The Measurement of Angiotensin-Converting Enzyme in Human Serum

Andrew P. Kenny

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The Measurement of Angiotensin-Convertible Enzyme in Human Serum

Andrew P. Kenny

1997
The Measurement of Angiotensin -Converting Enzyme in Human Serum.

By

Andrew P. Kenny FAMLS, DMLM.

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Declaration

I hereby declare that the work detailed in this thesis is the result of my own investigations. No part of this work has been, or is being, submitted in candidature for any other degree.

Andrew P. Kenny

June 1997.
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<td>CSF</td>
<td>Cerebro-spinal fluid.</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation.</td>
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<td>FAP</td>
<td>Furyclylphenylalanine</td>
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<td>FAPGG</td>
<td>Furyclylphenylalanylglycylglycine</td>
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<tr>
<td>Y-GT</td>
<td>L-Y-Glutamyl transferase (EC 2.3.3.3.)</td>
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<td>IFCC</td>
<td>International Federation of Clinical Chemistry</td>
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<td>SACE</td>
<td>Serum Angiotensin Converting Enzyme</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)methylamine</td>
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<td>QC</td>
<td>Quality Control</td>
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Abstract.

The measurement of Angiotensin Converting Enzyme in Human Serum.

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The measurement of angiotensin converting enzyme using the substrate FAPGG was investigated with a view to optimising its performance. It was found that a 100 mmol/l triethanolamine-HCl buffer gave a significantly higher absorbance change than the existing TRIS-HCl in the method. As this buffer has not been previously described in the measurement of ACE it was decided to introduce this buffer into a kinetic procedure and evaluate the modified method. The reference interval for the triethanolamine Method was determined by measuring the ACE activity on 200 serum from blood donors and a significant difference (P < 0.05) in the SACE activity of male and female donors was observed.

The modified method was found to correlate well with the existing TRIS CL assay on the Hitachi 704. The purchase by the laboratory of the Hitachi 911 provided an opportunity of running the ACE assay as a kinetic method over a longer incubation period. With the improved performance characteristics of the modified method, the possibility of detecting measurable amounts of ACE in CSF was determined but was found to be too insensitive to measure such low activity.

The potential interference by bilirubin haemoglobin and lipids was investigated. The effect therapeutic ACE inhibitors on the assay was investigated. A dose related response was seen in relation to the ACE inhibitors lisinopril and captopril was seen but inhibition of ACE by captopril was found to decrease on storage at 4°C. A sample would need to
be analysed within a few hours in order to give an accurate indication of
the degree of inhibition. The potential to use SACE as a bioassay of ACE
inhibitors or as a test of compliance is discussed. The millimolar
absorptivity of FAPGG on three instruments was evaluated and compared
with calibration as a way of measuring ace activity.
Chapter 1

Introduction.
1.1 **Preamble.**

Angiotensin-converting enzyme (ACE, EC 3.4.15.1, dipeptidyl carboxypeptidase) is a membrane bound glycoprotein located mainly on capillary endothelium(1). The enzyme cleaves a dipeptide Histidyl-Leucine from the carboxy terminus of the decapptide Angiotensin I and converts it into the powerful vasoconstrictor and salt retaining compound Angiotensin II(2). ACE also inactivates the circulating potent vasodilator bradykinin by cleavage of a dipeptide moiety(3). Because of its action on angiotensin I and bradykinin ACE plays a crucial role in blood pressure regulation(4). The importance of ACE in the regulation of blood pressure is reflected by the popularity of ACE-inhibitors in the treatment of hypertension and congestive heart failure.

The main source of the enzyme is vascular endothelial cells and it is found in particularly high concentrations in lung and kidney vasculature. Van Sande et al (5) found that the highest specific activities in humans were in several tissues of the intestinal and urogenital tract. The highest levels of all were in benign prostatic hyperplastic tissue. Normal prostrate tissue and prostatic adenocarcinoma had a much lower activity. ACE is also found in epithelial cells(6,7). The presence of ACE in the brush border of the proximal convoluted tubule of the kidney and of the intestine suggests a possible detoxifying role for ACE(8). Another source of ACE is from cells derived from the monocyte / macrophage system(9).

It was assumed that the rapid conversion of angiotensin I to angiotensin II took place in the circulation until Ng and Vane (10) recognised that the enzyme activity in plasma was insufficient to account for the speed of *in vivo* conversion. It was demonstrated that most conversion occurred during a single passage through the lungs where the enzyme is located on the
luminal surface of the pulmonary endothelium (11). The fact that blood pressure is maintained during cardio-pulmonary bypass indicates that ACE is also present in non pulmonary vascular beds(12).

1.2 **ACE as an Indicator of Disease:**

In 1975 Lieberman found a possible clinical role for SACE, while investigating the cause of low blood pressure in patients with cystic fibrosis(13). He discovered unexpectedly high SACE activity in fifteen patients with sarcoidosis who were included in a control group. This finding was subsequently confirmed by other workers(14). When it was discovered it was hoped that SACE would provide a specific biochemical marker that would assist in the diagnosis of sarcoidosis.

1.3 **Sarcoidosis**

This a disease of unknown aetiology marked by the presence of noncaseating, epithelioid cell granulomas in multiple organs and tissues. All parts of the body can be affected but it is the lung that is most frequently involved(8). The granuloma formation has been shown to be the result of a cell mediated immunological response, possibly to various allergens. This starts as a cellular inflammatory reaction involving activated helper T-lymphocytes and monocytes in the involved tissues. The lymphokine products of the activated cells induce the accumulation and transformation of phagocytic macrophages from the blood and tissues into storage and secretory cells (epithelioid cell) which form the granulomas. The final stage of this reaction is either the complete resolution of the granuloma or evolution into fibrinous scar tissue. A major aim in the
treatment of this disease, in addition to the resolution of the granulomas, is the prevention of fibrin deposition.

The raised SACE activity in sarcoidosis is due to activation of the monocyte / macrophage system which provide the epithelioid cells from which the granuloma is formed. Circulating blood monocytes, the precursors of the epithelioid cells found in the sarcoid granuloma contain very little ACE, but on culture demonstrate the capacity to secrete the enzyme\(^{(15)}\). The transformation from monocyte to epithelioid cell is accompanied by the induction of angiotensin converting enzyme, possibly due to soluble mediators produced by T Lymphocytes\(^{(16)}\).

Immunoflorescence studies show both Angiotensin II and ACE in the epithelioid cells of granulomas\(^{(17)}\). The synthesis of ACE in the monocyte in culture is modulated by the T. Lymphocyte. In the sarcoid granuloma ACE is most abundant in the peripheral part where T. Lymphocytes and epithelioid cells are in close contact\(^{(18)}\).

The diagnosis of sarcoidosis depends on the exclusion of other granulomatous diseases and the demonstration of the characteristic granulomas on biopsy of the affected tissues. The clinical signs are Erythema Nodosum (an inflammatory disease of skin and underlying tissues) and Lymphadenopathy an inflammation of the lymph nodes. The radiographic appearance in a chest x-ray of bilateral hilar gland enlargement and pulmonary infiltration, are also important diagnostic features of the disease. The definitive diagnosis of the disease is made by a positive biopsy of the affected organ. When superficial or palpable lesions (e.g. Skin, Lymph nodes) are present then a biopsy of the location is positive in 87% of cases\(^{(19)}\). Another diagnostic test of benefit is the Kveim-Siltzbach test where a granulomatous reaction appears four weeks after the intradermal injection of sarcoid spleen or lymph node tissue. This test is
positive in 50 - 60% of patients with sarcoidosis, but reactions do occur in other diseases as well(19).

1.4 SACE as a Diagnostic Test in Sarcoidosis

The actual percentage of patients with sarcoidosis presenting with an elevated SACE varies from 45% to 100% in different studies(8). The reason for this may be due to the presence of different admixtures of chronic and active sarcoid disease in these studies. If the SACE level is assayed too early in the disease process then the SACE may well be in the healthy reference range. Similarly if the granuloma has progressed to the fibrotic scar stage then the SACE may not be elevated. So the actual percentage elevated in a particular group will depend on the relative numbers of active and chronic cases in each study.

1.5 The Specificity of an Elevated SACE.

Elevated levels of SACE are not specific for a diagnosis of Sarcoidosis as a number of other conditions can also present with increased levels. It is important that the clinician suspecting Sarcoidosis excludes these conditions as a possible source of an increased SACE. In all but the most typical of cases the diagnosis should be confirmed by biopsy of the affected area.

1.6 SACE in Diseases which may be Confused with Sarcoidosis

SACE increases can be found in non-sarcoidotic pulmonary granulomatous diseases such as silicosis and asbestosis (20,21,22). Since alveolar macrophages are a potential source of SACE then its elevation may be due to either a numerical or functional enhancement of the macrophages. Berylliosis
may be a rare type of sarcoidosis where the initiating antigen can be seen as beryllium crystals(23).

It is important to rule out a number of infections as a source of an increase in SACE. Respiratory conditions such as acute histoplasmosis(24) and coccidioidomycosis which are both systemic fungal diseases can give rise to increased SACE. Pulmonary tuberculosis especially milliary can present with increased levels(25). Lepromatous Leprosy also presents with increased SACE levels in up to 53% of cases(26,27). In liver disease such as Primary biliary cirrhosis up to 28% of patients may have increased SACE(28). Some lymphomas such as Hodgkin's disease(8) and Lennert's Lymphoma (23,29) have been reported to have increased levels. Both of these must be considered in the differential diagnosis of sarcoidosis.

1.7 SACE in Other Conditions.

SACE is increased in up to 25% of patients with alcoholic liver disease (28) and in 24-32% of patients with Diabetes Mellitus(30,31).

Increased levels can also be seen in non granulomatous disorders such as hyperthyroidism. The thyroid hormones regulate the metabolism of vascular endothelial cells. In hyperthyroidism the increased levels of these hormones cause an increased metabolism of vascular endothelial cells which in turn causes an increase in SACE(32).

Very high levels of SACE can be found in extra thoracic diseases such as Gaucher's disease(33). The origin of the SACE appears to be the Gaucher cell which like the epithelioid cell of sarcoidosis, is derived from circulating macrophages, and is converted into a cell which stores glucocerebroside as part of this condition.
An increased serum ACE has also been reported in patients with human immuno-deficiency virus (HIV) infection. The serum ACE activity appears to correlate with the progression of the associated syndrome (34).

1.8 The Clinical Significance of low SACE.

SACE activity in serum has been reported to be decreased in chronic asthmatics (35,36) chronic obstructive pulmonary disease (12) and adult respiratory distress syndrome (37). A low activity of the enzyme has also been reported to be associated with a poor prognosis in lung cancer (38,39) and a pre-operative SACE level may be useful as a prognostic indicator (40).

There is a widespread use of therapeutic ACE inhibitors in the treatment of hypertension and congestive heart failure. A low SACE activity may be associated with the presence of one of these drugs in the serum of a patient. Gorski et al (41) have found that the measurement of ACE activity using the artificial substrate, Furylacrylylphenylalanylglucyglycine (FAPGG), provides a reliable indicator of changes in conversion of angiotensin I to angiotensin II in vivo during therapy with ACE inhibitors.

1.9 The Value of Sequential Measurement of SACE in Sarcoidosis.

The use of serial measurement SACE over a period of time show a relationship between changing enzyme activity and the clinical and radiological findings. As the disease goes into remission or is treated with corticosteroids then the raised SACE levels decrease towards normal levels. The measurement of SACE activity over a period of time is of value in identifying if the sarcoidosis is becoming active again. A relapse is characterised by increase in SACE activity, and the level of increase will give an indication of the severity of the disease. There is a positive correlation between SACE
and pulmonary disease activity as assessed by chest radiographs(25). The use of SACE activity provides a simple method of monitoring progression of the disease and also gives a measure of both pulmonary and extrathoracic sarcoid activity. There are other tests to assess the severity of the disease such as gallium scans and broncho-alveolar lavage fluid (BAL) T-lymphocyte profiles but these are more invasive and are not widely available and would not be applicable to serial measurement(42).

