OPTIMUM TIMING FOR VITAMIN A SUPPLEMENTATION IN CHILDREN WITH DIARRHOEA

By

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DEDICATION

I would like to dedicate this thesis to my Mother and my Father who always believed in me and encouraged me to realise my dreams and to achieve my aims in life.

DECLARATION

This study represents the original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.
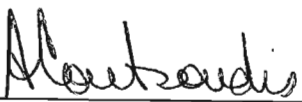
The research described in this study was carried out in:

- The Department of Chemical Pathology, School of Pathology and Laboratory Medicine;
- The Department of Paediatrics and Child Health, School of Clinical Sciences; under the supervision of Professor Anna Coutsoudis.

Karin Inga Elson

I hereby certify that the above statement is correct.

Prof. Anna Coutsoudis

ACKNOWLEDGEMENTS

SUMMARY

Vitamin A has well recognised benefits for the reduction in severity of diarrhoeal episodes but the impact of therapeutic doses given during diarrhoea on the biochemical and clinical outcomes is less clear. In this study these potential therapeutic benefits were investigated to establish the optimum time for vitamin A supplementation to improve vitamin A status. Establishing the optimum time for vitamin A supplementation during an infectious stage would improve cost-effectiveness and clinical benefit.

Young children (174) between the ages of 3 and 60 months with severe diarrhoea were randomised in a double - blinded placebo controlled trial into one of 2 groups. The 1st group received 60 mg of retinol as retinyl palmitate on admission during the acute diarrhoeal stage. The 2nd group received the same dose of vitamin A once symptoms had resolved, usually between 3 - 7 days. At each of these two time points, children not receiving vitamin A were given an identical placebo dose. Baseline (day 0) and day 3 serum samples were collected for vitamin A, retinol binding protein (RBP) and other biochemical markers. At four and eight weeks after discharge both morbidity and weight gain were recorded. The modified dose response test (MRDR) was conducted at the eight - week follow - up to estimate vitamin A liver stores.

Initially, most of the children presented with watery diarrhoea and dehydration and were clinically very ill. At day 3 plasma retinol concentrations improved in both groups viz. from 0.57μmol/L to 0.97μmol/L in the 1st group and from 0.49 μmol/L to 0.90 μmol/L in the 2nd group. Similar improvements were

found in retinol binding protein viz. 21.28 mg/L to 31.06 mg/L in the 1st group and 17.05 mg/L to 24.80 mg/L in the 2nd group. At 8 weeks there was also no significant difference between the two groups either for serum retinol (0.69 μmol/L and 0.73 μmol/L respectively) nor for MRDR ratios (0.036 and 0.049 respectively).

The MRDR results at 8 weeks indicated that these children did not have depleted vitamin A liver stores and that the low serum retinol levels seen at baseline were probably due to the acute phase response during an infectious episode.

The results of these analyses showed no significant difference between the two treatment groups thus indicating that there was no benefit to giving vitamin A on recovery from an infectious episode instead of on admission, as is currently practised.

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CHAPTER 1

1.0   INTRODUCTION AND JUSTIFICATION OF STUDY

Improving vitamin A status of young children in low income countries has been associated with a 23% reduction in mortality of children aged 6 months to 5 years (Beaton *et al*, 1993).   This effect was shown regardless of the nature of the prophylactic interventions (whether high dose supplement, low dose supplement, or increased frequency of eating vitamin A - rich foods) provided that vitamin A status was improved (Beaton *et al*, 1993; Fawzi *et al*, 1995; English *et al*, 1997).

These results have encouraged promotion of vitamin A supplementation programmes to young children through all available channels (WHO/UNICEF/IVACG, 1998).   One such channel is any contact these children may have with the health services which is not only a convenient opportunity but may also reach sick children from the poorer sections of the population who are malnourished and more at risk of deficiency.   Infection itself may decrease vitamin A status (Rhaman *et al*, 1996) which strengthens the rationale for supplementation at this time.   Infection may also reduce the bioavailability of vitamin A as vitamin A levels are affected by infection in varying degrees and the degree of the decline is related to the severity and duration of the illness (Mitra, 1998a).   Acute phase proteins are elevated, during illness, and are associated with low retinol levels (Filteau *et al*, 1993).

Although intestinal malabsorption of vitamin A is probably not a major concern except in very severe illness (Mansour *et al*, 1979; Ahmed *et al*, 1993), urinary excretion of retinol bound to retinol binding protein (RBP) may have a

negative impact on vitamin A status (Mitra *et al*, 1998b). Therefore, for improving vitamin A status, it may be preferable to delay supplementation of a child with diarrhoea until after diarrhoea symptoms have resolved.

Such a delay in supplementation may however, limit the usefulness of vitamin A as an adjunct treatment for the presenting diarrhoea illness. Therapeutic benefits of vitamin A for diarrhoea are much less well established than prophylactic benefits. It is likely that the variable results of therapeutic trials are due to variations among the populations, causative pathogens, and complications and severity of the illness.

The best time for supplementing children with acute diarrhoea is not currently known, therefore this study was designed to determine whether vitamin A supplementation should be given to children on admission or after diarrhoea symptoms have resolved.

Most studies measure effectiveness of vitamin A supplementation by measuring serum retinol, however this is limited by the fact that this is only an indicator of circulating vitamin A and not liver stores (except in severe deficiencies). In addition, the carrier protein of serum retinol, RBP, is an acute phase reactant and is lowered during infection. Therefore low serum retinol concentrations are not necessarily indicative of vitamin A status. To compensate for this, the relative dose response tests, which are proxy measures of vitamin A stores, are now becoming more widely used.

In this study we therefore chose to use the modified relative dose response to determine vitamin A status as it was believed to be the most accurate measure of vitamin A status, since it is an indirect measure of liver stores of vitamin A.

Young children with severe diarrhoea were randomised to receive 60 mg retinol palmitate during acute diarrhoea or once the symptoms had resolved, usually after 3 - 7 days, and placebo at the other time point. Plasma retinol, retinol binding protein and other biochemical markers were measured at days 0 and 3. At a four and eight - week follow - up after discharge the morbidity and weight gain was monitored. At eight weeks the children also had a modified relative dose response test to measure vitamin A liver stores.

CHAPTER 2

2.0   GENERAL BACKGROUND

2.1   HISTORY OF VITAMIN A

Hippocrates first mentioned that vitamin A was used for the treatment of nightblindness as far back as 3500 years ago.   In the 18th and 19th century physicians recognised the link between vitamin A deficiency (VAD) and corneal problems and other systemic diseases.   By mid 1800's there was enough proof to demonstrate that diet played an important role.   This was demonstrated in a study, by Magendie, in 1816 showing that dogs starved on sugar and distilled water developed perforating corneal ulcers resembling those in "ill-nourished infants" (Sommer and West, 1996).   An observational study by Hubbenet on children at a French orphanage, suggested that corneal complications in the children could be attributed to a poor diet (Sommer and West, 1996).   It took another 100 years before investigators realised that these changes were caused by the lack of a specific nutrient, vitamin A.

A number of studies were carried out by researchers on dietary deficiencies, which by 1920 showed convincingly that VAD resulted in growth retardation and reduced resistance to infection which subsequently resulted in ocular changes.   Xerophthalmia was also being recognised throughout the world and cod - liver oil was a recognised cure for nightblindness and xerosis conjuctivae (Sommer & West, 1996).

During the 20th century numerous studies, including animal models, were carried out on varying aspects of vitamin A deficiency.   Much attention was paid

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to xerophthalmia, however other complications occurring in VAD were also investigated. The scientists who led the way in this research pathway were Hopkins, McCollum, Osborne and Mendel, carrying out numerous studies on animals to determine the cause and effect of VAD (Sommer and West, 1996). Block, a paediatrician in Denmark, did numerous studies showing the aetiology, prevention and cure of diseases related to vitamin A deficiency (Sommer and West, 1996). Blegvad also carried out studies using children with xerosis conjunctivae and keratomalacia. He found that the children who received Vitamin A recovered while the others either died or became totally or partially blinded (Blegvad, 1924).

McCollum discovered fat soluble vitamin A in 1913 and Moore demonstrated the conversion of carotene to vitamin A. The structural formulae of β - carotene and vitamin A were later established by Paul Karrer and his colleagues at the University of Zurich between 1928 - 1931. Karrer was awarded the Nobel Prize for chemistry in 1937. In 1945 - 46, β - carotene and vitamin A was synthesised by Otto Isler and his team at Roche in Basel, and official production of commercially available vitamin A started in 1948 (McLaren and Frigg, 2001).

Numerous animal studies, clinical observational and supplementation studies were set up to examine the causes and consequences of VAD in more depth. From the observational studies it was noted that a correlation existed between xerophthalmia and vitamin A deficiency and there was evidence that also linked xerophthalmia to high mortality rates (Sommer 1982a; Sommer et al, 1987; de Sole et al, 1987). This later led to the investigation of vitamin A status among these populations and subsequently the introduction of supplementation trials.

2.2   SOURCE OF VITAMIN A

Vitamin A is a fat soluble vitamin.   It is found mainly in animal products such as liver, meat and to a small extent in kidneys, fish liver oils, butter and milk, as preformed Vitamin A.   Provitamin A carotenoids are the group of carotenoids that may be converted into vitamin A and are found in coloured fruits and orange and green - coloured vegetables.   The colour of the carotenoid is masked in the green leafy vegetables by the presence of chlorophyll.   The transformation of provitamin A to vitamin A occurs in the intestinal walls of animals and in the liver of humans.

There are approximately 600 carotenoids in nature and only three of these are important to humans: β - carotene, α - carotene, and β - cryptoxanthin.   Of these, β - carotene is the major provitamin A component of most carotenoid - containing foods and can provide adequate vitamin A nutrition (Tang *et al*, 1999).

Supplementation with both vitamin A or β - carotene seem to be an effective way of eliminating vitamin A deficiency.   This was shown in a randomised, blinded study on children aged 2 - 15, in a rural area in Senegal, using vitamin A and β -carotene treatment for 7 weeks.   There was an improvement of 51.2% and 50% respectively, in eye cytology (Carlier *et al*, 1993).   The benefits of vitamin A and β - carotene supplementation were also demonstrated in a community - based trial on pregnant women a few years later in Nepal.   After weekly dosing of 7 000 retinol equivalents (RE) of vitamin A or β - carotene given during pregnancy there was a reduction in the incidence of xeropthalmia in the first 6 months postpartum of 50 - 60% using vitamin A and 30 - 40% using β - carotene (Christian *et al*, 1998).

2.3 CHEMISTRY OF VITAMIN A AND RETINOL BINDING PROTEIN

Vitamin A is a subgroup of a class of compounds known as retinoids.   All retinoids are derived from a monocyclic parent compound containing five carbon - carbon double bonds and a functional group at the acyclic end (Figure 2.1).   The β - ionone derivatives (other than carotenoids) that have the biological activity of all - *trans* retinol are generically referred to as vitamin A.
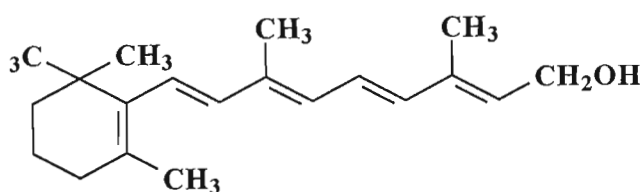
Fig. 2.1   all - *trans* retinol

Vitamin A has a molecular weight of 286,44 Daltons (Da) and is an alcohol also known as retinol.   It is found in nature in the all - *trans* form and exists as pale yellow crystals that are soluble in fats.   Provitamin A are compounds that can be converted into Vitamin A, one such provitamin A being β - carotene which can be converted into retinal (vitamin A aldehyde) and then into retinol (vitamin A).

For provitamin A activity to occur, the compound must include at least one unsubstituted β - ionone ring and a polyene side chain, with the other end of the molecule having a cyclic or acyclic structure.   It may be lengthened but not shortened to less than an 11 - carbon polyene chain.
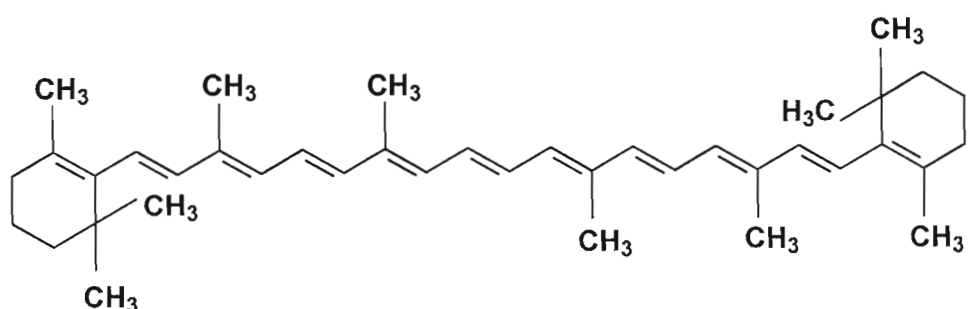
Fig. 2.2 β - Carotene

Carotenoids are biologically less active than retinol and their dietary sources are also less efficiently processed and absorbed from the gut. One molecule of beta - carotene (Figure 2.2) produces two molecules of Vitamin A (Figure 2.1). β - carotene was considered to be one sixth as effective as retinol itself. This is known as the Retinol Activity Equivalence (RAE). The Joint FAO/WHO Expert Group (1976) introduced the term Retinol Equivalent (RE) and assigned the value of 1/6 for β - carotene and 1/12 for the other provitamin A carotenoids, of the value of preformed vitamin A (Table 2.1).

