

EXPERIMENTAL STUDY

O-glycosylation delays the clearance of human IGF-binding protein-6 from the circulation

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Abstract

Objective: The actions of insulin-like growth factors (IGF-I and IGF-II) are modulated by a family of six structurally related, high-affinity binding proteins (IGFBPs 1–6). IGFBP-6, an O-linked glycoprotein, preferentially binds IGF-II and inhibits its actions. The aim of this study was to investigate whether O-glycosylation modulates the pharmacokinetics of IGFBP-6.

Design and Methods: The pharmacokinetic profiles of ^{125}I -labelled glycosylated (g) and non-glycosylated (n-g) recombinant human IGFBP-6 were studied following intravenous bolus administration in anaesthetised rats.

Results: The redistribution half-life of gIGFBP-6 was 2.3-fold greater than that of n-gIGFBP-6 (14.4 ± 1.2 vs 6.3 ± 1.5 min, $P = 0.006$). The elimination half-life of gIGFBP-6 was 21-fold greater than that of n-gIGFBP-6 (584.2 ± 130.2 vs 28.0 ± 4.2 min, $P = 0.019$). The effect of O-glycosylation on IGFBP-6 pharmacokinetics was not due to inhibition of intravascular proteolysis. Radioactivity was found in stomach, kidneys, lung, spleen, heart and liver but not brain 4 h after injection of g or n-gIGFBP-6.

Conclusions: O-glycosylation delays the clearance of IGFBP-6 from the circulation and may therefore contribute to its role as a circulating inhibitor of IGF-II actions.

European Journal of Endocrinology 142 512–516

Introduction

Insulin-like growth factors (IGF-I and IGF-II) are important mediators of physiological growth (1). Circulating and tissue IGFs are largely bound to a family of six structurally related high-affinity binding proteins (IGFBPs 1–6) (1, 2). IGFBPs are synthesised by most cell types, and may inhibit or potentiate IGF actions.

Within the circulation, about 75% of IGFs are bound in a 150 kDa ternary complex containing IGFBP-3 or -5 and an acid labile subunit (3, 4). Most remaining IGFs are bound to other IGFBPs, including IGFBP-6, in a 50 kDa binary complex whereas levels of free IGFs are very low (5). IGFs in ternary complexes are retained in the circulation (6), whereas IGFs in binary complexes can cross the capillary endothelial barrier. Whereas the circulating half-lives of free IGFs are ~15 min, this increases to 30–50 min in binary complexes, and 12–15 h in ternary complexes (6). The ternary complex therefore acts as a reservoir of circulating IGFs, whereas IGFBPs in binary complexes may assist in transport and targeting of IGFs to tissues (5).

IGFBP-6 binds IGF-II with higher affinity than other IGFBPs and has a distinctive 20- to 100-fold preference for IGF-II over IGF-I. IGFBP-6 is a relatively specific inhibitor of IGF-II actions (7). Expression of IGFBP-6 is associated with non-proliferative states (7)

and is induced by differentiating agents such as retinoic acid (8). These properties suggest that the main role of IGFBP-6 is to inhibit IGF-II-induced proliferation, which may be very important given that IGF-II has been implicated as a tumour growth factor (9). IGFBP-6 is found in human serum at a concentration of ~10 nM, which is similar to that of IGFBP-2 and which exceeds that of IGFBP-1 (5). By contrast, circulating IGFBP-3 levels are ~100 nM (5).

Human IGFBP-6 is O-glycosylated on five Ser/Thr residues within the non-conserved mid region of its amino acid sequence (10, 11). The role of O-glycosylation of IGFBP-6 has not been completely defined. Glycosylation increases the circulating half-life of a number of glycoproteins such as erythropoietin (12), tissue plasminogen activator (13) and human luteinizing hormone (14). We therefore investigated the effect of O-glycosylation on the pharmacokinetics of IGFBP-6 in the rat.

Materials and methods

IGFBP-6

Recombinant human glycosylated IGFBP-6 (gIGFBP-6) was expressed and purified from Chinese hamster ovary

(CHO) cells as described previously (11). The IGFBP-6 used for experiments was >99% pure as determined by amino-terminal Edman sequencing.