1.10 Monitoring the Treatment of Sarcoidosis with Corticosteroids.

Corticosteroids are used in the treatment of sarcoidosis and their administration usually results in an improvement of the condition. There is a strong relationship between corticosteroid therapy and SACE activity. Elevated levels of SACE activity fall towards normal after the patient is put on corticosteroid therapy and will usually come back into the reference range in 4-10 weeks(43). The effect of corticosteroids on SACE is not a direct inhibition of the enzyme, but a reflection of the effect of corticosteroid on the epithelioid cell granulomas(25). The use of serial measurement of SACE activity in the patient on corticosteroids will indicate if the dosage is adequate or needs adjustment. If there is a persistently elevated or a progressive rise in the level of SACE activity then an increase in the dosage may be required to adequately control the disease. On the other hand a falling SACE activity is a good prognostic sign and indicates that the corticosteroids are having the desired effect.
1.11 Methods used to Measure ACE

The first methods reported for the assay of ACE utilised physiological substrates such as angiotensin I (44), but suffered from interference by other peptidases which degraded both substrate and product.

Artificial substrates using N-substituted tripeptides were developed which were specifically hydrolysed by the enzyme. These model substrates include Hippuryl-L-Histidyl-L-Leucine (HHL) which was developed to measure ACE in rabbit lung in the original manual method of Cushman and Chung (45).

This method is based on the hydrolysis of the substrate to produce hippuric acid and the dipeptide histidyl-leucine. The hippurate is extracted and measured spectrophotometrically at 228 nm. The method was subsequently modified by Lieberman (46) to assay serum ACE by measuring the histidyl-leucine formed fluorometrically. An automated modification of this method has been reported (47) which uses a series of enzymes to directly measure the quantity of His-Leu formed.

Neels et al (48) used the substrate Hippuryl - Glycyl - Glycine (Hip-Gly-Gly). In this method the enzyme hydrolyses the substrate to produce hippuric acid and glycyl - glycine. After deproteinisation the Gly-Gly is derivatised using a borate buffered trinitrobenzenesulfonate solution to form trinitrophenyl-glycylglycine the absorbance of which is measured against a serum blank at 420 nm.

Alternatives to this extraction and derivatization have been proposed. These include the measurement of the hippuric acid formed by HPLC (49) or the use of a radiolabelled substrate and measurement of the products of the reaction in a radiometric assay (50, 51).

A modification of this method has been reported (52) which involves coupling an indicator reaction, catalysed by the enzyme γ glutamyltransferase.
The rate limiting substrate for the γGT is the Gly-Gly product of the ACE catalysed reaction. The addition of the indicator reaction increased the sensitivity of the method and made it more suitable for automation(53).

In 1977 Holmquist et al (54) developed a method using a synthetic substrate Furylacrylylphenylalanylglucylglycine (FAPGG), and was modified by Ronca Testoni (55) to measure ACE in serum. This method used a single stable reagent which could easily be run on automated analysers(56).

1.12 Iso-Enzymes of ACE.

There are two main iso enzymes of ACE which differ in their molecular weight and Zinc content. Lung ACE contained 2.35 atoms of zinc per molecule (M(r) 147,000), Kidney ACE has a greater amount of zinc per molecule at 2.58 but the same molecular weight. In contrast the testicular ACE contains 1.58 atoms of zinc per molecule and had a lower molecular weight(M(r)=80,000)(54). Immunofluorescent staining techniques showed the testicular ACE to be localised to the lumen of the seminiferous tubules while the endothelial ACE antiserum showed this isoenzyme to be located only in blood vessels.

1.13 Objectives of this Research Project.

The initial object of this project was to take a critical look at the assay of Angiotensin-Converting Enzyme in relation to different clinical conditions. When investigating the literature on the subject there seemed to be great diversity of methods. Even when the methods use the same substrate the reference interval quoted can vary considerably(48,55,56,61,71,72,92,93).

It was decided that rather then just looking at different disease states by measuring SACE by the current method, the method itself would be
fully investigated and optimised. As there is no reference method published for the measurement of SACE, it was decided to use the method of Maguire and Price (56) as the reference method for this investigation. The method, which used FAPGG as the substrate in a TRIS-HCl buffer at pH 8.3, was in use for a number of years in this laboratory and it was essential that any new method would be compared to it as a reference.

1.14 Aims to be met by Research.

Selection of assay buffer and method validation;

1. To improve the sensitivity of the SACE by looking at different buffer systems and by modifying the assay to run as a kinetic assay on the Hitachi 911.

3. To fully define the assay in terms of sensitivity, accuracy, precision, linearity and stability of the reagent.

4. To investigate the effect of Bilirubin, Haemolysis and Lipaemia on the assay.

5. To determine if the method is sensitive enough to measure SACE in other biological fluids such as Cerebrospinal Fluid.

Investigation into the reference interval;

6. To determine a reference interval for our local population and investigate any difference between male and female SACE levels.
7. To look critically at the method and determine why the published reference ranges differ to such a degree.

8. To investigate the millimolar absorptivity of the substrate FAPGG on three instruments.

The effect of therapeutic ACE inhibitors on the ACE assay;

9. To investigate the effect of different therapeutic ACE inhibitors on the assay. If the inhibition was dose related then to investigate the possibility of using the method as a bioassay for the concentration of ACE inhibitors in patient samples.
Chapter 2.

*Materials and Methods.*
2.1 Chemicals.

Tris(hydroxymethyl)methylamine, Sodium Chloride, Hydrochloric Acid, Sodium Azide and Ethanolamine were purchased from BDH Laboratory Supplies, Poole, Dorset, BH15 1TD, England

Triethanolamine, Furylacrylylphenylalanylglycylglycine (FAPGG) and Furylacrylylphenylalanine (FAP), were purchased from Sigma Chemical Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, England

Boric Acid was purchased from May and Baker Ltd, Dagenham, England.

All water used in this project was of reagent grade and produced with a Prima Reverse Osmosis system

2.2 Instrumentation.

Shimadzu
UV-160A UV-VIS Recording Spectrophotometer
Shimadzu Corporation
Analytical Instruments Division
Kyoto, Japan.

Hitachi
704 Automatic Analyser and 911 Automatic Analyser
Hitachi Ltd.
Tokyo, Japan.

pH Meter
PHM 84 research pH meter
Radiometer
Copenhagen
2.3 Clinical Samples.

GP samples. Initially 99 samples from patients referred by general practitioners for routine biochemical analysis were assayed for ACE activity by the TRIS Chloride method on the Hitachi 704. Ethical principles were implemented and only serum which was superfluous to diagnosis was used and full confidentiality was maintained. The clinical details given on the forms were checked and any patient referred for sarcoidosis, respiratory problems, renal investigation or diabetes were excluded from the evaluation. It was felt that this would give a good indication of the level of ACE activity found in the general population.

Blood Donors. Serum was collected from 200 blood donors, 100 female and 100 male. The serum was separated and stored at -20°C prior to analysis. When thawed the tubes were mixed and centrifuged again before analysis. These were used for comparison studies and for reference range calculation.

Serum Samples: Serum submitted to the laboratory for routine SACE analysis. These samples were separated and stored at 4°C prior to analysis. Prior to assay a 500 μL aliquot of each sample was transferred to a microfuge tube and spun at 10,000 rpm to provide a clear sample for analysis. After analysis by the TRIS-Chloride method the samples were frozen at -20°C for long term storage.

CSF specimens. Cerebrospinal fluid (CSF) samples of patients being screened for Neurosarcoidosis. Blood stained samples were not assayed because of contamination with serum ACE, which is significantly higher than that found in CSF.
Quality Control - Normal and Abnormal controls were purchased from Sigma Diagnostics. These are a lyophilised preparation containing porcine ACE in a buffered human serum base with stabilisers and preservatives added.

Calibration Material. - Initially the Sigma Abnormal control was used as a calibrant but this was subsequently replaced by a specific ACE calibrator from Sigma Diagnostics. Like the controls this is also a Human serum base containing porcine ACE. These were reconstituted and aliquots stored frozen at -20°C prior to use.

2.4 Ace Inhibitors.

In order to investigate the inhibitory effect of the most commonly prescribed ACE Inhibitors, samples of the following drugs were obtained from The Pharmacy Cork University Hospital. The quantity of the drug in each tablet is also indicated.

- **Capoten**
  - Bristol-Myers Squibb,
  - Contains 12.5 mg of Captopril

- **Innovace**
  - Merx Sharp and Dohme
  - Contains 2.5 mg Enalapril maleate.

- **Accupro**
  - Parke-Davis
  - Contains 5mg Quinapril

- **Zestril**
  - Zeneca
  - Contains 5mg Lisinopril

- **Tritace**
  - Hoechst Marion Roussel
  - Contains 1.25 mg Ramipril

- **Coversyl**
  - Servier
  - Contains 4 mg Perindopril.

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Solubility of the Ace Inhibitors:

Captopril, Quinapril, Lisinopril, Ramipril and Perindopril are quoted as being water soluble (58). Enalapril in only sparingly soluble in water but is freely soluble in Methyl Alcohol. These tablets were crushed and dissolved in either 70 mls of water or methanol according to their solubility. Varying quantities of each drug were then added to a high ACE serum pool in order to investigate potential interference from these drugs.

2.5 Materials for Investigation of Interference on the Method.

**Intralipid:** 20% fat emulsion was purchased from Kabi Pharmacia Ltd, Milton Keynes, Buks, MK5 8PH. Aliquots of the 20% intralipid were added to a high ACE pool in order to mimic various degrees of lipaemia.

**Bilirubin.** Crystalline mixed isomers from bovine gallstones was purchased from Sigma Diagnostics. A stock solution containing 6000 μmol/L of bilirubin was prepared by dissolving in dimethylsulphoxide (4.7 mol/L) and sodium carbonate (70 mmol/L) (59). Varying quantities of this stock were then added to a high ACE pool. The total bilirubin of each spiked sample was determined by the potassium ferricyanide (60) method on the Hitachi 911.

**Haemoglobin** Lithium heparin samples with normal ACE levels were centrifuged and the plasma separated. The cell pellets were then frozen and thawed in order to haemolysye the red cells. The haemolysate was centrifuged again in order to remove cellular debris. Varying dilutions of this stock were then spiked into a high ACE pool. The haemoglobin was quantified in each dilution as the cyanmethaemoglobin derivative on the Technicon H2, haematology analyser.
2.6 Methods:

The assay for serum ACE is based on the continuous monitoring spectrophotometric method of Maguire and Price(56). This method uses the artificial substrate developed by Holmquist et al, Furylacyrlphenylalanylglcyglylglyicine (FAPGG)(54).

The principle of the method is as follows:

\[
\text{Furylacyrlphenylalanylglcyglylglyicine (FAPGG)} \\
\downarrow \text{ACE} \\
\downarrow \\
\text{Furylacyrlphenylalanine (FAP),} \\
+ \\
\text{Glycylglycin (GG)}
\]

The hydrolysis of FAPGG results in a decrease in absorbance. The maximum difference between substrate and product has been reported to occur at 328 nm (61). The Hitachi 704 has a limited number of wavelengths to choose from. So the closest available wavelength of 340 nm was selected. A secondary or blanking wavelength of 660 nm was also selected to minimise interference.
2.7 **Buffers Evaluated**

**50 mmol Tris Chloride buffer.**

To 800 mL of water was added 23.4 g of sodium chloride, 6.1 g TRIS(hydroxymethyl)methylamine, and 100 mg sodium azide as a preservative. After mixing to dissolve, the pH of the buffer was adjusted to 8.3 with 1N hydrochloric acid. The final volume was adjusted to 1000 ml. This buffer contains 50 mmol TRIS, 400 mmol of sodium chloride and 1.54 mmol Sodium Azide per litre. Additional TRIS-HCl buffers containing 100 and 150 mmol of TRIS(hydroxymethyl)methylamine of molecular weight 121.14 g/M was used to prepare the buffers. The required weight of TRIS along with 23.4 g NaCL and 100 mg Sodium Azide were added to 800 mL of water. The buffers were mixed to dissolve and the pH adjusted to 8.3 at 20°C using 1N HCl. The final volume was adjusted to 1000 ml with water.