Table 2.1   Vitamin A Conversion Table (McLaren & Frigg, 1997).

1 Retinol Equivalent (μg RE)	= 6μg β - carotene
	= 12μg other provitamin A carotenoids
	= 3.33 IU vitamin A (VA) activity from retinol
	= 10 IU VA activity from beta - carotene
	= 5 IU VA activity from other provitamin A carotenoids

The above conversions were done on very scanty evidence (Hume and Krebs, 1949) and remained unchallenged for many years. This value is now under discussion and further research has been carried out to reassign a more accurate value. Research seems to indicate that the conversion factor is much higher than 6:1 as shown in a study by de Pee (1998) where the value calculated for β - carotene : retinol equivalence in leafy vegetables and carrots was as high as 26:1. To enable a more accurate and efficient way of evaluating bioavailability, a new method of assessment is currently being developed utilising extrinsic - stable - isotope - labelled vitamin A (Parker *et al,* 1999).

At the recent International Vitamin A Consultative Group Meeting (IVACG), in Vietnam, the latest equivalence factors were discussed. These new RAE values were drawn up by the US National Academy of Sciences. The new values suggest that 1μg retinol is equivalent to 12μg dietary β - carotene; and 24μg for the other provitamin A carotenoids (α - carotene and β - cryptoxanthin) (West, 2001).

In almost all the tissues vitamin A functions are carried out by the acid form, retinoic acid, and more specifically all - *trans* - retinoic acid (Figure 2.3).
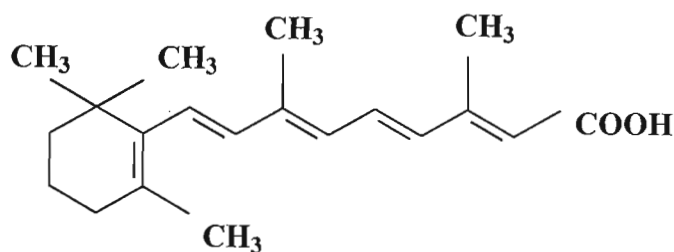
Fig. 2.3 all - *trans* retinoic acid

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Didehydroretinol has a molecular mass of 284,44 Da and is chemically very similar to the vitamin A structure, the only difference being the extra double bond (Figure 2.4).
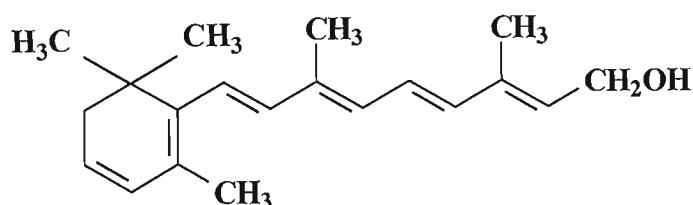
Fig. 2.4   3,4 – didehydroretinol

Plasma retinol binding protein was first discovered by Kanai *et al* in 1968. This three dimensional structure was later resolved by Newcomer *et al* in 1984 and the protein was found to have a unique, predominantly β - sheeted, structural fold.

Retinol binding protein (RBP) has been fully characterised as a single polypeptide chain with 182 amino acids in a known sequence, and a molecular weight of 21 230 Da. The structure of the RBP molecule (Figure 2.5) is three dimensional with a central core which is made up of eight antiparallel β strands forming a flattened hollow cone which contains a specialised pocket (hydrophobic pocket) into which fits the fat - soluble retinol (Sivaprasadarao and Findlay, 1994). The RBP is a member of a family known as the lipocalin superfamily.
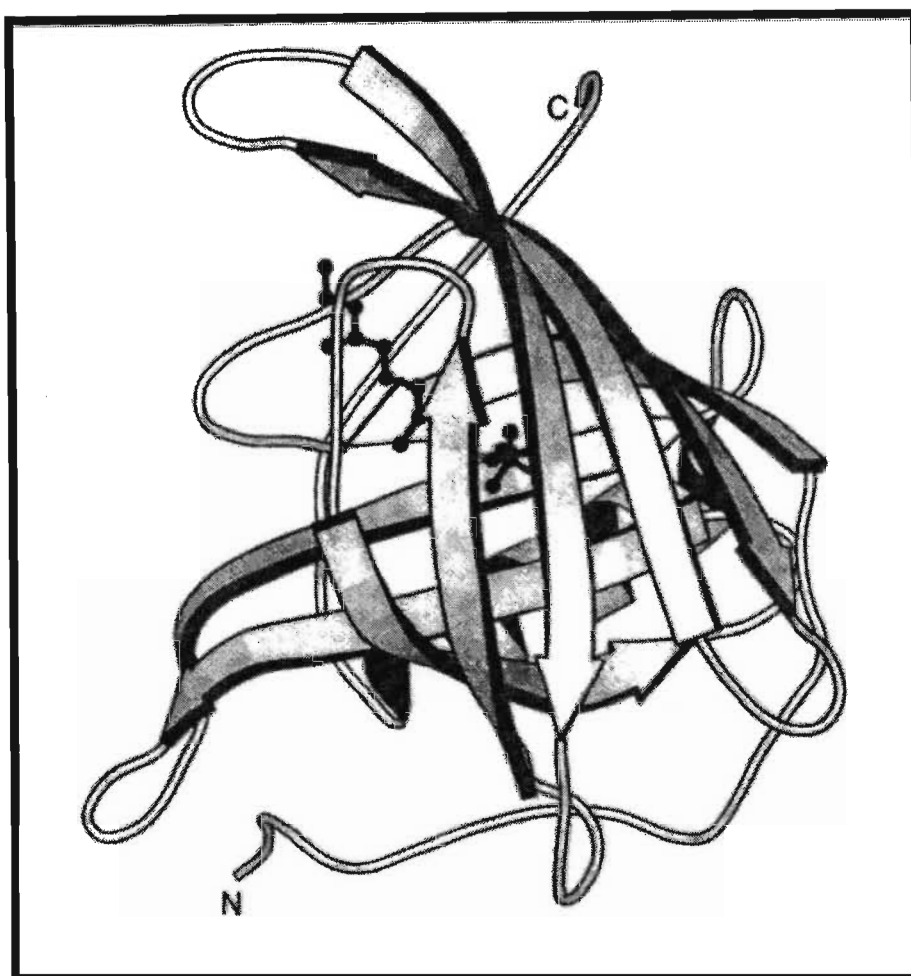
Fig. 2.5 **Schematic drawing of retinol binding protein (RBP) three - dimensional structure. Derived from the crystal structure of human retinol binding protein (RBP) (Zanotti G, *et al,* 1993). Prepared using the molecular graphics program MOLSCRIPT (Kraulis P, 1991).**

2.4   METABOLISM OF VITAMIN A AND RETINOL BINDING PROTEIN

The intake of vitamin A in the diet results in about 10% not being absorbed, 20% appears in the faeces through the bile, 17% is excreted in the urine, 3% is released as CO_2, and 50% is stored in the liver (Olson, 1994).   A simplified schematic outline of these metabolic pathways is shown in Figure 2.6.

Dietary preformed vitamin A and carotenoids are released from digested protein in the stomach by proteolysis.   Aggregates are formed with lipids and they pass into the upper small intestine.   Dietary fat, protein and their hydrolytic products stimulate the secretion of bile, through the secretion of a hormone known as cholecystokinin.   This emulsifies the lipids and promotes the formation of micelles that have lipophilic groups on the inside and hydrophobic groups on the outside.   This facilitates the absorption of fats.

Bile salts stimulate pancreatic lipase and other esterases which hydrolyse retinyl esters in the intestinal mucosal cells.   Retinol is a product of this hydrolysis and is well absorbed by the mucosal cells (Figure 2.6).

Dietary Source

| RETINOL | RETINYL PALMITATE | BETA-CAROTENE |

Hydrolase
(pancreatic juice)

RETINAL

Dioxygenase
(intestinal muco

Intestine

*Metabolism &
absorption*

RETINOL

Aldehyde
reductase

Emulsification and adsorption
Re-esterification with palmitic acid

RETINOL & ESTER

Liver

storage

Blood

transport

RBP : RETINOL
RBP : RETINOL : TRANSTHYRETIN

Periphery

utilization

| Epithelial cells | Other target cells | Photoreceptors |

Fig. 2.6   Schematic diagram of vitamin A metabolism (Sommer, 1995).

Over half of ingested retinol (70%-90%) is absorbed in the small intestine and transported to the liver where it is stored primarily as retinyl palmitate (Figure 2.7).
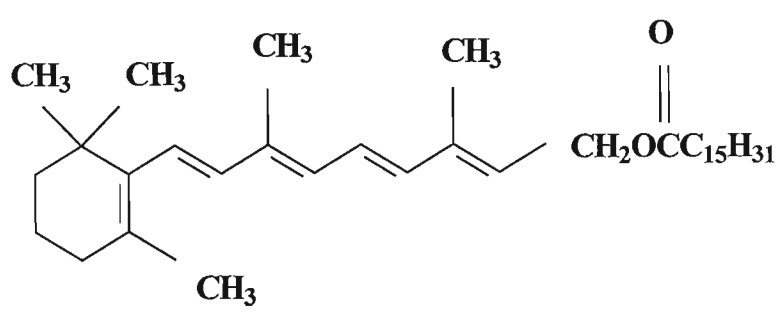
Fig. 2.7   retinyl palmitate

Retinol is esterified before it is incorporated into the chylomicrons (Figure 2.8) by an enzyme known as lecithin:retinol acyltransferase (LRAT) which is also responsible for delivering the retinol to the chylomicrons.
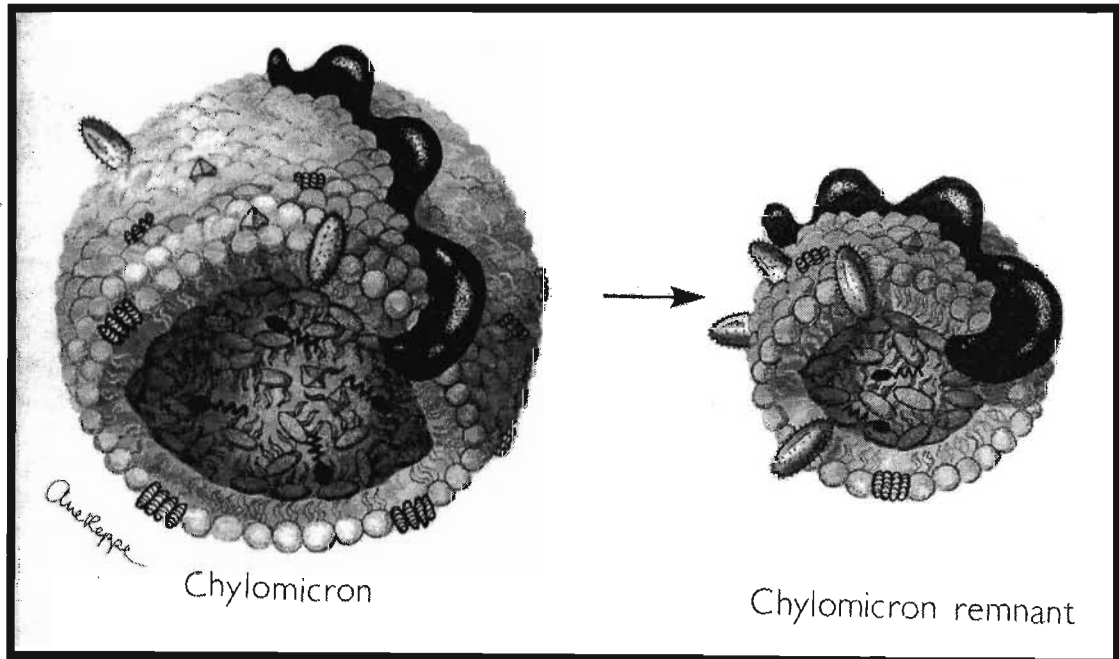
Fig. 2.8   Schematic drawing of chylomicron and chylomicron remnant (Blomhoff, 1994).

