

V. Agon, C.J. Howe, R. Kazlauskas, G.J. Trout

Aspects of the detection of insulin abuse in sport

Australian Sports Drug Testing Laboratory, National Measurement Institute, Sydney,
Australia

Abstract

Mass spectrometry-based approaches to the detection and quantitation of exogenous insulin in antidoping samples have been previously developed. However their cost means that application to a large number of samples is difficult to achieve.

Insulin secreted by the pancreatic beta cells is initially produced as proinsulin which is cleaved to produce the alpha and beta chains of the insulin molecule. The remaining centre portion of the proinsulin sequence forms C-peptide. The presence of insulin in the absence of C-peptide or proinsulin therefore suggests that the insulin is of exogenous origin. Well characterised relatively inexpensive immunoassays are available for all three analytes. This offers the prospect of a ratio or multivariate index to distinguish exogenous from endogenous insulin.

A study has been carried out using normal subjects to establish reference ranges. In addition administration trials of insulin have been carried out using both diabetic and non-diabetic subjects.

Introduction

This paper presents some preliminary results of a study designed to determine whether it was possible to develop a test which could detect the abuse of human insulin in a sports doping context. Insulin is on the World Anti-Doping Agency (WADA) List of prohibited substances (WADA 2008) and has been prohibited for some years. Whilst tests using liquid chromatography mass spectrometry (LC/MS) have been described for synthetic insulins (Thevis et al 2006), they are expensive to implement and are not capable of detecting the abuse of human sequence insulin. Endogenous insulin is produced initially as proinsulin which cleaves to produce insulin and C-peptide. Normally all three species can be detected in the blood stream using relatively inexpensive immunoassays (Clark 1999). If insulin is injected it would be expected that, shortly after the injection, the insulin level would have

increased whilst the C-peptide and proinsulin levels would not and in fact may fall. Thus measuring the insulin to C-peptide ratio or insulin to proinsulin ratio could form the basis of a test for detecting the abuse of insulin. The study was set up as follows

- establish reference ranges for assays for proinsulin, insulin, and C-peptide in serum;
- carry out administration trials of insulin to diabetic and non-diabetic subjects; and
- carry out a glucose tolerance test on the non-diabetic subjects.

Materials and Methods

The insulin administration was carried out after approval by the Human Research Ethics Committee of the Sydney South West Area Health Service (Approval Number: CH62/6/2006-066-P Liu).

The blood glucose levels were measured using an ACCU-CHEK® Advantage meter from Roche Australia

The assays used were: for Insulin - DPC Immulite, for C-peptide - DPC Immulite, and for Proinsulin - Mercodia Proinsulin ELISA.

The neutral human insulin solution used for injection was Actrapid® (Novo Nordisk Pharmaceuticals, Australia).

Reference ranges for insulin, proinsulin and C-peptide were established for the assays by measuring the values found in serum collected from over 300 subjects.

The administration study was designed to determine the effects of an insulin injection on glucose, insulin, proinsulin and C-peptide levels in normal and diabetic subjects. The normal subjects (three male and three female) were given a bolus intravenous injection of 0.5 IU/kg Actrapid® after fasting overnight. Blood samples were just taken prior to the insulin injection and at 15, 30, 45, 60, 120, 180, and 240 minutes after the injection. An additional sample was collected on the morning of the following day. The normal subjects underwent a similar protocol three times separated by at least one week. On one visit they were given a placebo injection of saline and on the other there was no injection but rather a dose of 75 g of oral glucose. Diabetic subjects underwent a similar collection protocol but self injected their normal insulin dose after nine hours fasting and then had their normal meal.

Results and Discussion

The values found for the insulin and C-peptide concentrations in normal subjects were highly variable. The values found for the insulin to C-peptide ratio were somewhat more consistent with no subject giving a value greater than 0.24. (Figure 1) Accordingly a value of 0.25 for the insulin to C-peptide ratio was chosen as the upper limit of normal values for this preliminary study.