**Tris Borate Buffer**

A range of buffers containing 50, 100 and 150 mmol of Tris(hydroxymethyl)methylamine of molecular weight 121.14 g/M were prepared. The required weight of TRIS along with 23.4 g NaCL and 100 mg sodium azide were added to 800 ml of water. The buffers were mixed to dissolve and the pH adjusted to 8.3 at 20°C using boric acid. The final volume was adjusted to 1000 ml with water.

**Ethanolamine**

A range of buffers containing 50, 100 and 150 mmol of Ethanolamine - (2-Aminoethanol) of molecular weight 61.08 g/Mol, density = 1.02 g/mL were prepared. The required weight along with 23.4 g NaCL and 100 mg Sodium Azide were added to 800 mL of water. The buffers were mixed to dissolve and the pH
adjusted to 8.3 at 20°C using 1 M HCl. The final volume was adjusted to 1000 mL with water.

**Triethanolamine**

A range of buffers containing 50, 75, 100, 125, 150, 175 and 200 mmol of Triethanolamine (2,2',2''-Nitrilotriethanol) of molecular weight 149.2 g/Mol, density = 1.12 g/mL were prepared. The required weight along with 23.4 g NaCl and 100 mg Sodium Azide were added to 800 mL of water. The buffers were mixed to dissolve and the pH adjusted to 8.3 at 20°C using 1 M HCl. The final volume was adjusted to 1000 mL with water.

**2.8 Working Reagent Preparation for the Hitachi 704**

To 20 ml of buffer was added 5 mg FAPGG and mixed. The FAPGG takes a while to dissolve so needs to be mixed for a few minutes. Examine the reagent to ensure that all of the substrate has dissolved. This working reagent contains 0.625 mmol FAPGG/L, 400 mmol NaCl/L, 1.54 mmol Sodium Azide / L at pH 8.3 at 20°C.
2.9 The Method parameters for the Hitachi 704

Assay Code = 2 Point Readings at cycle 6 and 32

Sample Vol = 20 µL.
Reagent 1 Vol = 350 µL
Reagent 2 Vol = 0 µL

Wavelength primary = 340 nm
Wavelength secondary = 660 nm

Calib Method = Linear

Std 1 Conc = 0.0 IU/L (154 mmol NaCl/L)
Std 2 Conc = 72.0 IU/L (Sigma Abnormal Control)

Absorbance Limit = 0 Decreasing.
Temperature = 37°C

In the Hitachi 704 two point assay 20 µL of serum is pipetted followed by 350 µL of reagent and incubated at 37°C. The first reading is taken after 120 seconds at a primary wavelength of 340 nm and a secondary or blanking wavelength of 660 nm. The second reading is taken at 640 seconds. The difference between the first and the second reading is the Δ OD and on the 704 is given in 10⁻⁴ Absorbance units. The absorbance change of test is then compared with that of the calibrators to give an enzyme activity.

2.10 Statistical analysis

Statistical analysis of the data was accomplished with the Minitab computer software system(62). Graphical presentation of the data was with the Harvard Graphics computer programme(63).
Chapter 3.

Results.
3.1 Selection of buffer.

The original method (56) on the Hitachi 704 used a 50 mmol TRIS-HCl buffer of pH 8.3 with a substrate concentration of 0.625 mmol FAPGG/L. In the evaluation of the method for SACE it was decided to optimise the method in terms of type of buffer used, molarity and pH of the buffer. As well as the existing TRIS-HCl buffer, three other buffer systems were investigated at different molarities. These were:

TRIS - Borate buffer at 50, 100 and 150 mmol/L
Ethanolamine Buffer at 50, 100 and 150 mmol/L
Triethanolamine at 50, 75, 100, 125, 150 and 200 mmol/L

Each buffer was prepared to the required molarity and the sodium chloride and Azide added before adjusting to pH 8.3 at 20°C (equivalent to 8.2 at 37°C). The working reagent was then prepared by adding 5 mg of FAPGG to 20 mL of buffer and mixing well. It is important to mix thoroughly as the substrate is slow to dissolve. The final concentration of reagents in each buffer were then as follows: sodium chloride (400 mmol/L), FAPGG (0.625 mmol/L), sodium azide (1.54 mmol/L) and pH of 8.3 at 20°C.

The working reagent was run on the Hitachi 704 using the two point assay, the absorbance change of a 79 IU/L standard being used as an indicator or the activity of the enzyme in that buffer. As is demonstrated (Figure 1) the maximum activity was seen using a 100 mmol/L triethanolamine-HCl buffer pH 8.3. As this buffer system gave a significantly higher absorbance change than in TRIS chloride it was decided to incorporate this into the assay in order to improve the sensitivity of the method.
Figure 1. The effect of four different buffers on serum ACE activity. The results are expressed as a percentage change relative to the change in absorbance of the 79 IU ACE /L calibration standard in 100 mmol/L triethanolamine-HCl buffer pH = 8.3. Each point is a mean of duplicates.
3.2 Optimisation the pH of the Assay Buffer.

As Triethanolamine- HCl at a molarity of 100 mmol/L gave the highest enzyme activity of the buffers investigated it was decided to further optimise the buffer by looking at optimum pH for maximum enzyme activity.

In this experiment the concentration of sodium chloride (400 mmol/L), FAPGG (0.625 mmol/L), sodium azide (1.54 mmol/L) and triethanolamine (100 mmol/L) were all kept constant in each buffer. The pH of six buffers were adjusted with 1M HCl to between pH 7.80 and pH 8.60. The buffer pH which gave the maximal enzyme activity was determined by measuring the absorbance change on the 79 IU ACE /L standard, the result of this investigation is shown in figure 2. From this plot the optimum pH of the assay buffer was determined to be 8.3 at 20°C which is equivalent to 8.2 at the actual assay temperature of 37°C.
Figure 2. The effect of pH of a 100 mmol triethanolamine /L buffer on serum ACE activity. The results are expressed as a percentage of the maximal enzyme activity at a pH of 8.3. The specimen in this assay being the 79 IU ACE /L calibration standard. Each point is a mean of duplicates.
3.3 Chloride Concentration

As ACE is a halide requiring dipeptidase (4) it was decided to investigate the effect of chloride concentration of the reagent on the activity of the enzyme. The concentration of sodium chloride added to the reagent was evaluated between 0 and 500 mmol NaCl/L. The additions were made to a prepared reagent containing, FAPGG (0.625 mmol/l) and sodium azide (1.54 mmol/L) in a 100 mmol/L triethanolamine-HCl buffer pH 8.3. In the absence of the chloride ion there was a significant decrease in the activity of the enzyme and there was a progressive increase in enzyme activity as the concentration of chloride increased (Figure 3). The optimal chloride concentration in the reagent was found to be 400 mmol NaCl/L.
Figure 3. The effect of chloride concentration on serum ACE activity. The results are expressed as a percentage of the maximal enzyme activity at a chloride concentration of 400 mmol/L. The specimen in this assay being the 79 IU ACE/L calibration standard. Each point is a mean of duplicates.
3.4 Concentration of Substrate to be Used.

The concentration of substrate used in the assay was investigated by measuring the absorbance change of the 79 IU ACE/1 calibrator on the Hitachi 704 at increasing concentrations of the substrate FAPGG. The buffer used to dissolve the FAPGG contained sodium chloride (400 mmol/L), triethanolamine- (100 mmol/L), sodium azide (1.54 mmol/L) at a pH of 8.3 at 20°C. A total of nine different concentrations of FAPGG were assessed from 0.43 mmol/L to 1.0 mmol/L. The effect of FAPGG concentration on serum ACE activity was investigated (Figure 4) and the Michaelis Menten constant Km was calculated from the plot of the Lineweaver-Burk transformation of the data (65)(Figure 5).

The apparent Km calculated from this plot is 0.3125 mmol FAPGG/L. The concentration of FAPGG used in the current assay on the Hitachi 704 is 0.625 mmol/L or twice the Km. This was the concentration incorporated into the Triethanolamine-HCl method on the Hitachi 911.
Figure 4 The effect of the concentration of substrate in the reagent on serum ACE activity. Substrate concentrations ranged from 0.43 to 1.0 mmol FAPGG/L. Each point is a mean of duplicates.
The effect of the concentration of substrate in the reagent on serum ACE activity. Substrate concentrations ranged from 0.43 to 1.0 mmol FAPGG/L. Each point is a mean of duplicates and the data has been plotted in the form of a Lineweaver -Burk Transformation.
3.5 The Sensitivity of the ACE Method on the Hitachi 704.

In this experiment the sensitivity of both the TRIS chloride and triethanolamine buffers were assessed. A total of twenty samples of 154 mmol NaCl /L were analysed by both methods on the Hitachi 704. The standard deviation of the results were calculated and the sensitivity of each method was expressed as the least amount measurable that would be distinguished from zero. This is expressed as zero plus 2.5 times the standard deviation of the imprecision run of 20 samples (65). The results of the imprecision runs by both methods are shown in Table 1.

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Mean</th>
<th>Sd</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS HCl</td>
<td>20</td>
<td>0.8</td>
<td>1.96</td>
<td>4.9 IU ACE/L</td>
</tr>
<tr>
<td>Trieth-HCl</td>
<td>20</td>
<td>-1.3</td>
<td>4.97</td>
<td>12.4 IU ACE/L</td>
</tr>
</tbody>
</table>

Table 1 The sensitivity of both methods using 154 mmol NaCl /L as sample in an imprecision run. n = 20.
3.6 Imprecision on the Hitachi 704.

The imprecision of the two point assay using the 100 mmol triethanolamine /L buffer was determined on control materials obtained from Sigma. The within batch imprecision was evaluated by measuring the controls twenty times in one run. The between batch imprecision was calculated by running the controls on twenty separate days. The results of this evaluation are shown on Table 2.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (IU/L)</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within Batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>20</td>
<td>29.9</td>
<td>0.76</td>
<td>2.5</td>
</tr>
<tr>
<td>Control 2</td>
<td>20</td>
<td>105.8</td>
<td>1.12</td>
<td>1.06</td>
</tr>
<tr>
<td>Between Batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td>20</td>
<td>47.5</td>
<td>2.91</td>
<td>6.1</td>
</tr>
<tr>
<td>Control 4</td>
<td>20</td>
<td>107.2</td>
<td>3.61</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 2: The imprecision of the SACE assay on the Hitachi 704 using 100 mmol triethanolamine/L, pH 8.3. Four different commercial quality control samples were used to evaluate the within run and between batch imprecision.
3.7 Sample Preparation Prior to SACE Assay.

It is important that all serum or plasma samples be centrifuged again in a microfuge tube immediately before analysis of ACE activity. The reason for this second centrifugation is to spin down any material that may have come out of solution during storage. The effect of centrifugation on the imprecision of the measurement of SACE on a plasma pool was investigated and the results are shown in Table 3. Analysis of the data by the Mann-Whitney test indicated that there was a significant difference between the original results and the spun samples \( p = 0.006 \).

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>Mean</th>
<th>Range</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Centrifugation</td>
<td>20</td>
<td>24.6</td>
<td>21 - 33</td>
<td>3.4</td>
<td>13.8</td>
</tr>
<tr>
<td>After Centrifugation</td>
<td>20</td>
<td>21.8</td>
<td>19 - 25</td>
<td>1.6</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Table 3 The effect of Centrifugation on the within run Imprecision of a Plasma Pool \( N=20 \).
3.8 **Investigation of the Reference Interval for Serum ACE by the Modified Method.**

In our laboratory the quoted reference interval for serum ACE by the TRIS chloride method (56) on the Hitachi 704 is 0 - 45 IU ACE /L. As an initial test of the validity of this range it was decided to run 99 samples sent by general practitioners. It was felt that these would give a reliable indicator to the levels of ACE found in the general population. The clinical details of each patient were checked and patients under investigation for sarcoidosis, renal failure, respiratory problems and diabetes were not assayed.

The mean SACE value was found to be 39.3 IU ACE /L with a standard deviation of 15.3 IU ACE /L. When a histogram of the data was produced it showed a skewed distribution with 26 of the 99 samples having SACE activity greater than the top of the current reference interval(Figure 6). This data indicated that the currently quoted range may be too low for the local population and may be giving a higher percentage of abnormal results then is correct.