Recombinant human non-glycosylated IGFBP-6 (n-IGFBP-6) was expressed and purified from *E. coli* as previously described (11). n-IGFBP-6 had the same high-affinity IGF-binding characteristics as gIGFBP-6 (15). Further, the stoichiometrics of IGF binding to gIGFBP-6 and n-IGFBP-6 were similar (15). These observations indicate that n-IGFBP-6 was correctly folded, since correct folding is likely to be required for high-affinity binding.

Iodination of IGFBP-6

Both gIGFBP-6 and n-IGFBP-6 (2 µg) were iodinated to specific activities of 20–40 µCi/µg using chloramine-T. Iodinated IGFBP-6 was separated from free Na¹²⁵I by gel filtration using a P6-DG desalting column (Biorad, Richmond, CA, USA).

Pharmacokinetic studies

Male Sprague–Dawley rats ($n=8$, ~300 g) were anaesthetised with sodium brietal (4 mg/kg). Temperature was maintained with a heating pad at 37°C. Both the carotid artery and jugular vein were cannulated using polyethylene tubing (internal diameter 0.58 mm) with 23 gauge needles attached. Animals were allowed to recover for 30 min. Experiments were approved by the Austin and Repatriation Medical Centre Animal Welfare Committee.

Once the animals were cannulated, experiments were performed under anaesthesia. Pharmacokinetics were also assessed once in conscious rats with similar results (not shown). Iodinated IGFBP-6 (3×10^6 c.p.m.) was administered through the jugular vein cannula in a volume of 150 µl heparin saline containing 1% bovine serum albumin. Blood (150 µl) was periodically collected from the carotid artery after 1–180 min. After each blood sample was taken, an equivalent volume of heparin saline was injected to maintain plasma volume. Samples were stored on ice for at least 10 min prior to centrifugation at 3500 *g* for 10 min at 4°C. Sera (100 µl) were collected and precipitated with trichloroacetic acid (final concentration 15%), and radioactivity in the pellet was counted in a γ-counter. In one experiment, radioactivity in serum was counted. At the end of the study, rats were killed by injection of sodium pentobarbital, and brain, heart, kidney, liver, lungs, spleen and stomach were removed and washed in phosphate-buffered saline, weighed and radioactivity was counted as above.

To determine whether intravascular proteolysis contributed to clearance of IGFBP-6, serum samples were separated by sodium dodecyl sulfate-15%-polyacrylamide gel electrophoresis (SDS-15%-PAGE) followed by gel fixation and exposure to X-ray film.

Data obtained for gIGFBP-6 and n-IGFBP-6 were analysed using Prism (GraphPad Software Inc, San Diego, CA, USA). The calculated second-order kinetic curves for both gIGFBP-6 and n-IGFBP-6 fitted the acquired data with r values of ~0.99. Elimination and redistribution half-lives were calculated from these curves. The apparent volume of distribution at steady state (V_{ss}) was calculated using the following equation: $V_{ss} = (\text{Dose} \cdot \text{AUMC}) / (\text{AUC})^2$ where AUC and AUMC are the area under the concentration–time curve extrapolated to infinity and the area under the first moment curve respectively.

Statistics

Results are shown as means ± s.e.m. Pharmacokinetic parameters of gIGFBP-6 and n-IGFBP-6 were compared using unpaired *t*-tests.

Results

O-glycosylation prolongs the circulating half-life of IGFBP-6

The disappearance curves of ¹²⁵I-labelled gIGFBP-6 and n-IGFBP-6 from serum following intravenous administration into rats were indicative of a two-compartment model (Fig. 1). n-IGFBP-6 was cleared more rapidly from serum than gIGFBP-6 in both the redistribution and elimination phases. The redistribution half-life ($t_{1/2\alpha}$) of gIGFBP-6 was 2.3-fold greater than that of n-IGFBP-6 (Table 1, $P=0.006$), whereas the elimination half-life ($t_{1/2\beta}$) was 21-fold greater ($P=0.019$). The volumes of distribution of gIGFBP-6 and n-IGFBP-6 were both ~250 ml/kg (Table 1, $P=0.88$), consistent