The results from the blood glucose measurements from five administration subjects are shown in Figure 2. The solid lines are the blood glucose concentrations measured after the intravenous insulin administration whilst the dotted lines are the values obtained from the 75 g oral glucose administration. As expected the insulin injection induced hypoglycemia with all subjects recording at least one value below 3.5 mmol/L. The serum insulin levels rose immediately after the Actrapid® injection but the elevation observed was different for each subject. Figure 3 shows the serum insulin concentrations for two of the subjects. It was anticipated that natural insulin production would be suppressed by the injection and this was found with both proinsulin and C-peptide concentrations falling after the injection. The net effect was to increase the insulin to C-peptide ratio to values markedly above those found in normal subjects (Figure 4).

The oral glucose administration was given to determine what effect the rapid release of large amounts of natural insulin might have on the insulin to C-peptide ratio. Whilst insulin and C-peptide are produced in equimolar amounts by the pancreas their removal rates are different which explains why their serum concentrations are not the same. Figure 5 shows the measured insulin concentrations for one subject on each of the three visits. It can be seen that the oral glucose produced much greater concentrations of serum insulin than did the Actrapid® injection. This rapid rise in insulin concentration altered the insulin to C-peptide ratio but did not exceed the upper limit of 0.25 set by the normal subjects whereas the Actrapid® injection clearly did (Figure 6). Thus the measurement of both serum insulin and serum C-peptide has the potential to detect the abuse of insulin by normal subjects

At the time of writing this report five subjects had completed the study and the results obtained for the insulin to C-peptide measurements are shown in Figure 7. The ratio exceeded 0.25 for all but one of the subjects. As expected for a bolus intravenous injection the effect was short lived with all subjects returning to normal levels within two hours. For this trial the ethics committee required the insulin to be injected intravenously because of its short effective duration. However insulin is normally given subcutaneously where the initial

effect will be less than for an intravenous injection but the duration of the effect will be longer. The ethics approval for this trial did not permit subcutaneous injection of the normal subjects however one of the diabetic patients did inject 10 IU of Actrapid® subcutaneously as part of their normal regime. As expected his insulin to C-peptide ratio was always high being above 0.6 for the entire four hour session. In order to obtain some idea of how a normal subject might behave if given such an injection the serum insulin values for this diabetic subject were taken and divided by the mean, lowest, and highest C-peptide concentrations recorded at 30 minutes for the normal subjects. The assumption being made is that a larger dose of insulin (10 IU) given subcutaneously will have a similar suppressing effect on C-peptide and proinsulin as did the 4 IU dose given intravenously but that the suppression will last longer. The results are shown in Figure 8 where the indications are that a 10 IU subcutaneous dose of Actrapid® will result in elevated insulin to C-peptide ratios for some hours. However this ratio is likely to be rapidly lowered if natural insulin production is stimulated by the consumption of carbohydrates.

Conclusions

- The intravenous injection of insulin to fasting non-diabetic subjects causes a depression of blood glucose, a rise in serum insulin, and a fall in C-peptide and proinsulin.
- The ratio of insulin to C-peptide in the serum can rise to levels considerably above those found in a normal population.
- Such measurements could be used as the basis of a test for doping with insulin however the effect will only last for the duration of the elevated serum insulin from the injection.

Acknowledgements

- We thank Dr Peter Liu and Ms Amanda Idan of the ANZAC Institute for their help in carrying out the administration studies.
- This project was supported by the Australian Government through the Anti-Doping Research Program of the Department of Communications, Information Technology and the Arts.

References

World Anti-Doping Agency. The 2008 Prohibited List. International Standard, Montreal (2008) http://www.wada-ama.org/rtecontent/document/2008_List_En.pdf (access date 25.04.2008)

Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W. (2006) Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 78(6), 1897-1903.

Clark P M, (1999) Assays for Insulin, proinsulins(s) and C-peptide. *Ann. Clin. Biochem.* 36, 541-564.