It was decided that the true reference interval would need to be calculated. A total of 200 serum samples were provided by the local Blood Transfusion Service Board laboratory, and by definition were from healthy donors aged between 18 and 65.
Figure 6

Histogram of the distribution of serum ACE activity in serum samples sent by General Practitioners. The samples were assayed on the Hitachi 704 analyser using the TRIS Chloride method. The upper limit of the quoted reference range is indicated by the arrow.
3.9 The Evaluation of the Reference Interval for SACE by the Triethanolamine Method on the Hitachi 704.

The number of samples required for the evaluation of the reference interval in order to obtain stable upper and lower reference limits for a population is 200 subjects according to Lott et al(66). The International Federation of Clinical Chemistry recommend at least 120 subjects as the minimum number (67). In keeping with these recommendations a total of 200 serum samples were obtained from healthy blood donors between the age of 18 to 65. After centrifugation the serum was separated and stored at -20°C prior to analysis. There were 100 sera from male donors and 100 from female donors which would allow the investigation of male versus female reference interval differences. Before analysis the samples were thawed and mixed before centrifuging again to provide a non-turbid sample for assay of ACE activity. The sera were assayed by the 100 mmol triethanolamine /L pH 8.3 method on the Hitachi 704. Statistical analysis of the data was according to the guidelines set down by the International Federation of Clinical Chemistry as stated by Solberg (67,68).

3.10 Calculation of Reference Intervals.

In the estimation of the reference interval the non-parametric method was used in the calculation of the values as recommended by the IFCC(67). The parametric estimate of the reference interval has also been calculated for comparison purposes. A total of two hundred serum samples were analysed by the modified 100 mmol triethanolamine /L method on the Hitachi 704.
The results of this analysis are as follows.

<table>
<thead>
<tr>
<th>Number of samples:</th>
<th>n = 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>44.94 IU ACE /L</td>
</tr>
<tr>
<td>Std Deviation</td>
<td>15.58 IU ACE /L</td>
</tr>
<tr>
<td>Std Error of mean</td>
<td>1.1 IU ACE /L</td>
</tr>
</tbody>
</table>

Nonparametric estimate of the reference interval

If the data is ranked and then sorted then the 95 % interval can be calculated by determining the 2.5 and 97.5 % values. Using this nonparametric method the reference interval with the 0.90 confidence limits shown in brackets was determined to be 21 (18-23) to 81 (78-86) IU ACE/L.

Parametric estimate of the reference interval

The 200 values when plotted on a histogram (Figure 7) shows that the distribution is non-Gaussian. The distribution is skewed to the right and gives a coefficient of skewness Gs = 0.7374, Ss = 0.1732 and of kurtosis Gk = 0.3024, Sk = 0.3464. A Log10 transformation of the data was calculated to see if it would give a Gaussian distribution. The log10 transformed data was plotted on a histogram and appears to give a more Gaussian distribution(Figure 8). A normal probability plot (Figure 9) of the transformed data shows the line of best fit to be straight thereby indicating a Gaussian distribution of results.
The coefficient of skewness was $G_s = -0.128$, $S_s = 0.1732$ and kurtosis $G_k = -0.309$, $S_k = 0.3464$. These tests of the Gaussianity of the distribution indicated that there was not a significant degree of skewness or kurtosis in the transformed data.

The reference interval (with the 0.90 confidence interval for each limit in brackets) for the 200 serum samples was calculated using the Log$_{10}$ transformed data to be 21.4 (20.0-23.0) to 83.4 (77.8-89.3) IU ACE/L.
Figure 7  Histogram of the distribution of ACE activity in 200 sera from blood donors. The samples were assayed on the Hitachi 704 using a 100 mmol triethanolamine /L buffer pH 8.3
Figure 8  Histogram of the Log$_{10}$ transformed data of the distribution of ACE activity in 200 sera from blood donors. The samples were assayed on the Hitachi 704 using a 100 mmol triethanolamine/L buffer pH 8.3
Figure 9  Normal probability plot of the Log_{10} transformed data of the 200 blood donor SACE results. Each data point is marked with an asterix (*). Where more than one data point occurs in a location then the figure printed indicates the number of data points in that position.
3.11 Investigation into the Possibility of a Sex Linked Difference in the Reference Interval.

The sera from 100 male and 100 female blood donors were analysed in order to determine if there was a significant difference in the reference intervals of the two groups. The one-way analysis of variance on the log_{10} transformed data indicates that there is a significant difference between the populations at p = 0.016. As the p value is less then 0.05 then it is valid to say that there is a significant difference between the populations at this level of confidence.

Samples from female donors.

<table>
<thead>
<tr>
<th>Number of samples: n = 100,</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean = 42.39 IU ACE /L,</td>
</tr>
<tr>
<td>Standard error of mean = 1.44 IU ACE /L</td>
</tr>
<tr>
<td>Standard Deviation = 14.40 IU ACE /L</td>
</tr>
</tbody>
</table>

Parametric Estimate of the Reference Interval:

The values when plotted on a histogram shows that the distribution is non Gaussian(Figure 10). The distribution is skewed to the right and gives a coefficient of skewness Gs = 0.6607, Ss = 0.2449 and of kurtosis Gk = 0.4, Sk = 0.4899 . A Log_{10} transformation of the data was calculated to see if it would normalise the data. The log_{10} transformed data was plotted on a histogram and appears to give a more Gaussian distribution(Figure 11). A normal probability plot of the transformed data shows the line of best fit to be straight thereby indicating a Gaussian distribution of results(Figure 12). The coefficient of skewness was Gs = -0.185, Gs = 0.2449 and kurtosis Gk = -
0.309, Sk = 0.4899. These tests of the Gaussianity of the distribution indicated that there was not a significant degree of skewness or kurtosis in the transformed data.

The Reference interval (with the 0.90 confidence interval for each limit in brackets) for the 100 female samples was calculated using the Log10 transformed data to be 20.4 (18.5 - 22.5) to 78.6 (71.4 - 86.6) IU ACE/L.
Figure 10  Histogram of the distribution of ACE activity in 100 sera from female blood donors. The samples were assayed on the Hitachi 704 using a 100 mM triethanolamine buffer pH 8.3
Figure 11  Histogram of the Log\textsubscript{10} transformed data of the distribution of ACE activity in 100 sera from female blood donors. The samples were assayed on the Hitachi 704 using a 100 mM triethanolamine buffer pH 8.3
Figure 12  Normal probability plot of the Log10 transformed data of the ACE activity measured on 100 samples from female blood donors. Each data point is marked with an asterix (*). Where more then one data point occurs in a location then the figure printed indicates the number of data points in that position.
Serum Samples from Male donors.

Number of samples n = 100
Mean = 47.6 IU ACE/L,
Standard error of mean = 1.16 IU ACE/L
Standard Deviation = 16.2 IU ACE/L

Parametric estimate of the reference interval.

The distribution of results when plotted on a histogram (Figure 13) shows that the distribution is non Gaussian and is skewed to the right with a significant degree of kurtosis. The tests of the Gaussian nature of the distribution were as follows, coefficient of skewness Gs = 0.7397, Ss = 0.2449, and of kurtosis Gk = 2.97, Sk = 0.4899. A log_{10} transformation of the data was calculated to see if it would make the data Gaussian. The log_{10} transformed data was plotted on a histogram and gave a more Gaussian distribution(Figure 14). A normal probability plot(Figure 15) of the data shows the line of best fit to be straight thereby indicating a Gaussian distribution of results. The coefficient of skewness of the transformed data was Gs = -0.069, Ss = 0.2449, and of kurtosis Gk = -0.52, Sk = 0.4898. These tests of the Gaussianity of the transformed distribution indicate that the Log_{10} transformation of the data has made the distribution Gaussian.

As the untransformed data was not distributed in a Gaussian manner it was decided to use the Log_{10} transformed data in the calculation of the reference interval. The reference interval (with the 0.90 confidence interval for each limit in brackets) for the 100 male samples was calculated to be 23.0 (20.9 - 25.4) to 87.6 (80.2 - 96.4) IU ACE/L.
Figure 13  Histogram of the distribution of ACE activity in 100 sera from Male blood donors. The ACE activity was measured on the hitachi 704 using 100 mM triethanolamine buffer pH 8.3
Figure 14  Histogram of the Log10 transformed data of the distribution of ACE activity in 100 sera from male blood donors. The samples were assayed on the Hitachi 704 using a 100 mM triethanolamine buffer pH 8.3
Figure 15  Normal probability plot of the Log 10 transformed data of the 100 male blood donor SACE results. Each data point is marked with an asterix (*). Where more than one data point occurs in a location then the figure printed indicates the number of data points in that position.
3.12 Method Comparison

A total of 187 samples used to determine the reference interval for the Triethanolamine ACE method were also assayed by the TRIS Chloride in order to statistically compare the methods. As the TRIS HCl method (56) had been used by the laboratory for a number of years it was used as the reference method for the evaluation of the modified method. The scatter plot of the results is shown in Figure 16.

The result of the Mann-Whitney test on the data indicates that there is not a statistically significant difference between the methods at $p = 0.05$.

The linear regression line and the correlation coefficient are shown on Figure 16.
A total of 187 samples were assayed and the correlation of ACE activity values obtained from the TRIS chloride method (abscissa) and the triethanolamine Method (ordinate) are shown in this plot.

Equation: \[ \text{Triethanolamine} = -0.895 + 1.02 \times \text{TRIS-HCl} \]

Correlation: \( r = 0.985 \)

\( n = 187 \)
3.13 Adaptation of the Method to run on the Hitachi 911

During the course of this project the Hitachi 911 analyser was purchased by the laboratory. This system gave had a number of features that would potentially improve the assay. It was decided to run the ACE method on the system as a kinetic assay with a sample volume of 30 µl and a reagent volume of 250 µL. The variables method programmed into the instrument were as follows:

Test [Ace] [00386]

Standards: 1 Conc = 0.0 Pos 18 Sample Vol = 30
           2 Conc = 72.0 Pos 28 Sample Vol = 30

Assay Code [Rate-A ] [15]
Assay Point [10] - [48] - [0] - [0]
Wavelength: (2nd / Primary) [660]/[340]
Serum Vol. Normal 30 µL.
Serum Vol. Decrease 20 µL.
Serum Vol. Increase 50 µL.
ABS. Limit [6000][6000][Decrease]
Prozone Limit [0] [0] [Lower]
Reagent R1 [250] [0] [00386] [0]
Calibration Type [Linear ] [2] [2] [0] [ ]

The zero calibrator was 154 mmol NaCl / L
The serum calibrator was purchased from Sigma and the current lot number has a value of 48 IU ACE/L
Assay conditions

In this method 30 µL of sample pipetted and 250 µL containing 0.875 mmol FAPGG /L, 400 mmol NaCl /L, 1.54 mmol sodium azide in a 100 mmol triethanolamine /L buffer pH 8.3. This is incubated at 37°C for 10 read cycles (200 seconds), this allows the reaction to stabilise before kinetic measurements are taken. The first reading is taken at cycle 10 at a primary wavelength of 340 nm and a secondary wavelength of 660 nm. The final reading is taken after 48 cycles (960 seconds).

The absorbance change between cycle 10 and 48 is calculated and the average rate of change per minute used to calculate the enzyme activity. Typically the change per minute of the blank is $1 \times 10^{-4}$ Absorbance units and of the calibrator $70 \times 10^{-4}$ Absorbance units. A graph of the 48 individual reading of the zero calibrator and of a 72 IU ACE /L calibrator is shown in Figure 17.
Figure 17. The 48 absorbance readings of the zero calibrator ( ) and 72 IU ACE /L calibrator (+) taken at 20 second intervals. The first reading for the kinetic assay is taken at cycle 10, the last reading is taken at cycle 48, both of these times are marked on the graph.

* Blank = 0 IU ACE /L  + Std = 72 IU ACE /L
3.14 Method Validation on the Hitachi 911.

