGF-I values after G75 extraction for both adult as well as fetal ovine plasma (Fig. 6). Furthermore, the slope of the regression line was close to unity and the paired comparison of plasma extracts analysed at different dilutions showed parallelism for both G75- and AEC-extracted samples (Table 3). Comparable results were obtained with three different polyclonal antisera (Tables 3 and 4). These results demonstrate that AEC and G75 extractions of fetal and adult ovine plasma give very similar results and are equally valid. The validity of the AEC extraction for blood plasma was demonstrated in a variety of species including fetal and adult sheep, rat, mouse and man (Table 4). The recoveries of unlabelled \( \gamma \)-rhIGF-I added to plasma before AEC extraction and assay (using antiserum 878/4) were 89.2±5.3% \( (n=5) \) for adult sheep, 88.3±9.5% \( (n=5) \) for fetal sheep, 87.6±13.0% \( (n=10) \) for mice, 92.1±8.9% \( (n=8) \) for rat and 84.6±7.0% \( (n=5) \) for man. These results are similar to recoveries of \( \gamma \)-rhIGF-I added to plasma prior to G75 extraction and assay which were 108.5±6.7% \( (n=4) \) for adult sheep serum.
TABLE 3. Correlation of IGF-I values from ovine blood plasma extracted by Sephadex G-75 (G75) or acid–ethanol cryo-precipitation (AEC) using antisera 863/5 and C549/804

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>100 µl</th>
<th>50 µl</th>
<th>Paired comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>863/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult (n = 10)</td>
<td>0.98</td>
<td>0.92</td>
<td>0.98</td>
</tr>
<tr>
<td>Fetal (n = 10)</td>
<td>0.92</td>
<td>0.92</td>
<td>1.02</td>
</tr>
<tr>
<td>C549/804</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult (n = 10)</td>
<td>0.96</td>
<td>0.95</td>
<td>0.98</td>
</tr>
<tr>
<td>Fetal (n = 9)</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Extracted samples were analysed at a twofold difference in concentration (100 µl and 50 µl extract). The correlation coefficient (r), the intercept (a) and the slope (b) of the regression were calculated on IGF-I values obtained in the assay after either extraction technique of the same plasma samples. The probability (P) of the difference between IGF-I values obtained for the same extract analysed at two different concentrations was calculated by paired comparison. A statistically significant difference indicates non-parallelism.

TABLE 4. Correlation of IGF-I values from blood plasma of various species extracted by Sephadex G-75 (G75) or acid–ethanol cryo-precipitation (AEC) using antisera 878/4

<table>
<thead>
<tr>
<th>Species</th>
<th>100 µl</th>
<th>50 µl</th>
<th>Paired comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult (n = 9)</td>
<td>0.98</td>
<td>0.89</td>
<td>0.99</td>
</tr>
<tr>
<td>Fetal (n = 13)</td>
<td>0.99</td>
<td>0.99</td>
<td>12.2</td>
</tr>
<tr>
<td>Man (n = 9)</td>
<td>0.99</td>
<td>0.99</td>
<td>-2.8</td>
</tr>
<tr>
<td>Rat (n = 8)</td>
<td>0.98</td>
<td>0.98</td>
<td>-15.5</td>
</tr>
<tr>
<td>Mouse (n = 10)</td>
<td>0.88</td>
<td>0.88</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Extracted samples were analysed at a twofold difference in concentration (100 µl and 50 µl extract). The correlation coefficient (r), the intercept (a) and the slope (b) of the regression were calculated on IGF-I values obtained in the assay after either extraction technique of the same plasma samples. The probability (P) of the difference between IGF-I values obtained for the same extract analysed at two different concentrations was calculated by paired comparison. A statistically significant difference indicates non-parallelism.

DISCUSSION

Increasing interest in the physiological role of IGF-I and extensive use of IGF-I RIAs for clinical diagnosis has led to the application of some incompletely characterized or validated assay systems. In this report we have described solutions to some problems and pitfalls commonly observed in RIAs for IGF-I. A range of high-affinity polyclonal antisera for IGF-I, including those raised in our laboratory and the widely used polyclonal antiserum distributed by the NIADDK, appear valid across a wide range of species provided that appropriate extraction methods to remove the influence of IGFBPs are employed.