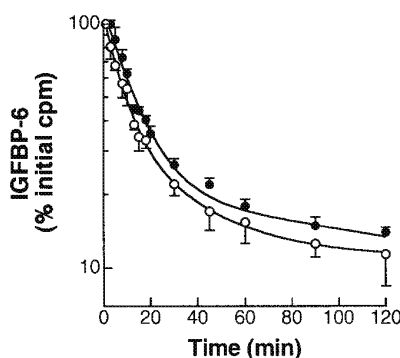


Figure 1 O-glycosylation prolongs the circulating half-life of IGFBP-6. Following intravenous administration of ¹²⁵I-labelled gIGFBP-6 (●) and n-IGFBP-6 (○), serum was removed at the indicated time-points and radioactivity counted. Results are shown as means ± s.e.m. of experiments performed with five and three rats for gIGFBP-6 and n-IGFBP-6 respectively.

Table 1 Pharmacokinetics of ^{125}I -labelled glycosylated and non-glycosylated IGFBP-6. Values are means $\pm$ S.E.M.

	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	V_{ss} (ml/kg)
Glycosylated IGFBP-6 ($n = 5$)	14.4 ± 1.2	584.2 ± 130.2	240.2 ± 30.6
Non-glycosylated IGFBP-6 ($n = 3$)	6.3 ± 1.5	28.0 ± 4.2	250.6 ± 60.7
<i>P</i> value (glycosylated vs non-glycosylated)	0.006	0.019	0.88

$t_{1/2\alpha}$, redistribution half-life; $t_{1/2\beta}$, elimination half-life; V_{ss} , volume of distribution.

with both forms of IGFBP-6 being distributed throughout the extracellular space.

SDS-PAGE and autoradiography of serum samples indicated that iodinated gIGFBP-6 and n-gIGFBP-6 remained intact, since fragments were not detected (Fig. 2). Intravascular proteolysis therefore does not underlie the faster clearance of n-gIGFBP-6.

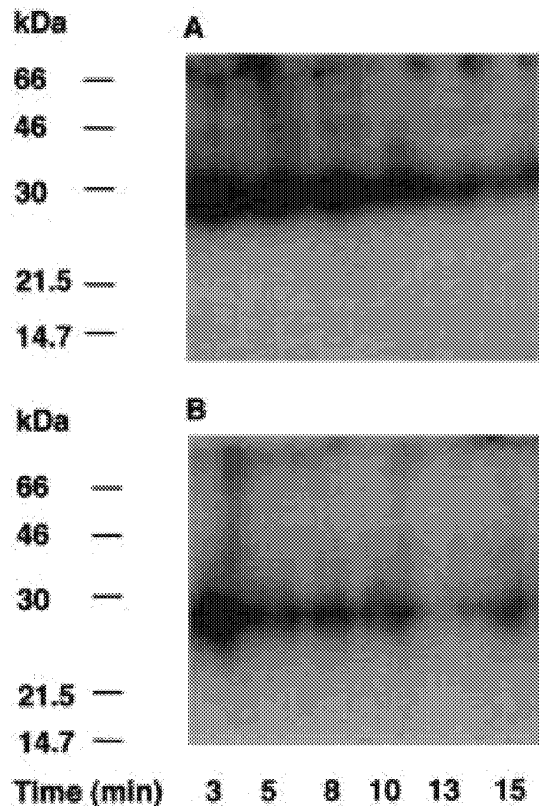


Figure 2 IGFBP-6 is not degraded in the circulation. Following intravenous administration of ^{125}I -labelled gIGFBP-6 (A) and n-gIGFBP-6 (B), serum was removed at the indicated time-points and analysed by SDS-15%-PAGE and autoradiography.

Localisation of extravascular [^{125}I]IGFBP-6 in the rat

The organ distribution of radioactivity was studied 4 h after intravenous administration of ^{125}I -labelled gIGFBP-6 and n-gIGFBP-6. The organ with the greatest radioactivity was stomach, which contained $59 \pm 6\%$ and $35 \pm 4\%$ of iodinated gIGFBP-6 ($n = 3$) and n-gIGFBP-6 ($n = 2$) respectively (Fig. 3, $P = 0.07$ gIGFBP-6 vs n-gIGFBP-6). Other organs with substantial radioactivity were kidney and lungs $>$ spleen $>$ heart $>$ liver. The distributions of gIGFBP-6 and n-gIGFBP-6 were similar except that n-gIGFBP-6 appeared to be localised to a greater extent in the lungs ($18 \pm 5\%$ vs $6 \pm 1\%$, $P = 0.05$). Neither gIGFBP-6 nor n-gIGFBP-6 crossed the blood-brain barrier as demonstrated by the absence of radioactivity in the brain.