Imprecision

The imprecision of the kinetic assay using the 100 mM triethanolamine buffer and 0.85 mmol FAPGG/L was determined on control materials obtained from Sigma. The within batch imprecision was evaluated by measuring the controls twenty times in one run. The between batch imprecision was calculated by running aliquots of the controls on twenty separate occasions.

The results of this evaluation are shown on table 4.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within Batch</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>20</td>
<td>39.0</td>
<td>0.83</td>
<td>2.1</td>
</tr>
<tr>
<td>Control 2</td>
<td>20</td>
<td>71.98</td>
<td>0.68</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Between Batch</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td>20</td>
<td>31.5</td>
<td>1.97</td>
<td>6.2</td>
</tr>
<tr>
<td>Control 4</td>
<td>20</td>
<td>69.0</td>
<td>1.87</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Table 4 The imprecision of the SACE assay on the Hitachi 911. The reagent used the 100 mmol triethanolamine/L buffer pH 8.3
The Sensitivity of the SACE Method on the Hitachi 911.

The sensitivity of the method was determined to be 3.21 IU ACE/L this is in contrast to 12.4 IU ACE/l for the same buffer on the Hitachi 704.

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Mean</th>
<th>Sd</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetic</td>
<td>20</td>
<td>0.405</td>
<td>1.284</td>
<td>3.21 IU ACE/L</td>
</tr>
</tbody>
</table>

Table 5 The sensitivity of the kinetic method on the Hitachi 911 using 154 mmol NaCl /L as sample in a precision run.

To further investigate the sensitivity of the method at low levels it was decided to dilute the low control and determine the recovery of the SACE. The Sigma normal control was initially diluted 1:4 with 154 mmol NaCl /L to give a value of 12.3 IU ACE /L. The diluted control was further diluted to give a range of values. The results of this investigation are shown in table 6.
Table 6. Recovery of SACE activity of diluted control sample by the kinetic 100 mmol Triethanolamine /L pH 8.3 method on the Hitachi 911.

### SACE activity in IU/L

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Target Value</th>
<th>Measured Value</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.7</td>
<td>4.8</td>
<td>129</td>
</tr>
<tr>
<td>2</td>
<td>4.9</td>
<td>6.0</td>
<td>122</td>
</tr>
<tr>
<td>3</td>
<td>7.4</td>
<td>8.3</td>
<td>112</td>
</tr>
<tr>
<td>4</td>
<td>9.8</td>
<td>9.8</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>11.1</td>
<td>11.1</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>12.3</td>
<td>13.1</td>
<td>106</td>
</tr>
</tbody>
</table>

**Linearity of the Method on the Hitachi 911**

The linearity of the Triethanolamine method was assessed by using two samples from sarcoid patients which had very high ACE activities as measured by the TRIS-HCl method. These samples were diluted with 154 mmol NaCl/L and analysed on the Hitachi 911 using the 100 mmol triethanolamine /L buffer. The results of this investigation are shown in table 7.

Any ACE activity result which was flagged by an instrument generated non-linearity error (Lin), was repeated as a reduced volume test. In this
case a sample volume of 20 μL instead of the normal 30 μL is used in the assay. The effect of taking the smaller sample volume is compensated for by the computer and the final result is printed as the corrected value.

<table>
<thead>
<tr>
<th>Dil: Target Value</th>
<th>Normal Volume.</th>
<th>Reduced Vol % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE activity in IU ACE /L</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sample 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>242</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>(Lin)</td>
<td>(Lin)</td>
</tr>
<tr>
<td>9/10</td>
<td>218</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>(Lin)</td>
<td>(Lin)</td>
</tr>
<tr>
<td>8/10</td>
<td>194</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/10</td>
<td>169</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/10</td>
<td>145</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sample 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>260</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>(Lin)</td>
<td>(Lin)</td>
</tr>
<tr>
<td>9/10</td>
<td>234</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td>(Lin)</td>
<td>(Lin)</td>
</tr>
<tr>
<td>8/10</td>
<td>208</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>(Lin)</td>
<td></td>
</tr>
<tr>
<td>7/10</td>
<td>182</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/10</td>
<td>156</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7  Linearity of the kinetic assay on the Hitachi 911. Two high ACE activity samples were diluted and assayed. Instrument flagged linearity errors are marked by (Lin)
3.15 The Effect of Bilirubin, Haemoglobin and Lipaemia on the assay.

**Bilirubin.**

Increasing concentrations of bilirubin were added to two serum pools. The samples were then analysed on the Hitachi 911 for SACE by the kinetic method, and bilirubin by the O'Leary method (60). Figure 18 shows that on both samples there was a gradual decrease in the measured SACE activity as the concentration of bilirubin increased. The increase observed on sample 1 at a bilirubin concentration of 600 μmol/L is a change of only one IU ACE/L on a value of 25 IU/L so may not be significant. The dilution effect of the addition of the bilirubin was compensated for by calculation. The addition of the stock bilirubin did not change the pH of the sample by more than 0.1 pH units. The addition of the bilirubin diluent (4.7 mol dimethylsulphoxide/L, 70 mmol sodium carbonate) did not affect the measurement of SACE.
Figure 18  The measurement of SACE activity in the presence of increasing bilirubin concentrations. Increasing concentrations of bilirubin were added to two serum pools. Sample 1 (•) had an ACE activity of 31.7 IU ACE / L and sample 2 (+) had a value of 45.7 IU ACE /L. The results are expressed as a percentage of the ACE activity of each sample before the addition of the chromophore.
Increasing concentrations of Haemoglobin were added to two serum pool. The samples were then analysed on the Hitachi 911 for SACE by the kinetic method, and on the Technicon H2 for haemoglobin. Figure 19 shows that on both samples there was a gradual decrease in the measured SACE activity as the concentration of haemoglobin increased. The dilution effect of the addition of the haemoglobin was compensated for by calculation.
The measurement of SACE activity in the presence of increasing concentration of haemoglobin to two serum pools. Sample 1 (*) had an ACE activity of 31.7 IU ACE / L and sample 2 (+) had a value of 45.7 IU ACE /L. The results are expressed as a percentage of the ACE activity of each sample before the addition of chromophore.
Increasing Lipaemia.

Increasing concentrations of lipid were added to two serum pools. The samples were then analysed on the Hitachi 911 for SACE by the kinetic method. Figure 20 shows that up to a total lipid concentration of 0.5 g/l there is not a significant interference. As the concentration increases above this value there is a reduction in the measured SACE activity. At concentrations above 2 g/l the absorbance of the reaction mixture was too high for the photometer of the 911 and flagged with an error. The dilution effect of the addition of the lipid was compensated for by calculation.
Figure 20  The measurement of SACE activity in the presence of increasing lipid concentrations. Lipid was added to two serum pools. Sample 1 (●) had an ACE activity of 31.7 IU ACE / L and sample 2 (+) had a value of 45.7 IU ACE /L. The results are expressed as a percentage of the ACE activity of each sample before the addition of chromophore.
3.16 The Stability of the Working FAPGG Reagent.

An accelerated stability study was carried out on the working reagent. The basis of this test is that stability of a reagent stored at 37°C for one week would be equivalent to storing the same reagent at 4°C for one year. This principle is commonly used by reagent manufacturers to assess the stability of individual batches of reagent. On day one the reagent was calibrated and controls run, the reagent was then taken from the analyser and stored at 37°C. On days 2, 9, 14 and 21, the reagent was taken from the waterbath and put back on the 911 in order to run a calibration and controls. The results of this evaluation are shown in figure 20.

There was no significant change in the calibration absorbance change or in the value of the controls. The results of this experiment indicated that the reagent could be prepared and would be stable for up to 3 years at 4°C. The actual on board stability of the reagent, which is stored at 4°C is at least three months, as assessed by day to day stability of the calibration and of the between batch imprecision.
Figure 21  Accelerated stability study of FAPGG reagent in 100 mmol triethanolamine buffer pH 8.3. The reagent was stored at 37°C for three weeks. The results are expressed as a percentage of the activity of the calibrator on day one.
In order to investigate the inhibitory effect, six of the most commonly prescribed ACE inhibitors were obtained from the Pharmacy, Cork University Hospital. Stock dilutions of these drugs were prepared as described in the materials and methods section and added to a serum pool. The effect of each drug at concentrations up to 200 nmol/L are shown in Figure 22. The dilution effect of the addition of the solution of drugs was compensated for by calculation.

The Effect of Storage of Serum at 4°C on the Inhibition of ACE by the ACE Inhibitor Captopril

Roulsten et al (69) demonstrated that there is a decrease in the degree of inhibition of ACE by Captopril on storage. This effect was investigated under our routine storage conditions of spinning and separation of the serum and storage for a few days at 4°C prior to analysis. Two samples were spiked with Captopril at concentration ranging from 0 to 100 nmol captopril /L. The ACE activity of the samples was measured within 30 minutes of the addition. The samples were subsequently stored for 24 hours at 4°C and then reanalysed for ACE activity. The result of this experiment is shown in Figure 23.
Figure 22  The effect of six commonly prescribed therapeutic ACE-inhibitors on the SACE activity of a serum pool with a value of 55.4 IU ACE/L.
Figure 23 The effect of storage at 4°C on the inhibitory effect of the ACE inhibitor captopril. Two sera were spiked with captopril and the ACE activity measured within 30 minutes. The samples were stored for 24 hours at 4°C and then analysed again for ACE activity. Sample 1 had an initial ACE activity of 64.8 IU/L and sample 2 had a value of 87 IU ACE/L
3.18 The Accuracy of the set Wavelength of the Shimadzu and Hitachi Photometers.

In order to accurately measure the millimolar absorptivity of the substrate FAPGG in was first necessary to determine if the 340 nm wavelength used was accurately calibrated on the systems. As it is not possible to put a filter in front of the photometer of the Hitachi systems and get a spectral scan, it was decided to use the Shimadzu as a reference for the photometric accuracy of the systems. In order to calibrate the Shimadzu spectrophotometer, three filters, which were originally applied to calibrate a Pye Unicam instrument. These filters were as follows:

1. Vycor 700571. This is filter which is optically clear from 300 nm to 800 nm. No peaks should be seen and is used as a check of light scatter.

2. Holmium 700570 This filter has 9 identifiable peaks at wavelengths from 241.6 to 637.6 nm.

3. Didymium 700871 This filter has 5 identifiable peaks at wavelengths from 572.4 to 807.5 nm.

The glass filters were inserted into the light path by placing them in the cuvette holder. A spectral scan for each filter was run, using air as a reference. The scan of each filter is shown in figures (24,25,26). The accuracy of the monochromator on the system was assessed by comparing the measured wavelength for each peak with the quoted target values (Table 8). The absorbance spectra for these filters were kindly provided by P.J Brennan and Co. Ltd., the Pye Unicam agent in Ireland.
Figure 24: Spectral scan of the VYCOR 700571 filter on the Shimadzu spectrophotometer.
Figure 25: Spectral scan of the HOLMIUM 700570 filter on the Shimadzu spectrophotometer.

Figure 26: Spectral scan of the DIDYMIUM 700871 filter on the Shimadzu spectrophotometer.
<table>
<thead>
<tr>
<th>Peak Number</th>
<th>Filter</th>
<th>Target</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Holmium</td>
<td>241.6 nm</td>
<td>not measured</td>
</tr>
<tr>
<td>2.</td>
<td>Holmium</td>
<td>279.3 nm</td>
<td>279.1 nm</td>
</tr>
<tr>
<td>3.</td>
<td>Holmium</td>
<td>287.6 nm</td>
<td>287.5 nm</td>
</tr>
<tr>
<td>4.</td>
<td>Holmium</td>
<td>333.8 nm</td>
<td>333.5 nm</td>
</tr>
<tr>
<td>5.</td>
<td>Holmium</td>
<td>360.9 nm</td>
<td>360.5 nm</td>
</tr>
<tr>
<td>6.</td>
<td>Holmium</td>
<td>418.7 nm</td>
<td>418.5 nm</td>
</tr>
<tr>
<td>7.</td>
<td>Holmium</td>
<td>453.3 nm</td>
<td>453.5 nm</td>
</tr>
<tr>
<td>8.</td>
<td>Holmium</td>
<td>536.3 nm</td>
<td>536.5 nm</td>
</tr>
<tr>
<td>9.</td>
<td>Didymium</td>
<td>572.4 nm</td>
<td>572.5 nm</td>
</tr>
<tr>
<td>10.</td>
<td>Didymium</td>
<td>585.4 nm</td>
<td>585.5 nm</td>
</tr>
<tr>
<td>11.</td>
<td>Holmium</td>
<td>637.6 nm</td>
<td>637.5 nm</td>
</tr>
<tr>
<td>12.</td>
<td>Didymium</td>
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<td>684.5 nm</td>
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<tr>
<td>13.</td>
<td>Didymium</td>
<td>740.4 nm</td>
<td>740.0 nm</td>
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<tr>
<td>14.</td>
<td>Didymium</td>
<td>807.5 nm</td>
<td>807.0 nm</td>
</tr>
</tbody>
</table>

Table 8  Measured and target wavelengths for peaks detected by running a spectral scan of the Pye Unicam Holmium and Didymium filters on the Shimadzu spectrophotometer.
3.19 Measurement of the Millimolar Absorptivity of FAPGG.