The chylomicrons pass into the lymph and general circulation and are broken down into remnants (Figure 2.8), where the retinyl esters remain, and are cleared by the liver. Work done by Blomhoff (1994) found that the chylomicrons also deliver the retinyl esters to the lungs and other tissues including some cancer cells. In the liver, vitamin A, as retinyl esters in the chylomicron remnants, are taken up by the hepatocytes. There the esters are hydrolysed and transferred to the endoplasmic reticulum. The retinol, when needed, combines with retinol binding protein (RBP) and after the complex enters the Golgi complex it is released into the bloodstream (Figure 2.9).
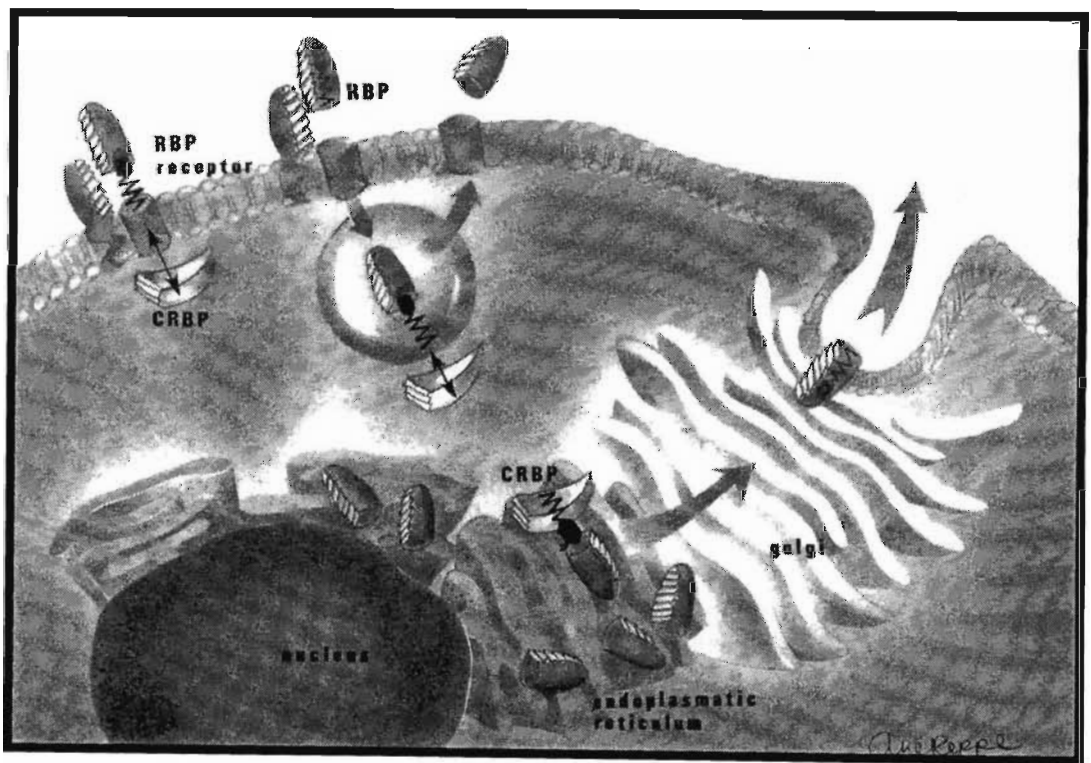
Fig. 2.9 Hypothesis for cellular RBP metabolism. RBP - retinol may be recognised by a cell surface receptor. Retinol may be transferred to CRBPs either at the cell surface or after internalisation into endosomes. CRBP may also deliver normal retinol to newly synthesised RBP in the endoplasmic reticulum (Blomhoff, 1994).

Retinol binding protein is a specific carrier protein in the liver and forms a 1:1 complex with vitamin A known as holo - RBP. The retinol free RBP is known as apo - RBP. The retinol - RBP complex combines with transthyretin (TTR), which is a large protein synthesised in the liver. Each retinol binding protein molecule interacts with three transthyretin molecules (Rostom et al, 1998). The formation of this complex reduces the loss of retinol in the glomerular filtrate (Naylor and Newcomer, 1999).

Studies in rat hepatocytes by Yamamoto et al. (1997) showed that the amount of retinol taken up by the RBP/TTR complex was nearly twofold that of the RBP alone. These results indicated the importance of TTR in retinol delivery especially to the vitamin A storing stellate cells in the non - parenchymal cell fraction of the liver.

The bound RBP is released from both the stellate and parenchymal cells in the liver. The main function of these stellate cells is the storage of RBP. Fifty to eighty percent of the vitamin A is stored in the liver of which 90% - 95% is in the stellate cells and 98% is in the form of retinyl esters mostly as palmitate. This store in humans is usually enough to last several months. However, in the absence of retinol due to vitamin A deficiency, the RBP accumulates in the liver and this is the basis of the relative dose response tests.

Retinol when released from the retinol - binding protein, needs a moderate local decrease of pH and dielectric constant in the proximity of the target membranes. This physiological function was simulated in vitro by Bychkova et al. (1998). The retinol is removed from the serum and utilised by target cells, such as retinal photoreceptors and the epithelial linings throughout the body. The

retinol when released from these tissues is in the form of holo - RBP, lipoprotein - bound retinyl esters or water - soluble retinyl β - glucuronides. All of these are transported back to the liver for recycling. When vitamin A intakes are low the efficiency of this recycling process increases.

When the intake of vitamin A is above the daily requirement needed (180 - 450 μg/day), the excess is stored in the liver and thus the liver stores are increased. When the intake is less, the liver stores are used to maintain the level required in the serum i.e. above 0.7 μmol/L. If this condition is prolonged then the liver stores are depleted and the serum retinol level drops. This results in clinical and physiological consequences. The RBP level in the serum also decreases as there is no vitamin A in the liver stores to transport. Normal concentrations of RBP in well - nourished adults are 1.9 - 2.4 μmol/L (40 - 50 μg/ml) and the value for children up to the age of puberty is about 60% of that of adults.

However the availability of stored vitamin A is also dependent on the child's nutritional and physical health status. Severely malnourished children synthesis RBP at a much reduced rate. Therefore retinol levels in the serum can be reduced even if the liver stores are high. A diseased liver will also result in reduced vitamin A stores as it cannot store as much retinol or metabolise as much RBP as a normal liver.

During infection the release and absorption of RBP and retinol may be reduced due to various predisposing factors related to the specific infection. This was demonstrated in a study using vitamin A deficient rats and endotoxin - induced inflammation. It was found that inflammation - induced hyporetinemia does not

imply vitamin A loss but redistribution of tissue vitamin A brought about by reduced

hepatic synthesis of retinol binding protein (Rolales & Ross, 1998b).

2.5 RECOMMENDED DOSAGE OF VITAMIN A

Acute ingestion of large amounts of preformed vitamin A can result in alarming side effects, such as increased intracranial pressure which can result in headaches, nausea, vomiting and visual disturbances (Swaminathan *et al*, 1970; Kusin *et al*, 1980; Floretino *et al*, 1990). One of the most common side effects of vitamin A supplementation in infants is the bulging fontanelle (West *et al*, 1992; West *et al*, 1995; Humphrey *et al*, 1996). This mechanism is not fully understood, however it is reversible (Humphrey *et al*, 1996). Chronic overdosing can also result in liver and bone damage, hair loss and skin changes (Persson *et al*, 1965; Seigel & Spackman, 1972; Mahoney *et al*, 1980).

Vitamin A deficiency is common among post partum women and consequently their infants living in deprived situations. Intervention at this stage can be beneficial to both mother and the unborn child as this improves her vitamin A status and that of her breast - fed infant. Supplementation has been recommended by WHO - UNICEF - IVACG since 1982 for post partum women with a vitamin A deficiency (Sommer, 1982a; WHO/UNICEF/IVACG, 1988 and 1997). The recommended dose of 200 000 IU has however been found to be too low and a much higher dose of 400 000 IU was found to be well tolerated (Iliff *et al*, 1999). However the WHO recommendation for mothers < 6 weeks post - partum is 200 000IU (WHO, 1998).

Infants are born with limited vitamin A stores however these stores are increased 10 fold during the first 6 months in infants who are breast - fed by well nourished mothers. Infants of mothers with low vitamin A levels receive "only sufficient" vitamin A to meet the basal needs with no surplus available for the liver

stores.   Studies done in Bangladesh and Indonesia by Stoltzfus *et al* (1993), Wahed *et al* (1997) and Rice *et al* (1999), reported that over 90% of the 6 month old babies had inadequate liver stores (defined as being RDR or MRDR positive).

Supplementation with vitamin A in children under 6 months is still very controversial and inconclusive in both the aspects of safety and benefit (West *et al*, 1992; Agoestina *et al*, 1994; West *et al,* 1995; Humphrey *et al*, 1996).   The recommended dose for infants that fall in the age group < 6 months is 50 000 IU and based on the findings from a trial in Nepal young infants under the age of 4 months should not be given more than 50 000 IU.   In one study it was found that the side effects were more pronounced in the children that were below 4 months of age, when the doses exceeded 50 000 IU for these infants (West *et al*, 1992). Another study at King Edward Hospital in Durban, South Africa, showed that three doses of 25 000IU of vitamin A given to low birth weight neonates over a period of 8 days was well tolerated and showed no side effects in any of the infants (Coutsoudis *et al,* 1996).

The earlier WHO recommendations for dosage of infants less than 6 months old that are not breast - fed was 50 000 IU, preferably at about 2 months of age, or two doses of 25 000IU given with an interval of a month or more in between. The supplementation dose for older children was 100 000 IU for children of 6 - 11 months and 200 000 IU for children 1 year and older, given every 3 - 6 months. (WHO, 1998).

Following a technical consultation to review recent studies, WHO now recommends 200 000 IU at delivery for the mother or within 6 weeks of delivery. Infants should receive 50 000IU at 6, 10, and 14 weeks (to coincide with

immunisation visits) and 100 000IU at 6 - 11 months, with a further 200 000IU every 4 - 6 months thereafter (WHO, 2000).

2.6   PHYSIOLOGICAL ROLE OF VITAMIN A

Vitamin A is commonly known as the anti - infective vitamin and has an essential role in vision and cellular differentiation.   Changes to the epithelial lining of vital organs occur in the early stages of vitamin A deficiency (Mc Cullough *et al,* 1999).

Vitamin A plays an important role in vision and is necessary for growth, normal development and differentiation of cells and tissues.   Vitamin A maintains the structure and function of the immune system and is responsible for the maintenance of general health and combating infections.

2.6.1 Vision

The best understood of the actions of vitamin A is its role in vision.   The 11 - *cis* form of retinal (Figure 2.10) is a component of the visual pigment rhodopsin, which is found in the rods of the retina.   When exposed to light the retinal changes to the 11 - *trans* form resulting in a series of changes occurring in the optic area, with the end result being the recognition of light by the brain.
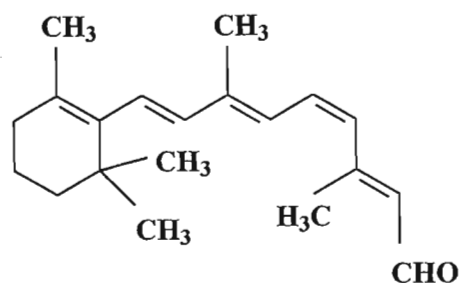
Fig. 2.10   11 - *cis* - retinal (plays an essential role in vision)

Animal research and clinical observations early in the twentieth century indicated that vitamin A deficiency resulted in ocular manifestations called xerophthalmia or "dry eye".   Retinoids have been shown to control the expression of the enzymes involved in the synthesis of glycoproteins (Vahlquist *et al*, 1994). Impairment of this function due to vitamin A deficiency may contribute to the lack of mucin secretion and liquefaction of the cornea seen in xerophthtalmia (McLaren, 2001).

Many studies have shown the effect of vitamin A deficiency on the eye morphology and the benefits of vitamin A supplementation in eye complications. Xerophthalmia left untreated may develop into keratomalacia and blindness (Chapter 3).

2.6.2 Cellular Differentiation

Vitamin A deficiency can result in the change of the cell morphology and kinetics, and the mucus secreting cells are replaced by keratin - producing cells in many tissues in the body.

This was observed in a study on rats where cells in the duodenum and colon were evaluated for changes due to vitamin A deficiency. A lower number of goblet cells were detected in the vitamin A deficient rats, and a higher number of proliferating cells in the duodenal crypt and villi in the control group, suggesting that vitamin A is associated with cell differentiation and epithelial metaplasia (Reifen *et al,* 1998). An overview of studies examining the effect of vitamin A on the epithelial integrity showed that vitamin A deficiency resulted in alterations in the epithelial lining of vital organs. Vitamin A supplementation of 60 mg of retinal palmitate acted as a barrier against these changes, and infection due to these changes (McCullough *et al,* 1999a).

The cellular retinol binding proteins serve as buffers and as "chaperones" during metabolism, and the nuclear receptors are now recognised to be the direct mediators of retinoid actions on the genome. Only two families of nuclear receptors have been cloned to date. Recently a new metabolite all - *trans* - oxo - retinol has been discovered. It functions as a receptor activator and differentiation agent and also binds and transactivates the retinoic acid receptors (RAR's) but not the retinoid X receptor (RXR's) (Achar, *et al,* 1996).