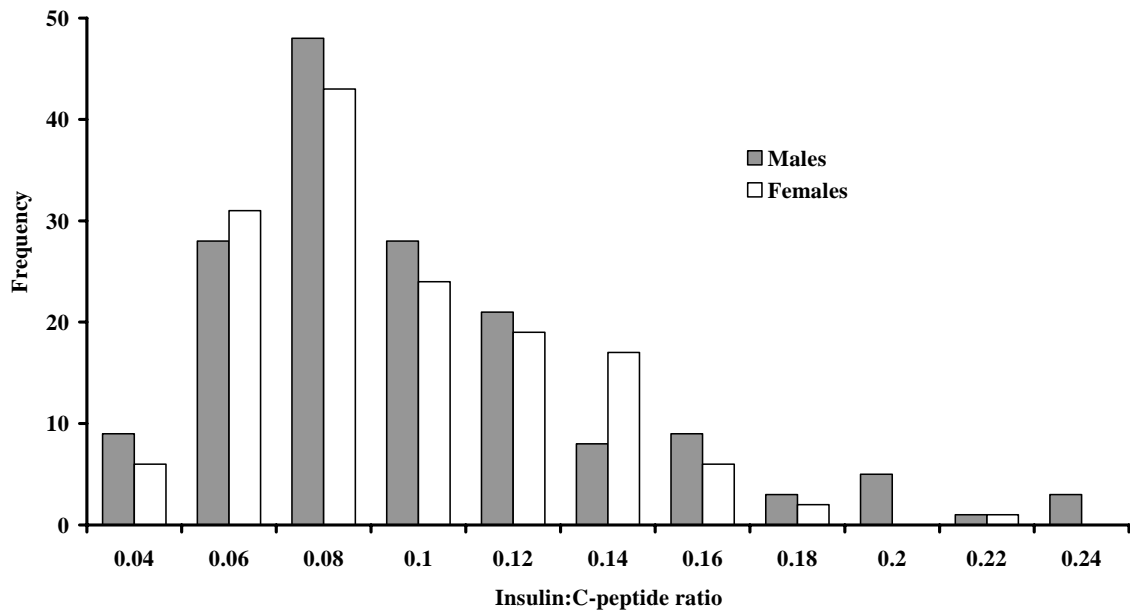


Figure 1. Insulin to C-peptide ratio found for a range of normal subjects (162 males and 149 females).

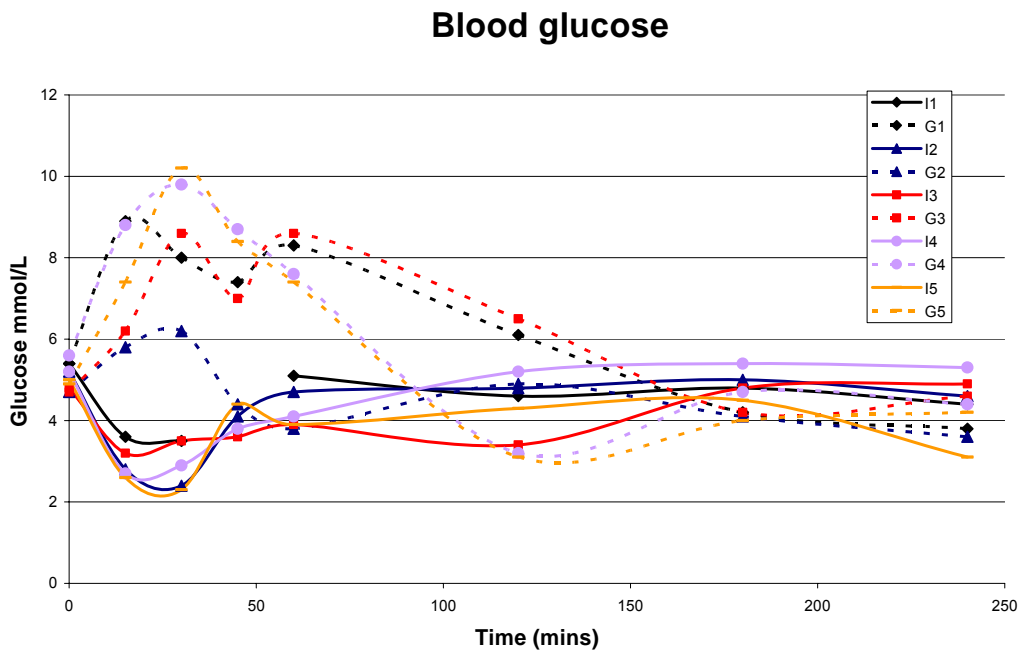


Figure 2. Blood glucose levels for 5 subjects after intravenous Actrapid® injection (I 1 to 5) and after oral glucose (G 1 to 5)