The preparation of IGF-I used as radioligand or standard in the assay is of greater significance than generally appreciated because IGF-I preparations are not always homogeneous (Buell et al. 1985; Meng et al. 1988). Frequent contaminants in recombinant-derived materials are misfolded forms (Meng et al. 1988) although other variants have also been reported (Ballard, Francis, Ross et al. 1987; Gellersfors, Axelsson, Helander et al. 1989). These IGF-I variants show markedly reduced ability to bind to IGFBPs (Hodgkinson et al. 1986b) and can lead to an underestimate of the amount of residual IGFBP in plasma extracts if size separation chromatography or ligand blotting techniques are used for the assessment of IGFBPs (authors’ unpublished results). In addition, our experiments show that IGF-I variants may demonstrate markedly diverse potencies with different antisera. Thus, the choice of reference standard could explain the greatly divergent range of plasma concentrations of IGF-I reported between different laboratories. The use of highly purified preparations of correctly folded IGF-I in conjunction with high-affinity antisera of high specificity for authentic IGF-I could minimize these potential problems.

A number of strategies have been developed to reduce interference of IGFBPs in the RIA, all of which utilize the dissociation of the IGFBPs and IGF-I under acid conditions (Daughaday & Rotwein, Journal of Endocrinology (1991) 128, 347–357).
The most commonly used extraction methodologies are: acidification of plasma samples before assay (Underwood, D’Ercole, Copeland et al. 1982), C18-silicate minicolumn-based extraction (e.g. Sep-Pak extraction) (Silbergeld, Litwin, Bruchis et al. 1986; Daughaday et al. 1987; Wallis et al. 1987), acid-ethanol extraction (Daughaday et al. 1987), acidification of plasma samples and Sephadex G-50 or G-75 column chromatography (Zapf, Walter & Froesch, 1981; Daughaday et al. 1987). Each of these procedures has been shown to increase the immunopotency of serum in the IGF-I RIA. Gel chromatography at acid pH has generally been accepted to be the most reliable method for removing IGFBP interference in the RIA (Daughaday & Rotwein, 1989). However, in order to circumvent such time-consuming separation techniques, AE extraction was developed for adult human plasma (Daughaday et al. 1980). More recently, Sep-Pak extraction has also become widely used (Daughaday et al. 1987). However, we found Sep-Pak extraction to have high variation in recovery of labelled y-rhIGF-I added to plasma before extraction.

It is without doubt that AE extraction has proved of great value in facilitating many studies and furthering our knowledge of the physiology of IGF-I. The AE extraction has, however, been used in situations where full validation has not always been performed. This has led to debate over the validity of AE extractions (Holland, Hosner, Niswender et al. 1988; Mesiano et al. 1988; Daughaday & Rotwein, 1989). Indeed, we observed that AE extraction, although possibly acceptable for adult human or adult sheep plasma, was not valid for fetal sheep plasma. The explanation is likely to be the marked difference in the IGF-I/IGFBP ratio between adult and fetal plasma (Butler & Gluckman, 1986; Hodkinson et al. 1989a).

The major interference observed in fetal sheep plasma extracted by the standard AE method appears to relate in part to the observation that AE-extracted plasma samples contain, in addition to 30–65 kDa IGFBPs, large molecular weight aggregates which interfere in the RIA. These aggregates lead to non-parallelism of displacement curves for AE-extracted plasma in comparison with the IGF-I standard. Our observation that these aggregates cryo-precipitate led to the development of the AEC method.

Since all extraction methodologies investigated in this report, including G75 extraction, leave some residual IGFBPs in the extract we assessed the degree of interference of IGFBP-1 as well as soluble IGFBP fractions obtained from fetal sheep plasma after acidification and size separation on Sephadex G-75. Our data show that IGFBP-1 does not interfere in the assay (using high-affinity antisera) unless concentrations markedly in excess of those commonly observed in plasma (Povoa et al. 1984; Cotterill et al. 1988) are present. Further, the residual IGFBP present in both G75- and AEC-extracted plasma did not interfere in the RIA employing a range of high-affinity polyclonal antibodies.

In summary we describe a cryo-precipitation step as a simple addition to AE extraction to eliminate aggregated proteins in plasma extracts. Detailed evaluation of AEC-extracted plasma shows that IGFBPs are also reduced and that the AEC-extracted plasma displaces completely parallel to y-rhIGF-I, rh-met-IGF-I, IRR IGF-I, oIGF-I and G75-extracted plasma. Furthermore, the high correlation with results obtained using G75 extraction demonstrates that AEC extraction gives valid estimates of IGF-I for blood plasma from a large number of species including sheep, rat, mouse and man. Finally, it should be noted that caution is necessary in extrapolating this approach to other biological fluids where the properties of the IGFBPs may be quite different (Breier, Gallaher, Gibson et al. 1990).

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Foundation of New Zealand, the Child Health Research Foundation and the Auckland Medical Research Foundation. The authors wish to thank Drs D. B. Burleigh, K. Mueller, W. Maerky and L. Moore, the National Institute for Biological Standards and Control for the provision of IGF-I preparations and Professor K. Hall for the donation of IGFBP-1. We also thank Drs L. E. Underwood and J. J. Van Wyk and the NIADDK for the provision of IGF-I antiserum.

REFERENCES