In the cells there are two distinct classes of retinoid - binding proteins known as cellular retinol - binding proteins and cellular retinoic acid - binding protein (CRBPs and CRABPs). The suggested roles of these cellular binding

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proteins are shown in Figure 2.11. Cellular retinol - binding - protein (CRBPII) is responsible for the transport of the lipid - soluble retinol molecule to retinoid - metabolising enzymes. Within the cells all - *trans* retinol in association with CRBP can be oxidised to all - *trans* retinioc acid and 9 - *cis* retinoic acid. These are then transported to the nucleus by CRABP where they are bound to one or more nuclear receptors (RARs and RXRs). The transport of retinol from the extracellular RBP to intracellular CRBP was demonstrated in a study by Sundaram *et al* (1998).
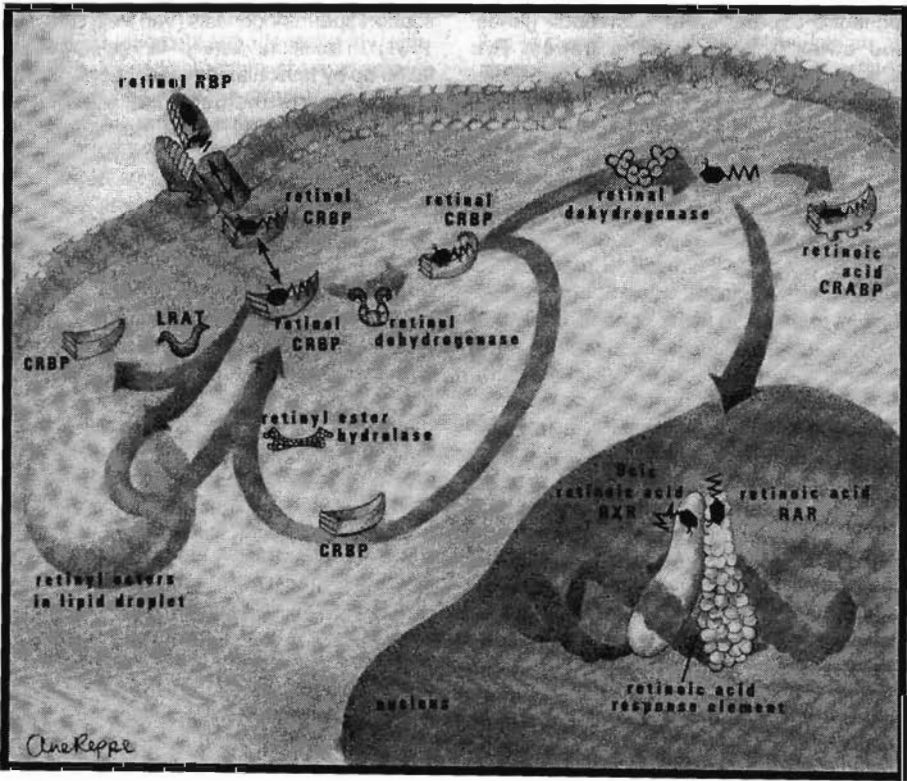
Fig. 2.11 Suggested roles of CRBPs and CRABPs (Blomhoff, 1994).

An important principle emerged from work by both Ong (Ong, 1994) and Napoli (Napoli, 1994) showing that specific binding proteins are responsible for delivering the retinol to the relevant enzymes thus limiting the level of free retinol in

the membranes. These binding proteins also protect the retinol from unwanted processing and damaging. It must be noted that free retinol which partitions into the membranes can if present in excessive amounts disrupt normal membrane structure and function.

Vitamin A, especially retinoic acid, plays an important role in reversing the effect vitamin A deficiency has on the tissues. This was demonstrated in a study on rats where a rat resection model showed that retinoic acid acts to modulate intestinal proliferation in the adapting small intestine (Wang *et al,* 1997).

2.6.3 Immunity

The association between vitamin A and infection resulted in the hypothesis that vitamin A was involved in the function of the immune system. This hypothesis has been supported by research which has shown that vitamin A plays an important role in maintaining immunocompetence.

Vitamin A plays a protective role (immune response) against infections caused by viruses and bacteria. Recent vitamin A supplementation trials suggest that the significant reductions in morbidity and mortality in different infectious diseases and immune responses vary considerably depending on the type of infection and the immune responses involved (Semba , 1999b).

There are two distinct responses to exposure to antigens; humoral and cellular (cell mediated immunity) (CMI). However there is an overlap between cell - mediated immunity and humoral immunity.

Both cellular and humoral immunity responses are affected by vitamin A deficiency (Smith *et al*, 1987) and resistance to infection has often been reduced by chronic vitamin A deficiency (Semba *et al*, 1994a; Semba *et al*, 1996). Humoral immunity results from antibody production mediated by B - lymphocytes, which is often T - lymphocyte dependent. These antibody responses can be due to successful vaccination or to recovery from infections such as measles, typhoid or chickenpox.

Vitamin A deficiency compromises the antibody response to certain types of antigens. This was demonstrated in various supplementation studies where vitamin A deficiency was responsible for reduced specific antibody response and that supplementation was able to reverse this immunodeficiency (Semba, 1994). One of these studies, a randomised, double - masked, placebo - controlled clinical trial, on 236 preschool children in Indonesia assessed the immune status in mild vitamin A deficiency. The immune response to tetanus immunisation was used as a measure of this immune competence. The results suggested that the children who were vitamin A deficient had depressed immunity whereas the immune response of children who had received vitamin A supplementation was significantly greater (Semba *et al*, 1992).

Cellular immunity (CMI) is mediated by T - lymphocytes and there are two main effector mechanisms: cytotoxic T - lymphocyte (CTL) responses, and delayed - type hypersensitivity (DTH) responses. The CD4$^+$ T cells provide help to cytotoxic T cells in CMI and to B cells in the humoral response, while interleukins or cytokines released by the T cells play an important role in antibody production and cell mediated reactions. Antibodies are also involved in some of the cytotoxicity mechanisms. The major effector cells in CMI are the cytoxic T

lymphocytes (CD8$^+$), natural killer (NK) cells and the macrophages that destroy infected or foreign cells. Many studies support the general hypothesis that lymphopoiesis and/or maturation of lymphocytes are altered by the lack of vitamin A. As demonstrated in a study on African children (4 - 24 months) suffering from measles complications, where supplementation with vitamin A (three doses equivalent to 30mg or 60mg) resulted in a significant increase in the lymphocyte counts (Coutsoudis *et al*, 1992). This was further validated in a vitamin A supplementation trial (200 000IU on two successive days) showing improvement in the immunological function in a group of children attending an AIDS clinic, when compared to the placebo group (Hussey *et al*, 1996). Another study of HIV positive children who received the influenza vaccination and vitamin A supplements showed a reduction in the HIV viral load 14 days after immunisation (Hanekom *et al*, 2000). Additionally a study in pre - school Indonesian children showed a relationship between low vitamin A and immunity where T - cells (CD4$^+$ and CD8$^+$ antigens) were lower in the children with xerophthalmia as compared to the non - xerophthalmic controls. However, after supplementation with vitamin A the proportion of CD4$^+$ and CD8$^+$ T cells and the percentage of naïve CD4$^+$ T lymphocytes increased in comparison to the placebo group (Semba *et al*, 1993).

The concept of improving vitamin A status in humans has gained momentum in recent years due to the success of controlled community based trials, and particularly the hospital based vitamin A supplementation trials in children with measles, an infection known to induce immunosuppression. In addition, vitamin A supplementation trials designed to examine morbidity and mortality have shown reductions in infections especially acute diarrhoeal disease.

2.6.4 Haemopoiesis

Vitamin A deficiency (VAD) has long been associated with iron deficiency anaemia. Anaemia observed in children with VAD can be due to the impaired release or transport of iron from the body stores; elimination of iron in the body stores due to infection; defective haematopoiesis within the bone marrow; or other factors. Vitamin A supplementation has been shown to have favourable effects on iron metabolism and the general nutritional status of children (Mejia and Arroyave, 1982). Another study (randomised, controlled) showed significant improvement in haemoglobin concentration (P<0.0001) in children in Tanzania who had been supplemented with vitamin A. There were also improvements in body weight and height in these vitamin A supplemented children (Mwanri et al, 2000).

In conclusion, despite the need for more detailed understanding of vitamin A - iron interrelationships, the improvement of vitamin A status should be considered along with iron supplementation in the control of nutritional anaemia (IVACG, 1998).

2.6.5 Growth

It has been shown that vitamin A has an effect on the growth and development of the child (Hadi et al, 2000). The effect on stunting has also been suggested in a few studies with vitamin A supplementation. A country - wide survey in Indonesia between 1978 - 1979 (Sommer, 1982a) observed stunting in children with corneal xerophthalmia. The effect of vitamin A supplementation on growth was demonstrated in a study on Bangladeshi children showing that vitamin

A alone was effective in terms of linear growth at 8 weeks, whereas vitamin A together with zinc increased weight gain (Roy *et al,* 1999a).

Retinoic acid plays an important "hormone - like" role in the control of growth and development of tissues in the musculo - skeletal system. One of the mechanisms that have an influence on growth, as demonstrated in this study, showed that both vitamin A and retinoic acid produced rapid release of cyclic AMP and human growth hormone secretion (Djakoure *et al,* 1996).

CHAPTER 3

3.0 ASSESSING VITAMIN A

Vitamin A status can be divided into five categories: deficient, marginal, satisfactory, subtoxic and toxic.

Assessing vitamin A stores in the liver is the most reliable way of assessment as the liver contains about 90% of the total body vitamin A. However, this is impractical for obvious reasons and not suitable for routine assessment. Serum retinol concentrations, using high performance liquid chromatography (HPLC), are the most commonly used, however, these only reflect the most severe deficiencies as shown in Figure 3.1.
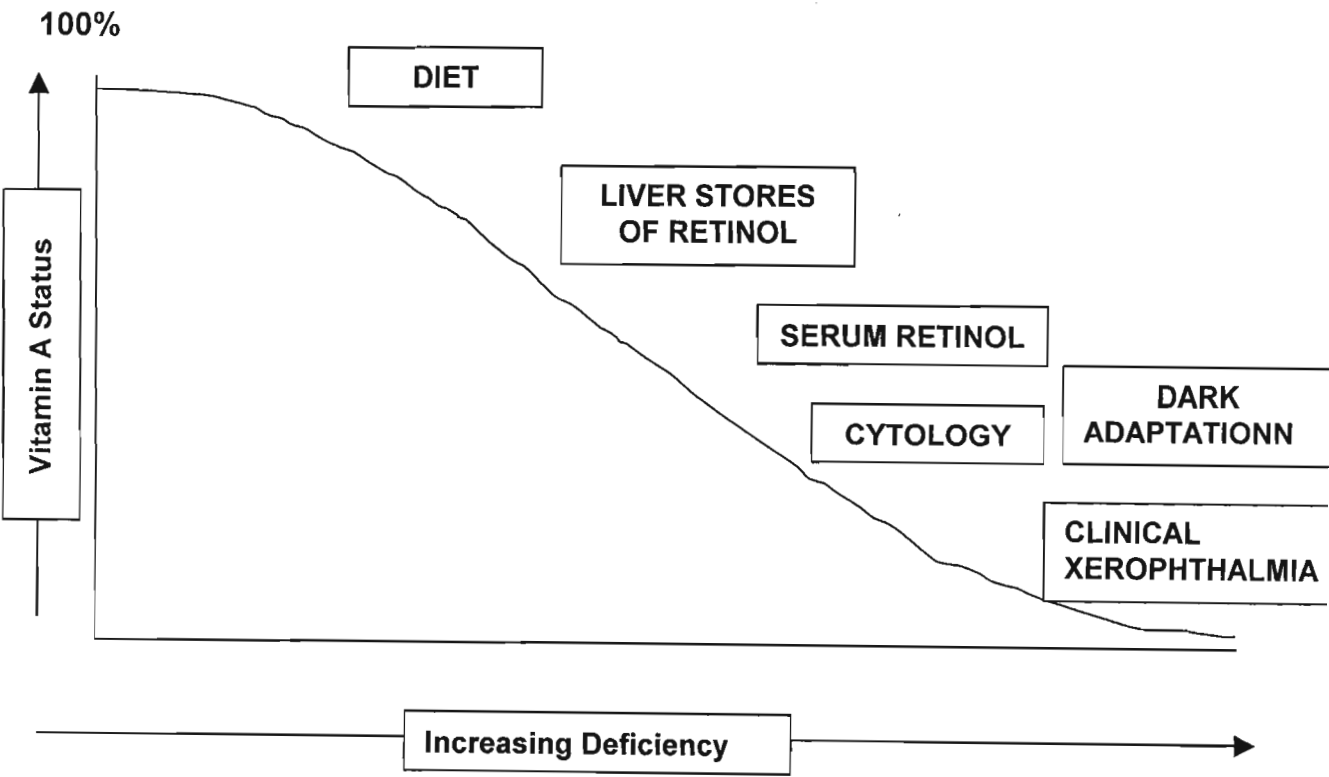
Fig. 3.1. Schematic relationship between the decline in vitamin A status and the onset of potentially relevant indicators of inadequate vitamin A nutriture. The shape of the curve is only illustrative (Sommer & West, 1996).