Discussion

In this study, we have shown that O-glycosylation prolongs the redistribution and elimination half-lives of IGFBP-6, which is consistent with the effects of glycosylation of other proteins (16). This effect may be mediated by a number of mechanisms. The mass contributed by glycans increases the circulating half-lives of glycoprotein hormones (17) and the molecular

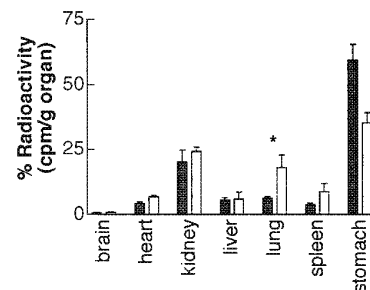


Figure 3 Organ distribution of intravenously administered [^{125}I]IGFBP-6. The distribution of ^{125}I -labelled gIGFBP-6 (shaded bars) and n-gIGFBP-6 (open bars) in rat tissues was measured by γ -counting of specific organs 4 h after intravenous administration. Results are shown as a percentage of total organ radioactivity corrected for organ weight. Results are shown as mean $\pm$ S.E.M. values for two to three separate experiments. * $P < 0.05$, glycosylated vs non-glycosylated.

weight of gIGFBP-6 is 10–20% greater than that of n-gIGFBP-6 (10, 11). Since IGFBP-6 has five closely spaced O-glycosylation sites (11), it is possible that this region adopts an extended, relatively rigid conformation (18) which may result in an increase in molecular size which is disproportionately large relative to the increase in molecular mass. This increased size may in turn result in delayed clearance from the circulation.

Another major factor responsible for the extended elimination half-life of glycoproteins is the presence of sialic acids on glycans, which inhibits binding to asialoglycoprotein clearance receptors (19). For example, desialylated erythropoietin (13, 20) and tissue plasminogen activator variants with decreased sialic acid content (21) have shorter half-lives than the equivalent fully sialylated glycoproteins. Sialic acids are components of the O-linked oligosaccharides of IGFBP-6 (10, 11) and may therefore contribute to the findings of the present study, although studies of asialoIGFBP-6 would be necessary to demonstrate this conclusively.

Tissue factor pathway inhibitor adheres to glycosaminoglycans on vessel walls and this is thought to be responsible for its relatively short half-life by aiding its redistribution to specific organs (22). This may also account for the shorter redistribution half-life of n-gIGFBP-6 than gIGFBP-6, since n-gIGFBP-6 binds to glycosaminoglycans to a greater extent than gIGFBP-6 (J. Marinaro, V. Russo & L. Bach, unpublished observations).

The above effects are explained by overall levels of glycosylation. Alternatively, individual glycans may have specific effects on pharmacokinetics (23), although demonstration of this would require more detailed studies of mutants in which specific O-glycosylation sites are altered.

Glycosylated IGFBP-6 used in this study was a recombinant protein purified from CHO cells (11). Glycoproteins may be differentially glycosylated by cells of different origins. The carbohydrate content of CHO-derived IGFBP-6 has been studied extensively (11), and hexoses, N-acetylhexosamines and sialic acids identified. Based on analytical studies and migration on SDS-PAGE, recombinant IGFBP-6 from this source has a similar carbohydrate content to IGFBP-6 purified from human cerebrospinal fluid (10, 11), although minor differences cannot be excluded. The pharmacokinetic properties of glycosylated IGFBP-6 in the present study are therefore likely to reflect those of native IGFBP-6.

The pharmacokinetics of IGFBP-1 and IGFBP-2 have been previously studied (24). Their redistribution half-lives were ~8 and ~14 min respectively, which are similar to those of n-g and gIGFBP-6 in the present study. The elimination half-lives of IGFBP-1 and IGFBP-2 were ~120 and ~144 min respectively, which are substantially shorter than that of gIGFBP-6. Although steady-state concentrations of IGFBP-6 are

relatively low compared with those of IGFBP-3 (5), its relatively long elimination half-life together with its IGF-II binding preference may result in its having a significant role in terms of IGF-II physiology. The rate of IGF-II synthesis is similar to IGF-I in man, yet serum levels of IGF-II are three to four times higher, due to free IGF-II levels being tenfold lower than those of free IGF-I (6). IGFBP-6 may therefore have a role in maintaining circulating IGF-II levels.