In this section of the project the measurement of the absorbance of both the substrate and product were investigated. The Shimadzu scanning spectrophotometer was used to look at the spectral scans of the substrate FAPGG and the product FAP using a 10 mm light path. The following spectral scans (27,28,29) give an indication of just how difficult it is to differentiate the substrate from the product at 340 nm. The wavelength used as the primary wavelength on both the Hitachi 911 and 704 is 340 nm. The importance of the accuracy of the monochromator on the instrument used is demonstrated in Figure 29. Neither FAPGG or FAP have an appreciable absorbance at the blanking wavelength of 660 nm.

The greatest difference between substrate and product was seen at 339 nm. The specifications for the Hitachi 911 state that the wavelength is 340 +/- 2 nm (85). There is a considerable difference between both the absolute absorbance and the difference between substrate and product from 338 to 342 nm. (Figure 29). It is important therefore to determine the Millimolar Absorptivity of FAPGG on each instrument at the wavelength delivered by that instrument.
Figure 27  Shows the absorbance spectrum of 0.1 mmol FAPGG / L in 100 mmol triethanolamine buffer / L pH 8.3, from 200 nm to 800 nm. This is the substrate for ACE in the assay.

Figure 28  Shows the absorbance spectrum of 0.1 mmol FAP / L in 100 mmol triethanolamine buffer / L pH 8.3, from 200 nm to 800 nm. This is the product of the hydrolysis of FAPGG by ACE.
Figure 29 The absorbance spectrum of 0.5 mmol FAPGG/L and 0.5 mmol FAP/L from 330 nm to 350 nm. The absolute difference between substrate and product at each wavelength is also shown.
Calculation of the Millimolar Absorptivity of FAPGG on Three Instruments.

The method used to determine the millimolar absorptivity in that suggested by Buttery and Gee (65). A 1.0 mmol FAPGG /L and a 1.0 mmol FAP /L were read at 340 nm on the Hitachi 911, Hitachi 704 and Shimadzu Spectrophotometer. The difference in absorbance between the two solutions is the measured and this is equivalent to the millimolar absorptivity at this wavelength with each instrument.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>n</th>
<th>FAPGG</th>
<th>FAP</th>
<th>Millimolar Absorptivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shimadzu</td>
<td>10</td>
<td>2.5000</td>
<td>1.6990</td>
<td>0.8010</td>
</tr>
<tr>
<td>Hitachi 704</td>
<td>3</td>
<td>1.9798</td>
<td>1.3552</td>
<td>1.041</td>
</tr>
<tr>
<td>Hitachi 911</td>
<td>10</td>
<td>1.6449</td>
<td>1.1242</td>
<td>0.867</td>
</tr>
</tbody>
</table>

Table 9  The millimolar absorptivity of FAPGG as measured on the Shimadzu Spectrophotometer, Hitachi 704 and the Hitachi 911.
The use of the Millimolar Absorptivity.

One International unit (IU) of ACE is that amount of enzyme that will hydrolyse 1 μmol of the substrate FAPGG into FAP and glycylglycine in one minute at 37°C under defined conditions including wave length which is assumed to be monochromatic. The enzyme activity in IU/L can be calculated using the millimolar absorptivity by use of the following equation

\[
\frac{\Delta A/\text{min} \times V_t \times 1000}{\Delta e \times V_s \times d}
\]

Where

- \( \Delta A/\text{min} \) = Absorbance change / minute
- \( V_t \) = Total assay volume
- \( \Delta e \) = Millimolar absorptivity
- \( V_s \) = Volume of Serum used.
- \( d \) = Path length in cm

Substituting the values for the method on the Hitachi 704 and 911 into this equation gives the following factors which can be used to calculate the ACE activity.

704 factor = -29619

911 factor = -17942

In the absorbance change per minute is calculated and multiplied by the relevant figure for the instrument thus the enzyme activity can be
calculated. If the absorbance changes of the calibration materials are substituted into the equation then the calculated values are twice the quoted values. On the 911 a control with a target value of 72 IU/L gives a calculated ACE activity of 142 IU/L. On the 704 the same ratio was observed.

This could indicate that the quoted target values are too low or alternatively the measured enzyme activity as measured with in house reagents is significantly higher then the method used by SIGMA to assign a value to the calibrator.

The use of a calibrator of a set value will make the methods appear to give the same values which in fact are reading significantly different.
Chapter 4

Discussion.
4.1 Preamble

The role of angiotensin-converting enzyme in the diagnosis of sarcoidosis was recognised by Lieberman in 1975 (13). This original finding was confirmed by many workers (14). This interest has lead to a diversity of methods being developed using different substrates and instrumentation. As can be seen from Table 10, there is a great variance in the reference interval quoted by different workers even when they are using the same substrate FAPGG.

<table>
<thead>
<tr>
<th>Ref No</th>
<th>Author</th>
<th>Year</th>
<th>Ref Interval</th>
<th>number of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>(55)</td>
<td>Ronca Testoni</td>
<td>1983</td>
<td>43 - 137</td>
<td>42</td>
</tr>
<tr>
<td>(71)</td>
<td>Harjane</td>
<td>1984</td>
<td>34 - 146</td>
<td>66</td>
</tr>
<tr>
<td>(48)</td>
<td>Neels</td>
<td>1984</td>
<td>33 - 148</td>
<td>90</td>
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<tr>
<td>(92)</td>
<td>Buttery</td>
<td>1985</td>
<td>32 - 105</td>
<td>54</td>
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<td>(93)</td>
<td>Hendriks</td>
<td>1985</td>
<td>18 - 93</td>
<td>97</td>
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<td>(73)</td>
<td>Buttery</td>
<td>1993</td>
<td>41 - 139</td>
<td>133</td>
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Table 10 Reference intervals quoted in the literature for SACE. All of the methods use the same substrate, FAPGG, at the same assay temperature of 37°C.
In this project it was proposed that the performance of the method in use in the Biochemistry Laboratory of the Cork University Hospital, be fully defined. The method is based on the hydrolysis of the substrate FAPGG originally discovered by Holmquist et al (54) and subsequently modified by Ronca Testoni (55) to measure ACE in serum. The current procedure is a modification of the method of Maguire and Price (56) and uses the substrate FAPGG in a 50 mmol TRIS-HCl/L buffer at pH 8.3 at 20°C.

The first task in modifying the existing TRIS-HCl buffered method was to investigate each aspect of the procedure with reference to improving the analytical performance. The different areas investigated included:

1. The selection of the buffer.
2. The optimum pH for the buffer.
3. The optimum chloride concentration to be used.
4. The concentration of the substrate FAPGG to be used.
5. Conversion of the existing method from a two point assay on the Hitachi 704 to a Kinetic Assay on the Hitachi 911.

4.2 Selection of the Buffer.

Many different buffer systems have been investigated to assess suitability for use in the assay of ACE; these included TRIS-HCl (56,61), borate-NaOH (55,71) and HEPES (50,72). Buttery and Stewart(73) found that borate-NaOH buffer gave a greater ACE activity than TRIS-HCl. This was contrary to the Ronca-Testoni(55) findings who found them to be equal. Beneteau et al (72) found HEPES at 25 mmol/L gave the highest activity whereas Buttery et al(73) found that 50 mmol HEPES/L gave an even higher activity.
but because it was near the limit of its buffering capacity at pH 8.2, at which point it would be unsuitable in the assay. In this project it was decided to look at a few different buffering systems that had not previously been described in order to see if these also gave an increased activity. An increased enzyme activity would give a greater absorbance change in the assay and so would lead to a more sensitive assay.

The buffers investigated were TRIS-HCL, TRIS-Borate, Triethanolamine-HCl and Ethanolamine - HCl. As can be seen in figure 1 the highest ACE activity was observed in a 100 mmol triethanolamine /L buffer at pH 8.3. As the increased enzyme activity in this buffer would lead to a more sensitive assay it was decided to incorporate it in a modified assay. This buffer had not been investigated in relation to the measurement of SACE in the literature so its introduction into the method made it unique in its own right.

Having introduced the buffer into the method the optimum pH for the measurement of SACE activity in this buffer was evaluated. As can be seen from Figure 2 the optimal pH for a 100 mmol/l Triethanolamine-HCl / L buffer was found to be 8.3 at 20°C, which is equivalent to 8.2 at the measurement temperature of SACE on the Hitachi 704 of 37°C. This finding is in accordance with the optimum pH that found by other workers (55,56,71,72,73).

4.3 Optimum Concentration of Chloride in the Reagent.

The enzyme Angiotensin-Converting Enzyme has been reported to be a halide dependant dipeptidase (4) which requires chloride for its activation. Chloride ions act as a possitive allosteric modifier of ACE and thereby lower the Km for the substrate. This halide dependence varies depending upon the substrate concentration tending to decrease as the Km decreases. The optimum
chloride concentration in the reagent was found to be 400 mmol NaCl /L (Figure 3). This is the same as recommended in the original method by Maguire and Price but is somewhat higher than that quoted by other workers (54,72) who found the optimum to be 300 mmol/L. Badminton et al (74) who used a HPLC separation of substrate and product found it to be 100 mmol NaCl/L. The optimum concentration of chloride is substrate dependant. Hurst et al (75) used Hippuryl-L-Histidyl-L-Leucine as substrate and found the optimum to be 800 mmol NaCl/L.

As the maximum enzyme activity was found with a concentration of 400 mmol NaCl/L, this is the concentration used in the final method.

4.4 Substrate Concentration

The effect of the substrate FAPGG concentration was investigated and the Michaelis Menten constant Km was calculated from the Lineweaver and Burk Plot (Figure 4). The apparent Km from this plot is calculated to be 0.3125 mmol FAPGG/1 which is close to the value of 0.31 mmol/1 quoted by Ronca Testoni(55). Ideally the concentration of the substrate should be at least ten times the Km(76). FAPGG has a high absorbance at 340 nm and a concentration of 3.125 mmol/L would be far outside the measurement limits of the photometer. The concentration of substrate used in the assay must be a compromise between the measurement limitations of the photometer and the enzyme kinetics of the assay. It is undesirable to have too low a concentration of substrate as a very high level of ACE activity in a sample may cause depletion of the available substrate and so lead to an incorrect calculation of enzyme activity. At the same time it is also incorrect to have a substrate concentration that gives a very high absorbance at which the photometer may give a non-linear response. In the method on the Hitachi 704.
a substrate concentration in the reagent of 0.625 mmol FAPGG /L was adopted.

When the method was adapted for the Hitachi 911 this concentration was increased to 0.875 mmol FAPGG /L, which is 2.8 times the Km. This was achieved as the larger sample to reagent ratio gives a substrate concentration in the final reaction mixture of 0.7812 mmol FAPGG/L or 2.5 times the Km. The fact that the Hitachi instruments use a 6 mm light path and have a very sensitive photometer would allow for the higher absorbance values to be measured accurately.

4.5 The Sensitivity of the method on the Hitachi 704.

The sensitivity of the original TRIS-HCl method as well as the Triethanolamine method was assessed and the results shown in table 2.