3.1 METHODS FOR ASSESSING VITAMIN A

3.1.1 Xeropthalmia

Xerophthalmia has long been associated with vitamin A deficiency and is responsible for 5 - 10 million cases of milder ocular diseases every year.

Depletion of vitamin A stores can result in xerophthalmia (Figures 3.2 to 3.10) as vitamin A affects the cells in the optical region. The epithelial cells of the conjunctiva in vitamin A deficiency are transformed from normal columnar to the stratified squamous type, with the resultant loss of goblet cells, formation of a granular cell layer and keratinisation of the surface. Clinically the manifestations are marked dryness and the affected area appears roughened with bubbles and droplets on the surface. Only the bulbar conjunctiva, and not the eyelid lining or the palpebral conjunctiva, are affected by xerosis. These changes in the cornea are known as keratomalacia (Figures 3.2 and 3.3). After recovery from vitamin A deficiency there remains scaring of the cornea of varying degrees and depth (Figure 3.9). In vitamin A deficiency the retina can also be affected resulting in nightblindness and there have been a few cases of structural damage to the rod cells (xerophthalmic fundus)(Figure 3.10).
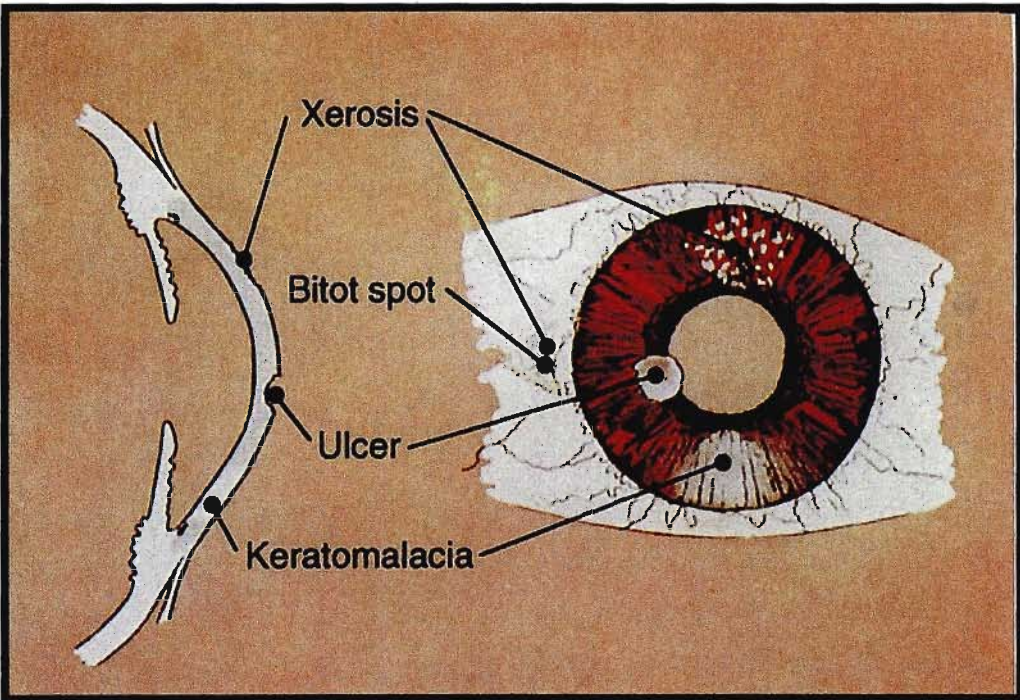
Fig. 3.2   Diagram indicating sites affected by xerophthalmia (Sommer, 1995).
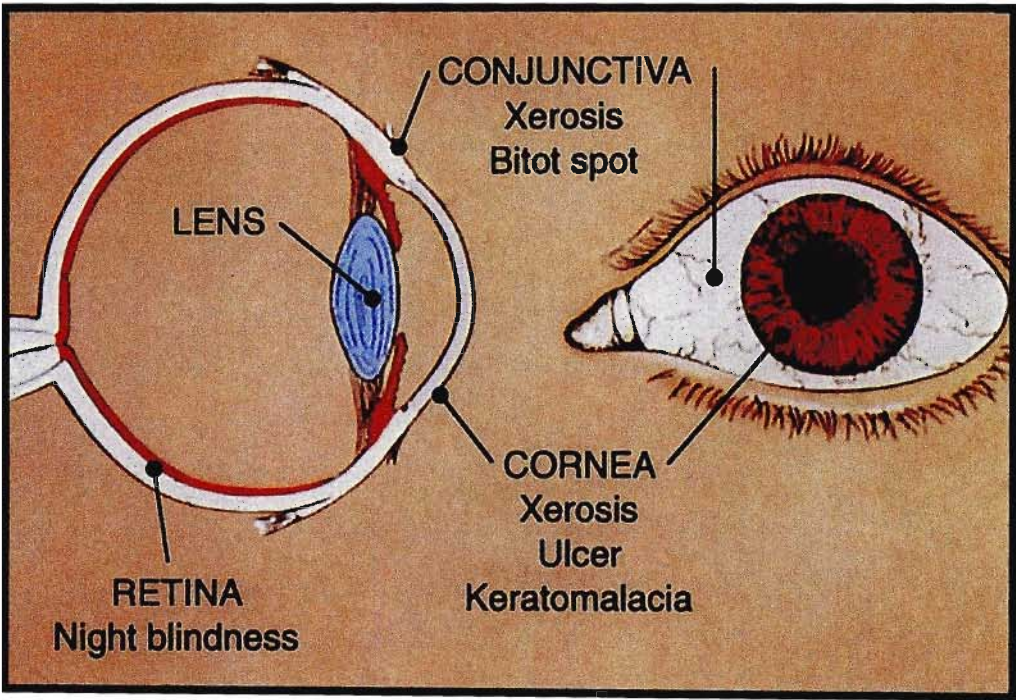
Fig. 3.3   Diagrammatic representation of xerophthalmia lesions (Sommer, 1995).

Fig 3.4. Temporal patch of conjunctival xerosis. (X1A)
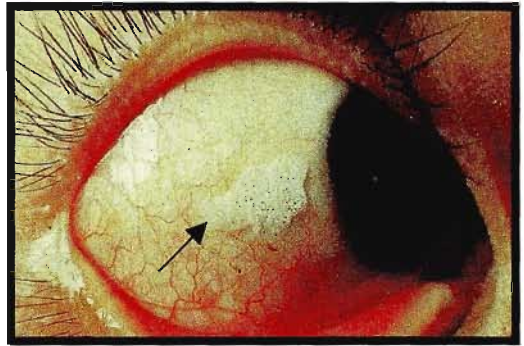
Fig 3.5. Typically foamy Bitot's spots. (X1B)
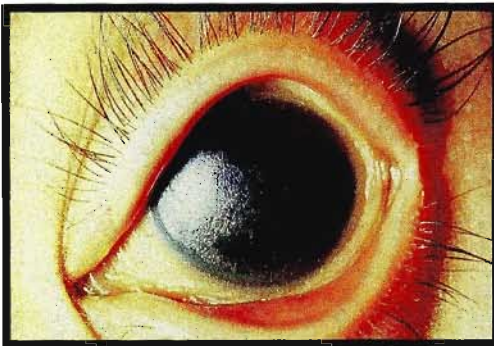
Fig 3.6. Dry, granular appearance of corneal xerosis. (X2)
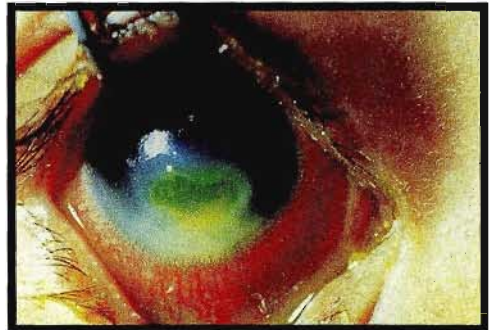
Fig 3.7. Larger, oval xerophthalmic ulcer stained with fluorescein. (X3A)
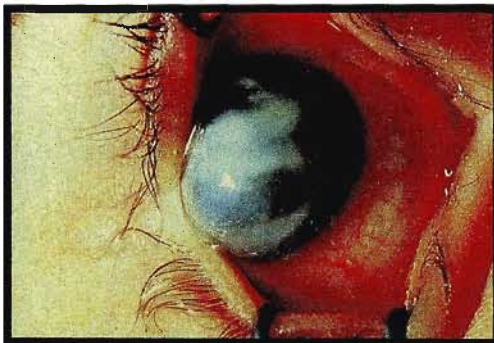
Fig 3.8. Necrosis/keratomalacia involving all of the cornea. (X3B)
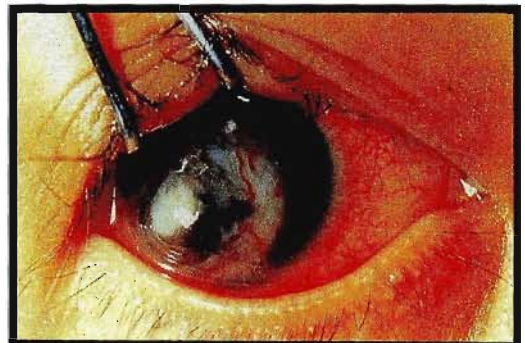
Fig 3.9. Healing of widespread necrosis may result in a weakened, anteriorly bowed, scarred corneal surface; a staphyloma. (XS)

Fig 3.10. White retinal specks characteristic of xerophthalmic fundus. (XF)

(Sommer A, 1995)

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All these eye changes are included in the term xerophthalmia and were classified by WHO in 1976 (WHO/USAID, 1976) and modified in 1982 (WHO Expert Group, 1982) (Table 3.1).

The ocular signs listed in the Table 3.1 are in order of severity. The retinal function is thus affected before xerosis affects the conjunctiva and then the cornea. Liquification of the cornea is usually a very late stage and corneal scars are not usually part of the deficiency process. Classification of xerophthalmia is as follows:

Table 3.1   Clinical Classification of Xerophthalmia (WHO, 1982)

Nightblindness (XN)
Conjunctival xerosis (X1A)
Bitot's spots (X1B)
Corneal xerosis (X2)
Corneal ulceration/keratomalacia < 1/3 corneal surface (X3A)
Corneal ulceration/keratomalacia ≥ 1/3 corneal surface (X3B)
Corneal scar (XS)
Xerophthalmia fundus (XF)

Xeropthalmia is most commonly a problem in children under 5 years of age. However clinical manifestations are uncommon in the first year of life even in vitamin A deficiency. This does not rule out the fact that vitamin A stores are depleted as corneal lesions are said to only develop late in the deficiency.

The policy adopted by WHO of giving a standard dose of 200 000 IU every six months (after the age of one year) was found to significantly reduce the occurrence of xeropthalmia (Sommer, 1989). It was also found that high - dose

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vitamin A therapy showed a reversal of the damage done to the goblet cells resulting in corneal healing (Sommer & Green, 1982b).

Xerophthalmia is now recognised as the late manifestation of severe vitamin A deficiency rather than an early, milder deficiency. It is also related to half a million or more cases of measles - related blindness and an equal number of cases of paediatric blindness worldwide (Sommer, 1998).

3.1.2 History of Nightblindness

Nightblindness is the earliest sign of vitamin A deficiency. A study of pregnant women in rural Nepal, found that nightblindness appeared to be related to vitamin A deficiency, anaemia and increased risk of morbidity (Christian P et al, 1996).

Vitamin A deficiency affects the production of rhodopsin by the rods, resulting in nightblindness. These are the sensory receptors of the retina responsible for vision under low levels of illumination. Some societies or cultures especially where vitamin A deficiency is endemic refer to the condition as "chicken eyes" (chickens lack rods and are thus night - blind). Nightblindness responds rapidly to vitamin A therapy, usually within 24 - 48 hours.

A history of nightblindness can easily be collected and is an indicator of vitamin A deficiency. It is often used as a rapid assessment on a population where it is suspected that vitamin A deficiency is a problem. Care must be taken to collect the history from a responsible adult who recognises that the child's behavior, after dusk or in a darkened room, is distinctly different from normal

children. The survey team must be familiar with local terminology so as to refer to these when conducting the interviews. This form of assessing vitamin A deficiency can be a more specific and sensitive index for vitamin A deficiency and xerophthalmia than the presence of Bitot's spots (Sommer *et al*, 1980).