Insulin in serum

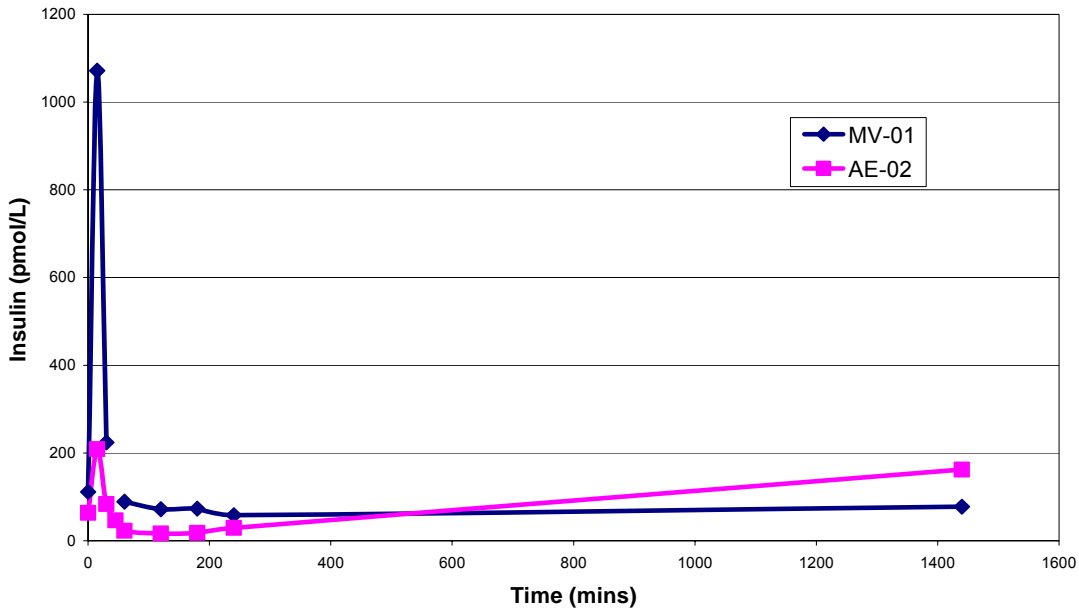


Figure 3. Serum insulin concentrations recorded for two non-diabetic subjects given a bolus intravenous injection of Actrapid® at time 0 minutes.