In contrast to the effects of O-glycosylation on the pharmacokinetics of IGFBP-6, N-glycosylation has no effect on the clearance of IGFBP-3 from the circulation (25). This may be because ternary complex formation is the major determinant of IGFBP-3 pharmacokinetics and binding of IGFBP-3:IGF complexes to the acid-labile subunit to form ternary complexes is unaffected by glycosylation (26).

The volumes of distribution of both gIGFBP-6 and n-gIGFBP-6 were similar to those of IGFBP-1 and IGFBP-2 (24), and indicate that IGFBP-6 is distributed throughout the vascular and extracellular compartments. This result is not surprising as binary IGFBP:IGF complexes are not restricted by endothelial barriers. In fact, IGFBPs may have a role in the specific targeting of IGFs to specific tissues (27–29). The organ distribution of ¹²⁵I-labelled IGFBP-3 (30) and IGFBP-6 were similar, with both IGFBPs being predominantly localised to the stomach and kidneys. However, it was not determined in either study whether the radioactivity present in the stomach was due to intact tracer or free iodine. High levels of radioactivity in the stomach may be a result of iodine deposition by the gastrointestinal iodide cycle subsequent to deiodination (31).

In summary, most previous studies of IGFBP-6 have emphasised its properties at the cellular level (7). However, IGFBP-6 may also have an important role in the regulation of circulating IGF-II because of its relatively long half-life due to O-linked glycosylation. O-glycosylation may therefore contribute to maintaining IGFBP-6 as a potent, high-affinity, circulating inhibitor of IGF-II actions.

Acknowledgements

This work was supported by grants from the Austin Hospital Medical Research Foundation and the Sir Edward Dunlop Medical Research Foundation. The authors would like to thank Nicole Farina for assistance with animal handling and Dr Maurice Fabiani for assistance in interpretation of pharmacokinetic data.

References

- 1 Jones JJ & Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* 1995 16 3–34.
- 2 Bach LA & Rechler MM. Insulin-like growth factor binding proteins. *Diabetes Reviews* 1995 3 38–61.