The advantage of this form of assessment is that it is non - invasive and some of the tests may be applied by non - specialised personnel. If the history is taken accurately it is a very reliable form of assessment.

3.1.3 Rapid Dark Adaptation Testing

This method of assessment was initially defined by Thornton in 1977 and modified by Vinton and Russell in 1981. Impaired dark adaptation is thought to be an early functional abnormality. The test is carried out in complete darkness and estimates the time for the retina to transfer from cone - mediated daytime (photopic) vision to rod - mediated night - time (scotopic) vision. After bleaching the retina with a bright light source, the time taken for each child to separate white and blue counters in dim light is recorded on at least three occasions and averaged. This relates to the time it takes for the retinal sensitivity to move from the red end of the spectrum to the blue end. This is also known as the Purkinje shift.

Field trials were conducted by Congdon using a portable dark adaptometer to measure the dark - adapted (DA) pupillary response in children and pregnant women. In this study it was found that women who received vitamin A supplementation versus placebo had significantly better DA scores (Congdon *et al*, 2000).

Further studies are under way in other vitamin A deprived populations and if these confirm the findings by Congdon, this method of vitamin A assessment may prove to be valuable, non - invasive, and practical in the field. On the negative side the testing requires sophisticated and expensive equipment and needs skilled ophthalmological staff. The subjects also need to be of an age and education as to co - operate fully in the testing.

3.1.4 Night Vision Threshold Test (NVTT)

This is a new simple inexpensive technique. This technique is based on the ability of the subject to see emitted light at different intensities. The test is carried out in a darkened room using a light emitting diode (LED) which emits a round illuminated spot of 30 cm in diameter on a wall lined with white paper. The subject adapts to the darkness for at least 10 minutes. This step can be done in groups. However each subject is tested separately while standing 10 feet away from the wall. The tester randomly directs the light from the NVTT in three different directions while decreasing the light intensity (Shrestha AK *et al,* 2000).

Each subject indicates their ability to see the emitted light and the tester determines the accuracy of the response from the subject by repeating the same intensity twice in random directions. The lowest light intensity accurately observed by the subject is the score (range being 1 - 5). The passing score is set at 5 and indicates the ability to see the lowest intensity of light.

This technique uses a simple and low cost instrument that can be readily used in the field. Preliminary testing has been carried out, however it showed that the Night Vision Threshold Test did not correlate well when compared with the

MRDR technique. It was noted that this could be due to the fact the study's sample size was too small and could have been increased (Shrestha AK *et al*, 2000). Further refinements are still required to determine the accuracy and efficiency of this form of assessment.

3.1.5 Conjunctival Impression Cytology (CIC)

Egbert *et al* (1977) and others described a method to do a simple conjunctival biopsy. A cellulose acetate filter is briefly applied to the conjunctival surface and removes the superficial layers of the conjunctival epithelium. By 1984 Hatchell and Sommer described ocular surface changes in rabbits subjected to a vitamin A deficient diet that manifested itself prior to changes of xeropthalmia indicating that CIC was a valuable form of assessment.

In the CIC test both eyes are sampled and the samples are taken from the inferior temporal conjunctiva. The samples are then placed in a fixative and stained with periodic acid - schiff and hematoxylin. A specimen is classified normal if at least five goblet cells are present on a sheet of normal epithelium and abnormal if there were fewer than five goblet cells on an abnormal epithelium sheet or if keratinized cells were present (Wittpenn *et al*, 1988).

CIC status is classified as normal if both disc specimens are "normal" and abnormal if both specimens are "abnormal" and borderline abnormal if one of the disc specimens was "abnormal".

Impression cytology appeared to detect vitamin A deficiency in a study on 148 Indonesian children when compared with low serum retinol levels

(Natadisastra *et al*, 1988). Conjunctival impression cytology was however found to be unreliable in another study conducted on Guatemalan children. The CIC did not identify the same group of children with vitamin A deficiency, as did the Relative Dose Response Test (RDR) (Gadomski *et al*, 1989). Another study on 178 preschool children in Northern Thailand, showed that the CIC assessment method was a poor indicator of vitamin A status (Fuchs *et al*, 1994). In a study comparing methods of assessing vitamin A (serum retinol, RDR and CIC) in children, in Belize, Central America, it was found that the CIC method of assessment was not successful in this population. It appears that the CIC method is more useful in populations with a large proportion of subjects having serum retinol levels < 0.35 μmol/L, and again less effective in populations with marginal vitamin A status. The CIC technique results may also be affected by inflammatory ocular diseases (Makdani *et al*, 1996).

Initially the CIC method for assessing vitamin A status was found to be promising however through a number of comparison trials it has been shown to be affected by too many external variables and has not compared favourably with the other forms of assessment methods that are now available.

3.1.6 Dietary Intake

Vitamin A intake should be the leading indicator to vitamin A status, as consuming too little vitamin A should presumably result in deficiency. Many studies have been done to validate this method of assessment (Olson, 1987).

There are several methods available using food frequency methods for assessing vitamin A. The Helen Keller International Food Frequency Method (HKI

– FFQ) takes into account the food intake over a period of seven days and includes 28 foods (de Pee *et al,* 1999). Another method is the Simplified Dietary Assessment (SDA) that takes into account locally available vitamin A enriched food, preparation methods and portion sizes (small, medium, large). This data is linked together to establish the vitamin A content per portion size and scores are assigned based on retinol equivalent content. Questionnaires can be developed and based on a list of foods using a 24 - hour recall period for short term (consumption index CI) intake and 7 - 30 day food intake for longer term (usual pattern of food consumption UPF).

These methods help to identify insufficient vitamin A intake in population groups, however they are not accurate enough to detect vitamin A deficiency individually. Presently there is uncertainty about the bioavailibility and bioconversion factors (Chapter 2) for provitamin A carotenoids which affects this form of assessment and until these matters are resolved results of field work should be treated with caution.

The advantage of this form of assessment is that it is non - invasive, inexpensive and are very simple to implement. Large numbers of subjects can be readily targeted and a profile of a population can be easily obtained.

3.1.7 Liver Biopsies

Under normal circumstances, 90% of vitamin A is stored in the liver as retinyl esters (Olson *et al*, 1987). In severely deficient individuals it may account for as little as 50% of the total stores (Olson *et al*, 1979). The liver stores directly reflect the body's ability to supply adequate vitamin A levels. Therefore the liver is probably the best indicator of vitamin A status. A concentration of 20 µg/g was a recommendation made by Olson *et al* (1987) as being "satisfactory" in that there were no clinical signs of a deficiency at this level.

Liver samples are generally taken from the central portion of the right lobe, either at the time of surgery or autopsy (Underwood *et al*, 1979; Amedee - Manesme *et al*, 1984a). The samples are homogenised and deproteinated and analysed using high performance liquid chromatography. However it has been found that there is considerable variation in the vitamin A distribution in the liver according to age and content (Olson *et al*, 1979). A study comparing micro - needle biopsy and macro - samples of human liver that were obtained at autopsy correlated well (r = 0.96) indicating that if carried out under proper clinical conditions by a specialist may provide independent confirmation of the other methods (Amedee - Manesme *et al*, 1984a).

Measurement of retinol stores using liver biopsies is obviously the most accurate way of determining vitamin A status of an individual however disadvantages to this form of assessment are that the people who are dying randomly and being autopsied are not necessarily representative of the target group needed. Another disadvantage is that it is an invasive form of assessment and cannot be carried out routinely on healthy persons.

3.1.8 Serum Retinol

Serum retinol is the most widely used, and has the longest history in the measurement of vitamin A. As early as 1926, Carr and Price described a method that was based on the formation of a blue colour following a reaction of antimony trichloride in chloroform, with vitamin A. However this method had a few problems in that large quantities of serum were required (3ml), antimony trichloride produced turbidity in the presence of small amounts of water, the blue colour which is measured lasts only for a short time which causes inaccuracy when reading the absorbance, and the reagents are dangerous to laboratory personnel as they are corrosive and poisonous.

This led to Bessey *et al* (1946) devising a microspectrophotometric method that has been widely used. More recently Neeld and Pearson (1963) and Bayfield (1971) developed a colorimetric method based on the Carr - Price method but using different reagents. They used trifluoracetic and trichloroacetic acid that did not exhibit interferences from endogenous compounds such as β - carotene and phytofluene.

In 1976 De Ruyter and De Leenheer published the first method using high performance liquid chromatography (HPLC). Today serum retinol is generally measured using HPLC as this method eliminates all the problems that existed previously. There are various HPLC techniques available however in this study we used the reverse phase technique and this is discussed in Chapter 5 (Laboratory Methods) in detail.

Serum retinol only detects vitamin A deficiency when the liver stores are very low, thus an isolated serum retinol value is not an accurate indicator of

vitamin A status. Serum retinol is best used to provide information about the status of a population and about the response to an intervention program. Serum retinol analysis using HPLC is an accurate and sensitive technique, but it requires very expensive equipment and specialised personnel.

Children with vitamin A levels of below 10ug/dl are considered deficient and levels between 10 - 19.9 μg/dl are considered low (Table 3.2). Normal levels for children are between 24 - 60 μg/dl i.e. 0,84 - 2,10 μmol/L. Vitamin A levels are expressed in molar terms in accord with the Systeme Internationale (SI). Thus, serum concentrations of retinol are given in micromolar terms (μmol/L) rather than in micrograms per decilitre (μg/dl) (28.57 μg/dl = 1 μmol/L).

Table 3.2   Serum Vitamin A levels and Classification of Vitamin A Status in Children (Sommer, 1996).

μg/dl	μmol/L	Vitamin A Status
≥ 20	≥ 0.7	Normal
10 - 19.9	0.35 - 0.69	Low
< 10	< 0.35	Deficient

3.1.9 Retinol Binding Protein

It has been found that retinol binding protein (RBP) is a good surrogate for measuring vitamin A (Almekinder *et al*, 2000; Gamble *et al*, 2001) in large population - based studies.

Human RBP is a single polypeptide chain with 182 amino acids and a molecular weight of 21 230. The 1:1 molar complex of all - *trans* retinol and RBP is called holo - RBP and is referred to simply as RBP. RBP is the carrier protein for retinol once it is released from the liver and is integrally related to retinol metabolism and demand. Levels of RBP are reduced in liver disease, retinol deficiency, malnutrition, zinc deficiency and diarrhoea (Velasquez - Melendez *et al*, 1996).

RBP can be measured in serum using an ELISA assay. The normal RBP concentration in the plasma of adults is 1.9 - 2.4 µmol/L (40 - 50 mg/L) with 80% to 90% of this existing as holo - RBP. In children up to the age of puberty their RBP values are approximately 60% that of adults viz. 1.14 - 1.44 µmol/L (24 - 30 mg/L).

There are many advantages using the RBP assessment method to measure vitamin A status as shown in one such study where high correlations between the RBP and serum retinol levels were seen. The RBP was also found to be stable when exposed to light and high temperatures (Blaner *et al*, 1999). Another advantage is that the analysis (ELISA) requires small amounts of serum (< 10 µl) and it is not necessary to use fasting blood (Mejia and Arroyave, 1983). It is also a less expensive and a simpler form of assessment, when compared with HPLC.

A simple, portable table microtechnique for measuring holo - RBP using a fluorometer has been introduced by Craft (1999). This technique uses capillary blood, however the hardware can also accommodate curvettes, to measure the vitamin A levels. In this study this technique compared favourably with serum retinol levels using HPLC.

3.1.10 Retinol Binding Protein (RBP)/Transthryretin Molar Ratio (TTR)

The RBP/TTR ratio is used to detect vitamin A deficiency during infection and inflammation. The molar ratio of RBP to transthrytretin (RBP:TTR) provides an indirect assessment of vitamin A status during inflammation. This was demonstrated in two studies, one using a rat model of endotoxin - induced inflammation, and a randomised, placebo - controlled trial of vitamin A supplemented children with measles. Comparisons were made on these two studies by Rosales and Ross (1998a) showing that a low molar ratio of RBP/TTR can distinguish vitamin A deficiency during infection.

In the acute phase response, RBP in the plasma falls, as it does in vitamin A deficiency. However the retinol is bound to transthyretin and it is the transthyretin that remains unaffected during vitamin A deficiency but falls in the acute phase response.

A study by Donnen (1999) showed no significant difference in the molar RBP:TTR ratio at three months in moderately malnourished Congolese children when comparisons were made between the groups who received 200 000IU vitamin A, 500 mg mebendazole, or no intervention. Another method failed to show significantly that RBP:TTR is sensitive and specific enough to be a useful

assessment tool when used to assess vitamin A status in South African children who had accidentally ingested kerosene (Filteau *et al*, 2000). This method therefore needs further investigation and validation as it has not been found to be consistently reliable.