Ratios of Insulin to C-peptide

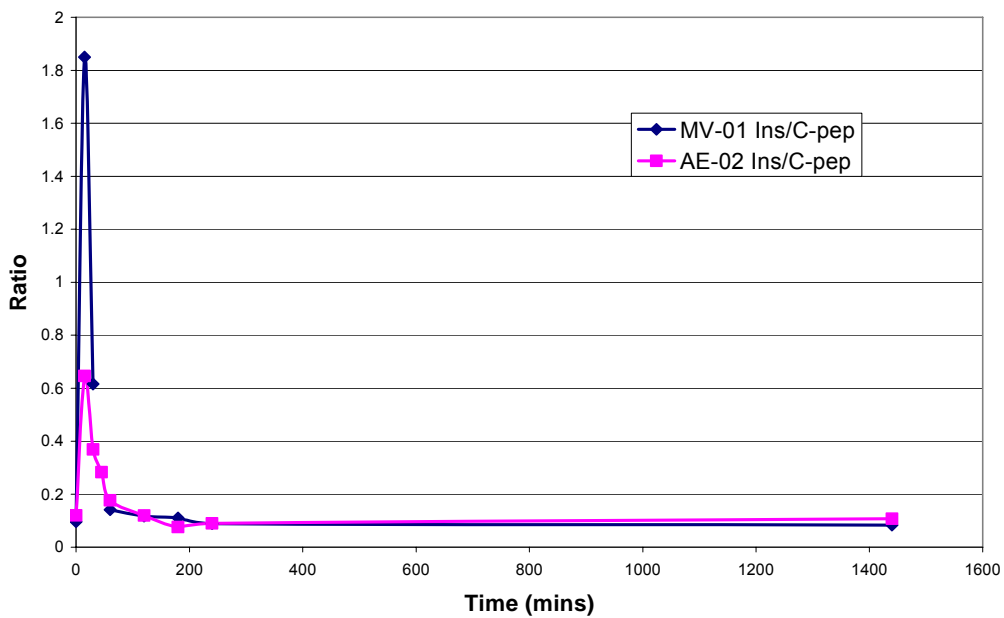


Figure 4. Ratio of insulin to C-peptide concentrations for two subjects given an intravenous injection of Actrapid® at time 0 minutes.

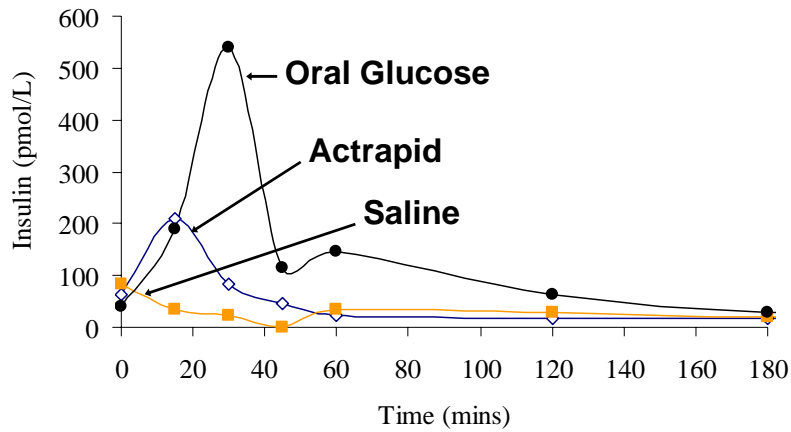


Figure 5. The effects of an Actrapid® injection, a saline injection, and the ingestion of oral glucose on serum insulin concentrations in a single subject on three separate occasions.

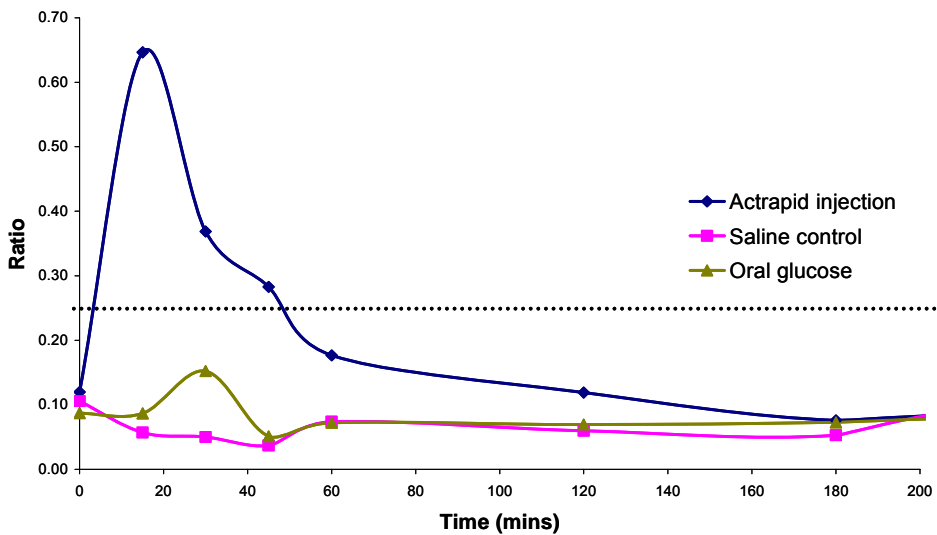


Figure 6. The effects of an Actrapid® injection, a saline injection, and the ingestion of oral glucose on the ratio of serum insulin to C-peptide in a single subject.