- 3 Baxter RC, Martin JL & Beniac VA. High molecular weight insulin-like growth factor binding protein complex. *Journal of Biological Chemistry* 1989 **264** 11843–11848.
- 4 Twigg SM & Baxter RC. Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. *Journal of Biological Chemistry* 1998 **273** 6074–6079.
- 5 Rajaram S, Baylink DJ & Mohan S. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocrine Reviews* 1997 **18** 801–831.
- 6 Guler HP, Zapf J, Schmid C & Froesch ER. Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinologica* 1989 **121** 753–758.
- 7 Bach LA. Insulin-like growth factor binding protein-6: the 'forgotten' binding protein? *Hormone and Metabolic Research* 1999 **31** 226–234.
- 8 Gabbits B & Canalis E. Retinoic acid stimulates the transcription of insulin-like growth factor binding protein-6 in skeletal cells. *Journal of Cell Physiology* 1996 **169** 15–22.
- 9 Toretzky JA & Helman LJ. Involvement of IGF-II in human cancer. *Journal of Endocrinology* 1996 **149** 367–372.
- 10 Bach LA, Thotakura NR & Rechler MM. Human insulin-like growth factor binding protein-6 is O-glycosylated. *Biochemical and Biophysical Research Communications* 1992 **186** 301–307.
- 11 Neumann GM, Marinaro JA & Bach LA. Identification of O-glycosylation sites and partial characterization of carbohydrate structure and disulfide linkages of human insulin-like growth factor binding protein-6. *Biochemistry* 1998 **37** 6572–6585.
- 12 Cole ES, Nichols EH, Poisson L, Harnois ML & Livingston DJ. *In vivo* clearance of tissue plasminogen activator: the complex role of sites of glycosylation and level of sialylation. *Fibrinolysis* 1993 **7** 15–22.
- 13 Briggs DW, Fisher JW & George WJ. Hepatic clearance of intact and desialylated erythropoietin. *American Journal of Physiology* 1974 **227** 1385–1388.
- 14 Burgon PG, Stanton PG & Robertson DM. *In vivo* bioactivities and clearance patterns of highly purified human luteinizing hormone isoforms. *Endocrinology* 1996 **137** 4827–4836.
- 15 Marinaro JA, Jamieson GP, Hogarth PM & Bach LA. Differential dissociation kinetics explain the binding preference of insulin-like growth factor binding protein-6 for insulin-like growth factor-II over insulin-like growth factor-I. *FEBS Letters* 1999 **450** 240–244.
- 16 Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 1993 **3** 97–130.
- 17 Baenziger JU. Glycosylation: to what end for the glycoprotein hormones? *Endocrinology* 1996 **137** 1520–1522.
- 18 Jentoft N. Why are proteins O-glycosylated? *Trends in Biochemical Sciences* 1990 **15** 291–294.
- 19 Morell AG, Gregoriadis G, Scheinberg IH, Hickman J & Ashwell G. The role of sialic acid in determining the survival of glycoproteins in the circulation. *Journal of Biological Chemistry* 1971 **246** 1461–1467.
- 20 Wasley LC, Timony G, Murtha P, Stoudemire J, Dorner AJ, Caro J *et al.* The importance of N- and O-linked oligosaccharides for the biosynthesis and *in vitro* and *in vivo* biologic activities of erythropoietin. *Blood* 1991 **77** 2624–2632.
- 21 Larsen, GR, Metzger M, Henson K, Blue Y & Horgan P. Pharmacokinetics and distribution analysis of variant forms of tissue-type plasminogen activator with prolonged clearance in rat. *Blood* 1989 **73** 1842–1850.
- 22 Holst J, Lindblad B, Westerlund G, Bregengaard C, Ezban M, Ostergaard PB *et al.* Pharmacokinetics and delayed experimental anti-thrombotic effect of two domain non-glycosylated tissue factor pathway inhibitor. *Thrombosis Research* 1996 **81** 461–470.
- 23 Bishop LA, Nguyen TV & Schofield PR. Both of the beta-subunit carbohydrate residues of follicle-stimulating hormone determine the metabolic clearance rate and *in vivo* potency. *Endocrinology* 1995 **136** 2635–2640.
- 24 Young SC, Miles MV & Clemmons DR. Determination of the pharmacokinetic profiles of insulin-like growth factor binding proteins-1 and -2 in rats. *Endocrinology* 1992 **131** 1867–1873.
- 25 Sommer A, Spratt SK, Tatsuno GP, Tressel T, Lee R & Maack CA. Properties of glycosylated and non-glycosylated human recombinant IGF binding protein-3 (IGFBP-3). *Growth Regulation* 1993 **3** 46–49.
- 26 Firth SM & Baxter RC. The role of glycosylation in the action of IGFBP-3. *Progress in Growth Factor Research* 1995 **6** 223–229.
- 27 Bar RS, Clemmons DR, Boes M, Busby WH Jr, Booth BA, Dake BL *et al.* Transcapillary permeability and subendothelial distribution of endothelial and amniotic fluid insulin-like growth factor binding proteins in the rat heart. *Endocrinology* 1990 **127** 1078–1086.
- 28 Boes M, Booth BA, Sandra A, Dake BL, Bergold A & Bar RS. Insulin-like growth factor binding protein (IGFBP)4 accounts for the connective tissue distribution of endothelial cell IGFBPs perfused through the isolated heart. *Endocrinology* 1992 **131** 327–330.
- 29 Sandra A, Boes M, Dake BL, Stokes JB & Bar RS. Infused IGF-I/IGFBP-3 complex causes glomerular localization of IGF-I in the rat kidney. *American Journal of Physiology* 1998 **275** E32–E37.
- 30 Arany E, Zabel P, Freeman D & Hill DJ. Elimination of radiolabelled recombinant human insulin-like growth factor binding protein-3 from the circulation, and its distribution amongst organs and tissues in adult male rats. *Regulatory Peptides* 1993 **48** 133–143.
- 31 Regoeczi E. *Iodine Labeled Plasma Proteins*. Boca Raton, FL, USA: CRC Press, 1987.

Received 2 September 1999

Accepted 3 December 1999