3.1.11   Dose Response Tests

The dose response tests measure the vitamin A levels in the liver. During vitamin A deficiency the concentrations of vitamin A fall steadily over a considerable period of time in the liver before the serum retinol levels begin to fall and even longer before any physiological changes begin to occur. This method of assessment therefore appears to be good measure of subclinical vitamin A deficiency.

The synthesis of *apo* - retinol binding protein in the liver is not controlled by vitamin A status. During vitamin A deficiency the RBP accumulates in the liver. Vitamin analogues such as dehydroretinol esters and 3,4 - didehydroretinol (DR) bind with the RBP in the liver and are then circulated in the plasma as *holo* - RBP. Therefore in vitamin A deficiency the retinol/vitamin A analogue complex value should be high and the opposite will occur if the patient has adequate stores.

3.1.11.1   Relative Dose Response Test

The use of dehydroretinol as a dose response test was first introduced in rats by Loerch and Underwood in 1979. The first dose response tests in humans used dehydroretinol and were known as relative dose response tests (RDR) (Flores *et al*, 1984; Amedee - Manesne *et al*, 1984b; Amdee - Manesme *et al*, 1987; Amatayakul *et al*, 1989). The RDR test indirectly measures vitamin A stores in the liver without the need of a liver biopsy.

A small standard oral dose of retinyl ester or dehydroretinyl ester dissolved in oil (450 - 1000 µg / 1.57 - 3.48 µmol) is given, (Flores *et al*, 1984) which results in the *holo* - RBP being released from the liver into the serum and transported to the target cells. The response of the individual to this dose of retinyl ester is measured in the serum 5 hours after administration. The dose has also been given intravenously as a water - dispersed suspension to children with liver disease (Amedee - Manesme *et al*, 1987). Two blood samples are needed, one at 0 hours and one at 5 hours, after dosage. The blood samples can be processed either by HPLC or a colorimetric method. The response is measured in the serum as a percentage. A response of 20% or higher shows inadequate liver stores (liver concentrations of < 0.07 µmol/g / < 20 µg/g). Negative values can be recorded and are expressed as 0% RDR and this shows adequate liver stores.

The RDR value is calculated by the following equation:

$$RDR = \frac{A_5 - A_0}{A_5} \times 100\%$$

where A_5 is the serum retinol concentration after 5 hours and A_0 is the baseline sample.

A study on children with persistent diarrhoea showed elevated RDR results with significantly low serum retinol levels, showing that the RDR levels responded to low vitamin A stores in the liver and were a more accurate marker for measuring vitamin A status (Usha *et al,* 1991). However another study showed unreliable RDR results when tested on 23 Belizean children. This highlighted the fact that some subjects may not metabolise sufficient retinol in the 5 hours given, which should result in elevated retinol levels and thus resulting in underestimating the deficiency due to inaccurate RDR scores (Apgar *et al,* 1996).

Disadvantages of this test are that two samples are required for analysis, and individual responses to the retinyl ester may differ resulting in unreliable results as shown in the above study.

3.1.11.2   Modified Relative Dose Response Test (MRDR)

The RDR has some disadvantages namely that the child has to be in the fasting state and that two blood samples must be drawn at 5 hour intervals. Tanumihardjo, Olson and collegues therefore developed the modified relative dose response (MRDR) that required only one sample (Tanumihardjo *et al,* 1987; 1988).

The MRDR assay is very similar to the RDR assay. 3,4 - Didehydroretinol (DR) is used as an indicator of retinol liver stores. A single dose of 3,4 - didehydroretinyl acetate (DRA) in oil is administered orally. Like retinol (R), DR binds with the accumulated *apo* - RBP in the liver and is released into the plasma as *holo* - RBP. A molar ratio of DR/R in serum is determined 4 - 6 hours after dosing by taking a single blood sample.

The MRDR result is calculated as follows:

$$MRDR = \frac{Serum\ DR\ Concentration}{Serum\ R\ Concentration}$$

The MRDR assay involves giving the children a single dose of 100 μg/kg body weight or adults a standard 2.5 mg dose of 3,4 - didehydroretinyl acetate dissolved in oil and then taking venous blood 4 - 6hours later. The oily solution is given to the child using an automatic positive displacement pipette. The tips of these pipettes are disposable and the volume range is also adjustable. The serum is then extracted using an ethanol/hexane extraction and analysed using HPLC with an ultraviolet spectrophotometer set at 350nm (optimum wavelength for DR).

During Vitamin A deficiency RBP builds up in the liver and cannot be released until vitamin A is available. Therefore when didehydroretinol is given in dose form to a patient with vitamin A deficiency the didehydroretinol binds with the RBP resulting in a large increase in didehydroretinol levels in the blood. However when the patient has adequate stores there is no build up of RBP and the DR is simply taken into storage. Therefore the DR/vitamin A ratio is inversely proportional to the severity of the vitamin A deficiency.

Children with a DR/R ratio of ≥ 0.060 are classified as being in a marginal vitamin A status. However the cut - off levels for MRDR are still being debated as it was shown that in well nourished children a cut - off of ≥ 0.03 was necessary. In this study on 24 well nourished American children it was found that only 3 children had a ratio above 0.03 and that the ratios plateaued out between 4 - 10 hours to 0.02. After vitamin A treatment the ratio decreased to 0.019. A tentative cut - off ratio for DR/R of ≥ 0.03 for well nourished children showed a satisfactory vitamin

A status (Tanumihardjo *et al*, 1990a). Another study carried out on Indonesian children who were at risk of being vitamin A deficient showed 62% as being tested above the cut - off of $\geq$ 0.03 at 5 hours, and these children were showing no clinical signs of vitamin A deficiency indicating the MRDR test is a good indication of sub clinical deficiency (Tanumihardjo *et al*, 1990b).

The MRDR method has been used extensively as a method of assessing vitamin A status. It has been found to be an accurate, sensitive and a good indicator of vitamin A deficiency even before clinical signs were evident (Spannaus - Martin *et al*, 1997; Rice *et al*, 1999; Rice *et al* 2000).

Refinements were made to this method in 1996 by Tanumihardjo and colleagues(1996a). These included, changes to the dosage of 3,4 - didehydroretinyl acetate (DRA), validation of the time between administering the oral dose and obtaining the blood sample and a stability study on didehydroretinol. The dosage was changed from dosing per kg body weight to standard graded doses of 5.3 μmol for children younger than 6 years, 7.0 μmol for children between 6 and 12 years and 8.8 μmol for adults and children > 12 years. The timing between administrating 3,4 - didehydroretinyl acetate and taking the blood sample was validated as being anywhere between 4 - 7 hours. DRA was found to be stable for $\geq$ 18 months when stored in amber vials at 2°C and at -20°C, and unstable at 22°C. This study showed that stability was affected more by temperature than exposure to light. This study simplified the MRDR technique and validated issues of uncertainty (Tanumihardjo *et al*, 1996a).

A study on Indonesian children, infected with ascaris lumbricoides who received vitamin A and albendazole, used serum retinol and MRDR to measure

vitamin A status. The MRDR test proved to be a better monitor of the effects of the treatment on vitamin A status than serum retinol levels alone (Tanumihardjo *et al*, 1996b). The MRDR value also proved to be a better indicator of vitamin A status when compared with serum retinol levels, in a study on lactating mothers in Indonesia (Tanumihardjo *et al*, 1996c).

Advantages of using the MRDR over other methods are that it is more sensitive because it is a proxy measurement of vitamin A stores in the liver and only one blood sample is required for analysis. The blood samples can be frozen and stored for analysis at a later stage and both the DR and retinol are analysed from this single blood sample. Another advantage is that the child can be dosed at home and brought to the clinic a few hours later.

Some disadvantages are that DR is not stable once extracted from the serum so it is necessary to protect samples from direct light and to inject them as soon after extraction as possible. Didehydroretinol is not commercially available and is expensive and needs to be synthesised before use.

HPLC is the usual method of analysis and although being an expensive form of analysis and needs trained personnel, it is still the most reliable, sensitive and accurate way to analyse both vitamin A and DR. MRDR holds considerable promise as a minimally invasive technique by which to assess the liver stores of vitamin A in individual children before clinical manifestations of vitamin A deficiency.

3.1.12 Isotope Dilution Method

The isotope dilution method is another method for assessing vitamin A stores. These deuterated - retinol - dilution techniques can also provide quantitative estimation of total body stores and detect changes in the vitamin A pool size in response to different intakes of vitamin A (Haskell *et al,* 1997; Haskell *et al,* 1999; Ribaya - Mercado *et al,* 1999).

In this technique, a precise amount of deuterated retinol viz. retinyl - d_4 acetate has to be ingested and allowed to reach pseudoequilibrium with the existing vitamin A body stores over seven days. This implies that the subject continues to eat a normal diet that provides a continuous dilution of vitamin A in the body pools by unlabeled vitamin A. Complete equilibrium between the labeled vitamin A and the unlabeled body stores is however never achieved. A blood sample is taken at this point and the extent of the dilution of the labeled tracer relates to the amount of endogenous reserves. This blood sample is, therefore, needed to determine the ratio between the labeled and unlabeled vitamin A. Vitamin A is extracted and the samples are purified using HPLC. The sample is then converted to *tert* - butyldimethylsilyl (tBDMS). These samples are stable at 4°C in the dark for 3 months. The extract is then analysed by gas chromatography/mass spectrometry (GC/MS) using selected ion monitoring. This method of converting retinol to its *tert* - butyldimethylsilyl ether has significantly improved sensitivity, reliability, precision and should allow smaller doses of isotopically labeled vitamin A to be administered to the patient (Handelman *et al,* 1993).

A new procedure using gas chromatography - electron capture negative chemical ionisation mass spectrometry measured the total reserves in 47 selected elderly Guatemalans aged between 60 - 81 years. The technique worked well and had a relatively small variance at the various time points (Tang *et al*, 1998).

Although a promising technique the applicability of this technique to population analysis is limited by the need for highly skilled personnel and expensive equipment.

3.1.13   Rapid vitamin A field test (Craft)

This new technique was introduced at the IVACG Meeting in March 1999 by Neal E Craft (1999). There is a necessity to develop a method to enable vitamin A analysis in the field. This method uses capillary blood and a fluorometer. The fluorometer weighs less than 7 kg and is able to use battery power. The mercury lamp has been replaced with a powerful phosphor - coated lamp. Results showed a linear response down to the deficient vitamin A levels and compared favourably to serum vitamin A samples measured on HPLC. Further validation is obviously required before this technique could be recommended.

3.1.14 Retinol analysis in dried blood spots (Craft)

This method uses dried blood spots collected on blood collection cards that are analysed using HPLC (Craft *et al*, 2000a). The samples are agitated in an ultrasonic bath into phosphate buffer containing antioxidants and metal chelator. The samples are denatured with ethanol and the retinol is extracted into hexane, which is evaporated under nitrogen and reconstituted in methanol before injecting onto a HPLC column for analysis. This method was validated using healthy volunteers and the results were comparable to serum retinol levels (Craft *et al*, 2000b).

There are many advantages to measuring vitamin A in dried blood spots rather than venous blood such as easier collection, transport, storage, easier accessibility to younger and more remote populations and the decreased risk of disease transmission.

3.2 SUMMARY AND CONCLUSION

There are many forms of assessment methods available, but it is necessary to determine what needs assessment and to select the correct indicator for that particular population group being studied. The first factor that needs consideration is whether assessment is at an individual level or at a population level as this will have an obvious influence on the choice of assessment method.

Many studies have compared methods of assessment and have found that some do not compare favourably. However, this can be due to a number of reasons that can then assist in choosing the best form of assessment.

Reasons for variation of results can be; differing vitamin A deficiency levels in the populations, source of vitamin A used for supplementation (β - carotene or retinyl palmitate), age of the population, health status of the population and location. The objective of assessing vitamin A effectively and accurately is to determine the specific problem area and to implement effective supplementation programmes.

CHAPTER 4

4.0 VITAMIN A AND INFECTION

There are two complementary hypotheses (Table 4.1) for the protective role of vitamin A against infection (Ross, 1996). One focuses on epithelial barriers and the other on immunological functions. The epithelial barriers provide a "first line of defense" in resistance to infection and are defined as an offensive role. The immune system is primarily a defensive response against infection once it has taken place and the ability to develop protection through antigen - specific memory.

Both these hypotheses are probably involved in the protective role against infection at different times and degrees during the infectious state and in different settings and surroundings.

Table 4.1   Hypotheses Concerning the Protective Role of Vitamin A (Ross AC, 1996)

Epithelial Barriers Hypothesis	Immunologic Response Hypothesis
Principally *offensive*: protects against Infection	Principally *defensive*: responds to pathogens or non-self antigens
Structural integrity paramount	Functional integrity emphasised; but cell differentiation also important
Predicts resistance to infection will be reduced by vitamin A deficiency	Predicts response to infections will be reduced by vitamin A
Predicted major effect: decreased incidence of infection	Predicted major effect: decreased duration or severity of infection

4.1 INFLUENCE OF INFECTION ON VITAMIN A STATUS

During infection, vitamin A status is affected due to the acute phase response. An infectious episode may result in serum retinol levels being lowered due to inflammation of the epithelial and absorption being affected. Various infectious episodes in children, such as chickenpox, bronchitis, upper respiratory infection, tonsillitis and diarrhoea have been shown to be associated with depleted vitamin A and retinol binding protein (RBP) levels, with the effects being more intense when accompanied with a fever (Arroyave and Calcano, 1979).