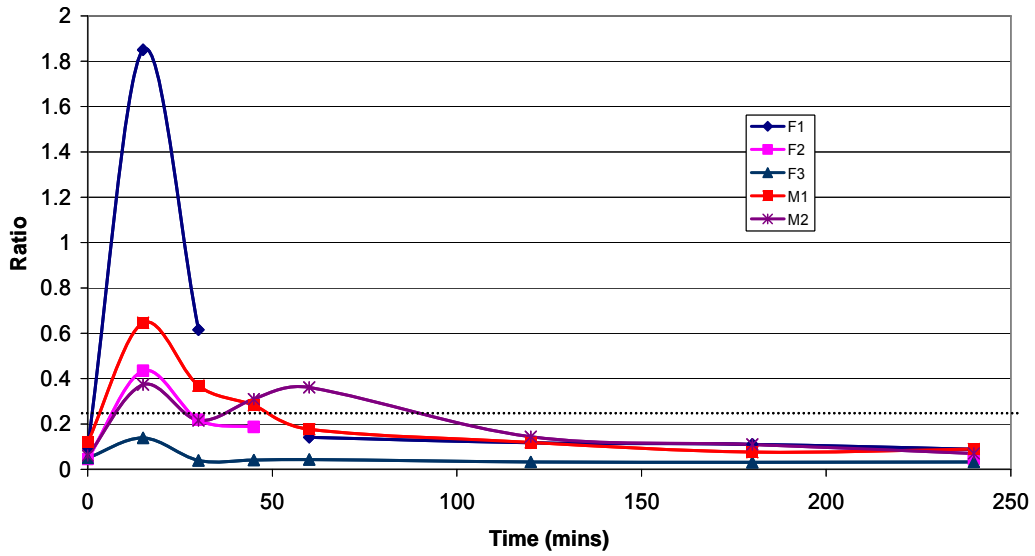


Figure 7. Insulin to C-peptide ratios for five non-diabetic subjects given an intravenous injection of Actrapid® at time 0.

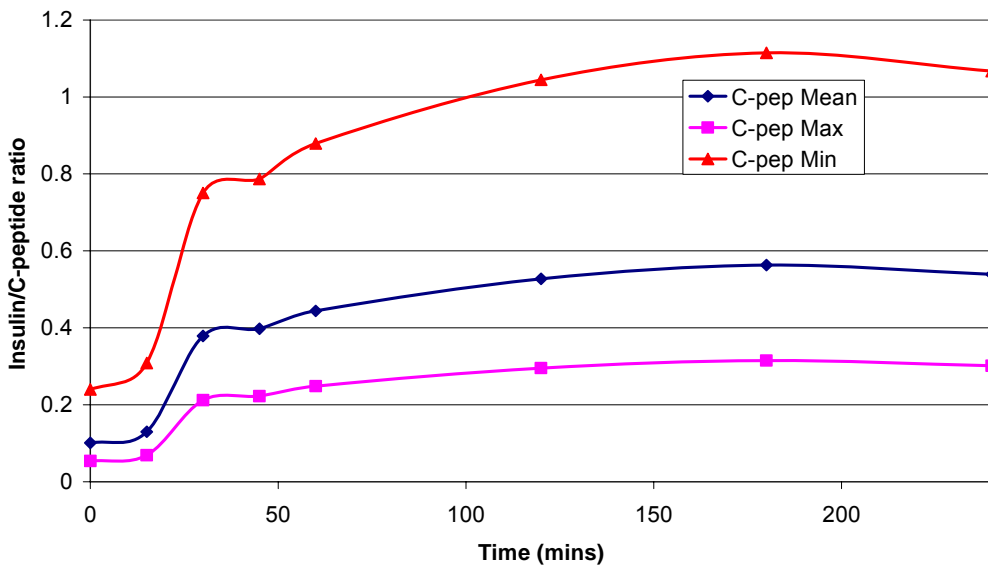


Figure 8. Predicted Insulin to C-peptide ratios for a subcutaneous injection of Actrapid® assuming suppression of C-peptide as occurred with the intravenous injection.