The results indicate that the sensitivity of both methods on the Hitachi 704 were poor with the new Triethanolamine method being less sensitive then the existing TRIS Chloride method.

A more sensitive assay was required in order to measure low ACE activity in serum and to potentially measure ACE in CSF and other biological fluids. Initially the method was tried as a kinetic assay on the 704 but there was no improvement in the sensitivity. It was then decided that the method would be transferred to the Hitachi 911 and a kinetic assay evaluated on this newer analyser.

4.6 The Imprecision of the method on the Hitachi 704.

The within batch and between batch imprecision were assessed by the Triethanolamine method on the 704. The results of this investigation are
shown in Table 2. The results indicate that the method has low imprecision with a within batch C.V. of less than 2.5% and a between batch C.V. of 6.1%.

4.7 The Evaluation of the Reference Interval.

The reference interval quoted for the TRIS-HCl method (54) currently in use is 0 - 45 IU ACE / L. This is considerably lower than that quoted by other workers using the same substrate (Table 10). An initial investigation using samples sent by general practitioners to the laboratory for routine biochemical analysis indicated that our quoted reference interval was possibly too low. Two hundred blood bank samples (one hundred from male and one hundred from female donors) were assayed by the new 100 mmol Triethanolamine /L method on the Hitachi 704.

Data analysis

The guidelines as set down by the International Federation of Clinical Chemistry were followed with regard to statistical analysis of the data. (67). In clinical practice it is usual to compare an observed patient's value with the corresponding reference interval which is bounded by a pair of reference limits. The interpercentile interval is recommended by the IFCC. (67). It is convention to define the reference interval as the central 95% interval bounded by the 2.5 and 97.5 percentiles. The interpercentile limit is used as a summary or description of the subset reference population. The precision of the percentiles as an estimate of the population is dependant on the number of samples. It is less precise with fewer observations, so it is necessary to also calculate the confidence interval of the percentile. If the assumption of random sampling is fulfilled then the confidence interval
gives the limits in which the true percentile is located with a specified degree of confidence (68). The 0.90 confidence interval of a reference limit would indicate that the true percentile will be found between these limits with a confidence of 0.90 if all the samples in a population were measured.

The analysis of the data in order to determine the interpercentile interval can be done by two methods.

Nonparametric method

The first and simplest method is recommended by the IFCC for general use (67). In the non-parametric estimation of the reference interval the values for the samples are ranked from lowest to highest and the 2.5 and 97.5 percentile are calculated. The non parametric method makes no assumptions about the type of distribution of the data and does not use estimates of distribution parameters. The percentiles are determined by cutting off the required percentage of values in each tail of the subset population.

Parametric Method.

The Parametric estimation technique requires that the data fit into a specific distribution type usually Gaussian. Apart from visual inspection of the data displayed on a histogram, a number of coefficients can also be calculated to indicate if the distribution is skewed (extended tails) or has a significant degree of kurtosis (the degree of peakedness). A distribution which does not fit into the normal Gaussian distribution can be converted to a more acceptable distribution (e.g. using logarithms of the measured data).

Parametric estimates of the fractiles are theoretically more precise with
smaller numbers of samples then those obtained by non-parametric methods, providing that the assumption of the distribution type is valid.

As there was sera from 100 male and 100 female blood donors in the study of the reference interval, it was decided to use this data to determine if there was evidence of a sex linked difference between the reference intervals. As there is 100 samples in each group which is less then the 120 samples recommended by the IFCC for determining the reference interval by the non-parametric method(67). As the parametric method is theoretically more precise using smaller sample numbers, it was decided that this method be used in the calculation of the reference intervals.

4.8 The measured Reference Interval.

The reference intervals of each group is summarised as follows.

Combined male and female samples
Reference interval (with the 0.90 confidence limits shown in brackets) was determined to be 21 (18-23) to 81 (78-86) IU ACE/L.

Samples from female donors.
Reference interval (with the 0.90 confidence interval for each limit in brackets): 20.4 (18.5 - 22.5) to 78.6 (71.4 - 86.6) IU ACE/L.

Serum Samples from Male donors.
Reference interval (with the 0.90 confidence interval for each limit in brackets): 23.0 (20.9 - 25.4) to 87.6 (80.2 - 96.4) IU ACE/L.

The combined reference range assumes that both female and male reference intervals would be subsets of the same reference interval. The ACE values of samples from 100 female and 100 male donors were separated into two populations and the reference interval for each population calculated. The
numbers analysed would be less than the 120 recommended by the IFCC (67) but will still give a good indication of a difference between the genders. A parametric analysis of the data was used as this is theoretically more accurate than non-parametric methods when dealing with smaller numbers of samples (68). When the data was subjected to statistical analysis, a significant difference was observed between the two populations. The male reference interval was found to be approximately ten percent higher than the female reference interval. Non-parametric analysis of the data using one way analysis of variance indicated a significant difference between the two populations \( p = 0.016 \).

This evidence of a sex-related difference agrees with the data of Beneteau-Burnat et al in 1990 (77) who demonstrated that the level of ACE activity in children is higher than adults. They also noted a significant difference in the ACE activity in the serum of boys vs girls in the 13 - 18 age group. The difference may be due to the steroid hormonal regulation of ACE synthesis, the testicular and epididymal form of ACE which develops at puberty is dependant on pituitary function. The development at puberty and the maintenance during adulthood of testicular ACE requires the presence of an intact pituitary and so is under endocrinological control (78). Lieberman (13) also noted a difference in ACE activity between samples from male and female subjects. These findings are contrary to Varela et al (38), Beneteau et al in 1985 (72) and Harjananne (71) who did not detect an age or sex related difference in ACE activity also using the substrate FAPGG.

The possibility of a sex-related difference must be considered when interpreting the significance of an elevated ACE activity. The influence of age on the distribution of results was not investigated as part of this project but would be worthy of further study.

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4.9 Method Comparison

The 100 mmol Triethanolamine /L method was compared with the reference method chosen which was the TRIS-HCl method of Maguire and Price(56).

When plotted on a graph (Figure 16) there appears to be very good correlation between the methods. The correlation coefficient $r = 0.985$, and the regression line is described by the equation:

$$\text{Triethanolamine} = -0.895 + 1.02 \text{TRIS-HCl}.$$ 

The use a non-parametric test, such as the Mann-Whitney to statistically compare the methods indicated that the results were not significantly different at $p = 0.05$

4.10 The Triethanolamine Method on the Hitachi 911.

During the course of this project the Hitachi 911 was purchased for the laboratory. This analyser was more modern then the Hitachi 704 and offered a number of features that could potentially improve the Triethanolamine Method for SACE.

1. The photometer of the new Hitachi 911 was more sensitive then that on a 10 year old Hitachi 704.

2. It is possible to run kinetic assays for up to 16 minutes on the 911. The longer measurement time would give a higher signal thereby improving the sensitivity of the assay.
3. The sample volume is more flexible on the 911.

4. The reagent volume can be reduced to 250 µL so that the sample to reagent ratio, with a normal serum volume of 30 µL, could be increased from 1:18.5 on the 704 to 1:10.7 on the 911.

5. Because of the improved photometer and increased sample to reagent ratio the concentration of FAPGG in the reagent was increased to 0.875 mmol/L. The final concentration in cuvette was 0.781 mmol/L. The method was adapted to run as a kinetic assay as described in the results section the performance of the kinetic assay

4.11 Method Evaluation on the Hitachi 911

Imprecision.

The imprecision on the Hitachi 911 gave acceptable results as can be seen from table 4. The within run imprecision was quite good with a CV of 2.1% at an ACE activity of 39 IU/L. The between run imprecision was higher at 6.2% at a value of 31.5 IU ACE/L but is acceptable. The results were similar to those seen on the Hitachi 704. (Table 2)

Sensitivity

The sensitivity of the modified method on the Hitachi 911 gave a much better performance than on the Hitachi 704 (Table 1). The least measurable amount was calculated to be 3.2 IU ACE/L (Table 5). This is a better sensitivity than either the TRIS-HCl or the Triethanolamine-HCl methods on
the Hitachi 704 (Table 2). This value was confirmed by dilution of a low control and preparation of a dilution curve (Table 6). The improved sensitivity would allow for the measurement of much lower concentrations in serum and offered the potential of the assay of ACE activity in other fluids such as CSF.

**Linearity**

The Hitachi 911 allows for three different sample volumes to be programmed into the parameter page of the method. The normal sample volume for ACE was set at 30 μL and was used for routine analysis. The increased volume option of 50 μL was used when an improved sensitivity was required on a sample. The final result is corrected to take the dilution into account. The final volume programmable is the reduced sample volume in this case a volume of only 20 μL can be used and so increases the linearity. The Hitachi 911 software takes the reduced volume into account and calculates the true enzyme activity.

The results of the linearity investigation as shown in Table 1, would indicate that the method is linear up to 168 IU ACE/L in the normal volume mode and up to 218 IU/L, if the sample is rerun in the reduced volume mode. Any sample presenting with a Lin flag should routinely be re-assayed in the reduced volume method and if it still flags should be repeated in dilution. The linearity on the system would be acceptable for most clinical samples as the number of samples seen with values greater than 168 IU ACE/L would be small. The fact that the Hitachi 911 is capable of detecting a non-linear response in a reaction will flag those samples that need repeating in reduced volume or in dilution.
4.12 The Effect of Chromophoric Interferents in the Assay.

The effect of three potential interferents on the measurement of ACE activity was evaluated. It is not uncommon to find a sample for serum ACE analysis that is haemolysed. Turbidity due to the presence of lipid may be found in sample taken immediately after a fatty meal or in a patient with a problem of lipid metabolism. An icteric sample can be seen from patients with obstructive jaundice, hepatic disease or pre-hepatic haemolytic diseases. It is important to evaluate these materials as a potential source of interference.

Another potential source of turbidity is when plasma samples become turbid on storage at 4°C due to fibrinogen coming out of solution. If these samples are assayed for SACE then a falsely elevated result may be seen. In one case a turbid plasma sample measured by the TRIS-HCl method on the Hitachi 704 gave a value of 286 IU ACE/l and after centrifuging gave a result of 106 IU ACE/l. A simple solution to this problem is to spin the sample in a microfuge tube immediately prior to analysis.

**Bilirubin**

The interference in the method by concentrations of up to 180 µmol Bilirubin/L was not significant. At concentrations in excess of this the measured ACE activity decreased as the concentration of bilirubin increased (Figure 18). From this data samples with bilirubin values less than 180 µmol Bilirubin/L would be acceptable for analysis.
**Haemoglobin**

The effect of increasing haemolysis was investigated and a significant interference was observed (Figure 19). Even 1 g Haemoglobin/L caused a significant decrease in measured ACE activity. As haemolysis also indicates a sample that has been mistreated it would be wise not to assay any sample that exhibits a significant degree of haemolysis.

**Lipid**

The effect of added lipid up to 1 g/L did not cause a significant interference in the assay (fig 20). At concentrations in excess of this there was a significant decrease in measured enzyme activity. The interference may be due to the turbidity of the sample at high concentrations of lipid. Re-spinning of the sample in a microfuge may help by causing the lipid to separate out to the top of the sample. The serum can then be pipetted from below this layer and if it does not exhibit a significant degree of turbidity it can be used in the assay.

**4.13 Stability of the Prepared Reagent.**

An accelerated stability test was carried out on the reagent. The results of the test (Figure 21) show that the reagent is theoretically liquid stable for up to three years. In practice a batch of reagent was prepared and was found to be stable when left on board the Hitachi 911 for a period of three months as assessed by the stability of the calibration from day to day.

The measurement of ACE activity in cerebrospinal fluid has been suggested as a diagnostic marker for Neurosarcoïdosis(79). The presence of measurable ACE activity in CSF may be of two origins. Firstly there may be diffusion of systemic ACE facilitated by a disturbance in the blood brain barrier. Secondly in neurosarcoïdosis when active sarcoid tissue is in contact with the CSF the ACE activity may be a better indication of disease activity than serum ACE(80). When sarcoïdosis involves the nervous system the clinical features include hydrocephalus, cerebellar signs and cranial nerve lesions. Histological confirmation of the disease may be difficult to obtain during life and may be prevented by the clinical need for corticoëstroid therapy. A reliable marker for this condition in CSF would be very useful in screening patients for this condition.

The reference interval quoted by Jones et al of 0 - 1.43 IU/L for a control group was measured by the fluorometric method of Friedland and Silverstine(44) modified for CSF. The ACE activity in the CSF of treated patients with Parkinson's disease has been investigated by Konings et al(81).

This method used the substrate benzyl-oxycarbonyl-phenylalanyl-histidyl-leucinein a method optimised for CSF analysis(73). With this method the reference interval was 0.63 to 0.99 IU ACE /L. As can be seen from the quoted reference intervals for these methods the amount of ACE in the CSF is very low in comparison to serum ACE levels. A method that is optimised for the measurement of the enzyme in serum will not necessarily be suitable for CSF.

The sensitivity of TRIS-HCl is 4.9 IU ACE /L and Triethanolamine-HCl is 12.4 IU ACE /L when run as a two point assay on the Hitachi 704. The Triethanolamine-HCl method on the Hitachi 911 was more sensitive at 3.21 IU ACE /L. Running the kinetic Triethanolamine-HCl method on the Hitachi 911 still did not give a method that was sensitive enough to measure accurately.
at a level of enzyme activity of less than 2 IU ACE/L. The question arises as to whether it is valid to try and measure the activity of the enzyme at such a level. The actual change in absorbance at the level of enzyme activity would be of the order of $1 \times 10^{-4}$ absorbance units per minute. At this level of enzyme activity it may be more accurate to develop a sensitive immunoassay which would measure the enzyme protein itself rather than trying to measure the activity.

4.15 The Effect of Therapeutic ACE Inhibitors on the Measurement of ACE Activity.

Therapeutic ACE inhibitors are used in the treatment of congestive heart failure and operate by lowering systemic arteriolar resistance through vasodilation which leads to a reduction in afterload. Their use in the treatment of hypertension is based on the dampening of the renin-angiotensin system by inhibiting the critical Angiotensin-1-Converting Enzyme which catalyses the production of angiotensin II a potent vasoconstrictor(74). ACE inhibitors are now widely used as a first line drug in the treatment of hypertension, therefore the effect of these therapeutic agents on the activity of ACE in serum was investigated.

The effect of six therapeutic ACE inhibitors on the ACE activity of a serum pool was investigated (figure 22). Two of the drugs, Lisiopril and Captopril gave an immediate inhibition of ACE activity in the serum pool, the degree of inhibition was directly related to the concentration of the drug in the serum.

The other drugs were found to be pro-drugs which require conversion in the body to their active metabolites. Enapril maleate is converted to its active metabolite enalaprilat while perindopril is converted into its active
metabolite perindopril. Quinapril Hydrochloride is converted to quinaprilat and ramipril is converted to its active metabolite ramiprilat (58). The fact that these drugs need to be metabolised in the body to produce the active metabolite would indicate that further study involving clinical subjects would be required to determine if there is a dose related inhibition by these drugs. Some work has already been done in this area by Gorski and Campbell (83) who found that the substrate FAPGG gave a reliable measure of changes in ACE activity in vivo in patients treated with the ACE inhibitors enapril and perindopril. Some further work would need to be done on the other drugs to see if the same effect could be demonstrated. If a direct dose related response could be demonstrated then it would provide a simple approach to monitoring ACE inhibitor therapy. The test would also be useful in determining compliance of the patient in taking the medication.

The Effect of Sample Storage on the Inhibition of ACE by Captopril.

The effect of storage of samples under routine conditions and their influence on the inhibition of ACE activity by the ACE inhibitor captopril was investigated. Under normal circumstances any sample received in the laboratory is centrifuged and stored at 4°C prior to analysis. In this experiment there was almost total inhibition of ACE activity in the serum samples when they were analysed immediately after the addition of the captopril (Figure 19).

A significant degree of this inhibitory effect was lost if the same samples are repeated 24 hours later. This effect was first demonstrated by Roulsten et al (69). They found that that ACE activity measured in serum increased with storage. This finding was subsequently confirmed by Kamoun et al (84) who suggested that the decrease in inhibition is due to a
dissociation between ACE and captopril which may be related to the sulfhydral group of captopril. They found the binding of captopril is stable in serum samples stored at -80°C (liquid nitrogen) for 12 days.

This effect would indicate a need to modify the way routine samples are handled if the method is to be used as a means of monitoring the inhibition of ACE in patients who are on captopril therapy. If the samples were analysed within a few hours then the ACE activity would correlate with the drug level. If the sample was stored then this effect would be reduced and so the measured ACE activity would be much higher.

The inhibitory effect of captopril resembles that of a naturally occurring ACE inhibitor found in the serum of approximately 25 per cent of the samples submitted for ACE assay(8). The role and significance of the natural inhibitor in not known. The effect of the natural ACE inhibitor is removed if the sample is diluted. At least an eightfold dilution of the serum sample is required during the assay to get a true estimate of ACE activity and to obtain an elevated ACE activity in approximately an additional 5-10 per cent of sarcoidosis patients(8).

4.16 The Measurement of the Millimolar Absorptivity of FAPGG.

As is demonstrated in table(11) there is a great deal of variation in the published values for the molar absorptivity of FAPGG. The molar absorptivity of FAPGG was measured according to the method of Buttery and GEE (70) on the Shimadzu spectrophotometer, the Hitachi 704 and Hitachi 911. The result of this experiment is shown in table 10. The Shimadzu gave a molar absorptivity of 0.810 , the Hitachi 704 gave a value of 1.041 while the Hitachi 911 gave a value of 0.867. The absorbtion of both FAPGG and FAP are very sensitive to differences in the set wavelength on the system. The wavelength fidelity of the Shimadzu was checked against filters which had
defined peaks (Table 8). The results of the investigation indicates that the wavelength accuracy of the Shimadzu is very good. The peaks detected are all within 0.5 nm of the target value. The result of this comparison of the theoretical peaks with the actually measured peaks indicate that the Shimadzu monochromator is very accurate in wavelength selection and can be used as a reference for the photometers for the Hitachi 704 and 911. The main interest here is to determine if wavelength selection on the systems are accurate at measuring a peak at 340 nm.

The Hitachi systems use a diffraction grating to produce individual wavelengths. The wavelength at 340 nm is quoted as being +/- 2 nm in the training manual (85). This range in the wavelength would emphasise the importance of determining the molar absorptivity on each individual instrument. If one system is reading 338 nm while another is reading 342 nm then the absorbances obtained and molar absorptivity calculated will also vary (Figure 29). It is therefore important to measure the molar absorptivity on each individual instrument and to check it again after any maintenance procedure on the photometer or diffraction grating. Once derived on an instrument it would be specific for that instrument and would not be interchangeable with other instruments.
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Table. 11  The millimolar absorptivity of the substrate FAPGG as quoted in the literature.

The use of the millimolar absorptivity in the measurement of ACE activity is well established as can be seen from the range of published values (Table 11). The millimolar absorptivity is very much dependant on the wavelength fidelity of the instrument used and if the set 340 nm wavelength on an instrument is actually 342 nm, this obviously will affect the calculation of the factor. As the millimolar absorptivity is directly used to calculate the measured enzyme activity then the variation seen in the quoted values may in some way explain the variation in reference intervals also (Table 10).
A second reason for the differences in the quoted reference intervals may be the difference in substrate concentrations in the different methods (55,56). As substrate concentration is often not greatly in excess of the $K_m$ of the enzyme, the activity of the enzyme is substrate dependant. Similarly the type of buffer used, concentration of chloride in the reagent can also influence the measured ACE activity.

As there are so many variables it is essential that there is an agreed standardisation of the method in terms of substrate concentration and the wave length used. Maguire and Price (86) suggested using a substrate concentration of 0.8 mmol FAPGG/L, a wave length of 340 nm, and with the correct molar absorptivity being established for each instrument. Roulsten (87) suggested that there could be a number of reasons for the difference in reference intervals. Firstly the relatively small numbers of samples used to establish the reference intervals, Secondly the presence of a significant lag phase in samples with low ACE activity so the setting of a time window in the assay is important. In order to get agreement between different methods it is necessary to calibrate against an external reference material as suggested by Roulston (87). This paper also supports the use of physicochemical tests of the wavelength fidelity of the photometer being used.

Buttery (88) also supports the use of an enzyme reference material to help with the standardisation of the method. If manufacturers can develop an appropriate enzyme reference material then it would aid in the standardisation of the method. The influence of the instrumentation used rather then the methodology was considered to be the main influence on the variation in the reference interval.

Moss (89) in his paper suggests the use of a uniform common calibrator in methods that are themselves different, rather then standardising the
methods to an agreed single method. This approach would have inherent risks as the calibrator would have to behave exactly the same in a number of methods. Evans (90) on the other hand felt that the unconditional use of an enzyme calibrator may provide an illusion of a number of methods giving similar values for specified reference or control materials, while samples containing therapeutic ACE inhibitors may yield unacceptable variations. Similarly Gerhardt (91) felt that the use of calibrators in different methods is an illusion as enzyme determinations by catalytic activity are method dependant. He suggests that method standardisation should be done by measuring the catalytic activity of the enzyme and comparison with continuous external quality assessment.

If a commercial enzyme calibrator is to be used on a number of different instruments then it is necessary that there is a degree of standardisation in the methods being used otherwise the calibration would be invalid. If the methods are the same then the use of a calibrator specifically prepared for that method will give accurate results. Its use will also compensate for any slight variations in the photometers of the various system.

It is necessary to standardise the method in terms of:

1. Buffer Used
2. pH of buffer
3. Wavelength used
4. Chloride Concentration in the reagent.
5. Use of an external reference material or millimolar absorptivity
Chapter 5

Conclusion
In Conclusion.

The introduction of the 100 mM triethanolamine buffer into the kinetic ACE method along with the optimisation of each of the assay components has resulted in an improved method. The evaluation has shown the modified method to perform better than the original method in terms of sensitivity, accuracy and imprecision. The reagent is liquid stable and is linear to 167 IU ACE/l in the normal mode or to 218 in the reduced volume mode.

With reference to the variation in reference interval quoted for different methods it is desirable that a standard method should be recommended. The ultimate objective would be a kinetic assay that has a common reference interval for assays using the same reagent on different analysers. A potential recommended method would be as follows

1. Buffer used i.e. TRIS HCl
2. Molarity and pH of buffer. i.e. 50 mmol/L pH 8.2 at 37°C
3. Substrate used - FAPGG
4. Concentration of substrate - 0.8 mmol/L
5. Saline concentration - 400 mmol/L
6. Wavelength of 340 nm
7. Determination of the correct millimolar absorptivity for each instrument.
8. If calibration material is used then the value assigned by the manufacturers must be determined using the same assay conditions as described.
9. All assays must be run at the same temperature of 37°C.
10. Each laboratory should participate in external quality assurance schemes.

Maguire and Price (86) also suggested using a 0.8 mmol FAPGG/L at a wavelength of 340 nm with the correct measurement of the millimolar absorptivity. They did not however make reference to the type or molarity of the buffer or to the assay temperature.

The diversity of methods available along with intra-instrument variation in wavelength fidelity would be the primary reason for the differences observed. The above recommendations would reduce the variability of the method and so lead to a convergence of reference intervals.

In reference to the assay of ACE in CSF the sensitivity of the spectrophotometric method in not good enough to measure the low activity present. The use of a sensitive immunoassay to measure the enzyme protein would be recommended.

Future work on ACE.

The question of using the SACE assay as a bioassay of therapeutic ACE inhibitors is worth further investigation. Some of the ACE inhibitors, as discussed, are pro-drugs that are converted to active metabolites in vivo. A study to investigate the inhibitory effects of these drugs would require a close liaison between laboratory and clinical staff in order to determine a dose response relationship. More work also needs to be done in relation to the male vs female reference intervals and to look into age related differences. The use of a serum calibrant vs millimolar absorptivity needs to be investigated fully in relation to different methods.
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