In a community based study, Indonesian pre - school children were examined at 3 month intervals for 18 months for signs of xerophthalmia and other infections. It was found that the children with diarrhoea and respiratory disease developed xerophthalmia at more than twice the rate of the healthier children (Sommer *et al*, 1987; de Sole *et al*, 1987).

A study of 137 children (72 acute diarrhoea; 65 healthy controls) in the city of Lima in Peru, was set up to examine the relationship between acute diarrhoea and vitamin A status. Serum retinol levels were significantly lower in children with diarrhoea suggesting that diarrhoea may lead to a lower circulating serum retinol concentration (Salazar - Lindo *et al,* 1993). The association between diarrhoea and low vitamin A and RBP levels was also shown in a cross - sectional study in Sao Paulo City on children with a history of diarrhoea (Velasquez - Melendez *et al,* 1996)

A study in Malatya, Turkey was set up to establish if vitamin A deficiency was a public health problem in this developing country. Serum retinol levels were measured in pre - school children who had recurrent acute respiratory infections

and diarrhoea and in a group of healthy children. It was found that the serum vitamin A was significantly lower in this infected group than in the control group of healthy children (Kucukbay et al, 1997).

A study on young children with inflammatory bowel disease showed a prevalence of low vitamin A levels (< 20 µg/dl) especially in children with active Crohn's disease (Bousvaros et al, 1998).

Another study on 90 children with dysentery (66 with shigellosis), hospitalised in Bangladesh, also showed that infection was associated with a significant decrease in serum retinol concentrations (0.36 µmol/L). This decrease was proportional with the severity of the disease (Mitra et al, 1998a).

One of the mechanisms involved in lowering vitamin A levels during infection could be increased excretion of retinol in the urine. This was demonstrated in a number of studies, where vitamin A and retinol binding protein loss was increased substantially during severe infections. It was thought that these losses were due to pathological changes associated with the febrile response (Stephensen et al, 1994). Similar results were found in another study on children hospitalised for treatment of acute diarrhoea; urinary loss of retinol was increased in the infectious group and not the healthy control group (Alvarez et al, 1995). Mitra et al (1998b) found that it was impaired tubular reabsorption of low - molecular - weight proteins, such as RBP transporting retinal, that appeared to be the cause of retinol urinary loss in this study.

In a letter reviewing these three studies the point was made that loss of retinol in urine was increased in the more severe infectious state such as sepsis, than in less severe infectious conditions such as diarrhoea and pneumonia. It

was recommended that these children be given vitamin A supplementation to replace this urinary loss during such infections (Mitra *et al,* 1998c).

4.2   ASSOCIATION BETWEEN VITAMIN A STATUS AND INFECTION

Vitamin A has been extensively researched in the last 15 years for its ability to combat and prevent against infection.   In the 1920's a theory emerged that vitamin A could be used in "anti - infective" therapy.   This idea was further investigated by Mellanby that led to a series of about 30 trials, trying to determine whether vitamin A could reduce morbidity and mortality caused by infections (Semba, 1999a).

The association of vitamin A status and infection has been noted by a number of researchers and the studies/observations discussed below will indicate that vitamin A deficiency causes pathological changes to the epithelial tissues and reduces resistance to infection.   Studies carried out examining this association looked at both morbidity and mortality rates in communities with vitamin A deficiency.   In the earlier studies, to be discussed, severe xerophthalmia (vitamin A deficiency) was associated with high mortality due to infections.   Several cross - sectional studies have found an association between vitamin A deficiency and chronic and acute illnesses such as tuberculosis, coughing and lower respiratory infections, gastroenteritis and diarrhoea, measles and urinary tract infections:-

Diarrhoea was commonly found to be associated with varying degrees of xerophthalmia and the prevalence appeared to increase with the severity of xerophthalmia, as shown in studies in Nepal (Brilliant, *et al,* 1985; Brilliant, 1988) and Indonesia (Sommer, 1982a).   At the Cicendo Eye Hospital, Indonesia,

gastroenteritis was three times more common in the more severe xerophthalmic cases than in the milder cases of eye disease, and twice as common in the corneal cases as in their controls (Sommer, 1982a). This was confirmed by a study in Truk, Micronesia, where the incidence of diarrhoea was almost three times higher in children with abnormal conjunctival impression cytology (Lloyd - Puryear *et al*, 1991).

The degree of vitamin A deficiency is often proportional to the severity of the disease showing an association between vitamin A status and mortality and morbidity. This was observed in a population - based study on 90 children who were admitted to a Bangladesh hospital with varying degrees of dysentery. It was found that the more severe the vitamin A deficiency, the more virulent the strain (Mitra *et al*, 1998a).

A number of studies show the association of xeropthalmia coexisting with diarrhoea and pneumonia in vitamin A deficient children (De Sole *et al*, 1987; Gujral *et al*, 1993; Ghana VAST Study, 1993). All these studies indicate a strong association between vitamin A deficiency, morbidity and mortality.

In 1995 Fawzi found that a vitamin A deficient diet was associated with an increase in coughing and fever in Sudanese children aged 6 months to 6 years. High infant mortality among infants born to HIV positive mothers has also been shown to be associated with vitamin A deficiency. In this study infants were monitored for 12 months and the mortality rate was higher in the infants whose mother's vitamin A levels were significantly lower (P < .0001) (Semba *et al*, 1995a).

4.3 VITAMIN A SUPPLEMENTATION AND EFFECT ON MORBIDITY AND MORTALITY

The observational findings showing an association between vitamin A deficiency and morbidity and mortality were later confirmed by controlled supplementation or dietary intervention trials which proved to be successful in combating and reducing childhood mortality and morbidity rates (Fawzi et al, 1993a; Glasziou et al, 1993).

Vitamin A supplementation has been studied in a number of childhood diseases such as:

> Diarrhoea
>
> Respiratory Infections
>
> Measles
>
> Malaria

4.3.1 Diarrhoea

Diarrhoea whether chronic or acute has a negative affect on vitamin A levels however through controlled intervention with vitamin A supplementation there may be a decrease in diarrhoeal mortality and morbidity in children.

Community based studies have shown the importance of vitamin A supplementation in children with vitamin A deficiency. This was shown in one such study carried out in villages in northeastern Thailand on 1 772 children (ages 1-8 years). Children with a history of diarrhoea had significantly lower vitamin A and retinol binding protein levels. Subsequent supplementation (200 000IU

vitamin A) of these children showed a 50% reduction in incidences of diarrhoea for a period of at least 2 months (Bloem *et al,* 1990).

Several other studies have shown that vitamin A supplementation resulted in marked decreases in mortality and morbidity (Sommer *et al,* 1986; Coutsoudis *et al,* 1991; Lie C *et al,* 1993; Barreto *et al,* 1994). A study a few years later in 900 Indian children between the ages of 1 - 5 years showed a reduction in severity and incidence of diarrhoeal episodes during supplementation with vitamin A (60 mg) in the non - breast - fed children but not in the breast - fed children. The reason for this could have been that the breast - fed children were receiving sufficient vitamin A from the mothers stores (Bhandari *et al*, 1997). A single dose of 200 000 IU vitamin A given to 83 children aged 1 - 7 years suffering with shigellosis, in Bangladesh, with no clinical signs of vitamin A deficiency, also showed reduced severity of the disease (Hossain *et al,* 1998).

In a South African study, HIV infected children, with diarrhoea, who received vitamin A supplementation of 200 000IU showed an improvement in diarrhoea, however there was no effect in the HIV uninfected children (Coutsoudis *et al,* 1995). This was validated by a later study in Tanzania by Fawzi *et al* (1999) where 687 children between the ages of 6 months to 5 years were admitted to hospital with pneumonia. Of the 648 children, 58 were HIV infected. The children received a dose of 400 000 IU vitamin A; there was a 92% reduction in diarrhoea related deaths and a 68% reduction in AIDS - related deaths. In a randomised, double - blind, placebo - controlled clinical study on hospitalised pre - school children in the Democratic Republic of Congo, one group received 200 000IU vitamin A and the second group 5000 IU of vitamin A daily until discharge. It was found that the low doses significantly reduced the incidence of severity of

diarrhoea in the severely malnourished children. Supplementation with high doses did not reduce morbidity in this population (Donnen *et al*, 1998).

This was further validated in a randomised, double - blind, placebo - controlled field trial in an Andean urban slum in 400 children (6 - 36 months) who were given low doses of vitamin A (10 000IU) for 40 weeks. This resulted in the risk of severe diarrhoea being reduced in the supplemented group of children between the ages of 18 to 23 months (Sempertegui *et al*, 1999).

All the above studies showed beneficial effects of vitamin A supplementation on diarrhoeal morbidity however there are some studies that reported no significant effects and in some there was increased prevalence of diarrhoea and respiratory symptoms after a 2 week period (Stansfield *et al*, 1993). A group of 1407 Indonesian pre - school children who received high doses (100 000/200 000IU) of vitamin A also showed no significant change in the incidence of diarrhoea. Vitamin A was seen to increase the incidence of diarrhoea in the children < 30 months of age, but reduced the incidence of diarrhoea in the older children (Dibley *et al*, 1996).

In the Ghana VAST Study (1993) there was no significant difference between the vitamin A and placebo groups in the prevalence of diarrhoea or acute respiratory infection however the supplemented group had far less clinic attendance's, hospital admissions and deaths. The mortality rate due to acute gastroenteritis was also lower in the vitamin A supplemented group.

Although a few studies have shown no effect on vitamin A supplementation on the incidence of diarrhoea in children, overall the data seems to suggest that

vitamin A supplementation is important to reduce the severity of the diarrhoeal episodes.

In a double - blind, randomised, placebo - controlled study in Tanzania, 660 children, between the ages of 6 and 60 months, who were hospitalised with pneumonia were given 200 000IU vitamin A. There was a significant reduction in the risk of severe watery diarrhoea, however the effect on acute diarrhoea and respiratory tract infection in the children who were not undernourished, increased (Fawzi et al, 2000).

The overall consensus amongst scientists and health officials is that vitamin A supplementation is important in preventing, managing and reducing the severity of subsequent diarrhoeal episodes (WHO/UNICEF/IVACG Task Force, 1997).

4.3.2 Respiratory Infections

Vitamin A deficiency predisposes the child to infection in the upper and lower airways as the epithelial lining in the trachea and bronchi are affected by changes such as decreased mucus secretion and loss of cilia. The process of trapping and clearing airborne pathogens is affected, and this results in a number of pathogens reaching the lungs.

Observational studies mentioned in the beginning of this section, show that respiratory infections are more prevalent in children suffering from xerophthalmia and the prevalence appears to increase with severity of eye disease.

In previous studies there have been conflicting results in studies carried out in variety of populations and areas. Ineffectiveness of therapeutic benefit of

vitamin A supplementation in children with respiratory tract infections was highlighted in a study on children in Guatemala city where 263 children between the ages of 3 - 48 months received 100 000/200 000 IU of vitamin A depending on age (Kjolhede et al, 1995). These findings were confirmed in a meta - analysis by the Vitamin A and Pneumonia Working Group (1995) showing little or no effect in mortality reduction, especially in the < 6 month age group.

A study on 165 infants, receiving 15mg of vitamin A (retinyl palmitate) or placebo, at immunisation contact, found that 61% of the infants remained deficient even after supplementation. These results suggested that even though the children were supplemented, due to persistent attacks of respiratory infection and fever, the vitamin A levels remained depleted (Rahman et al, 1996). This may have resulted because these children are in a continuous state of sub-clinical infection.

There was no effect on incidence and severity of acute respiratory infection in 269 children (aged 1 - 5 years) in Cikutra, Indonesia, who received 200 000IU vitamin A every 6 months. However there was a slight improvement in children under 5 years of age in the duration of the infection (Kartasasmita et al, 1995). Another study by Fawzi et al (1998) on 687 children in Tanzania who were admitted to hospital with non - measles pneumonia showed that large doses of vitamin A (100 000/200 000IU) had no effect on the severity of pneumonia. This was also the case in children aged 6 months to 4 years when given large doses of vitamin A (200 000/400 000IU) during an acute pneumonia state, on the subsequent morbidity and severe morbidity of children with marginal vitamin A deficiency (Nacul et al, 1998). In Durban, South Africa, a randomised placebo

controlled trial was done on low birth weight infants. It was found that giving 25 000 IU doses of vitamin A in the first few days of life had no effect on neonatal or post - neonatal respiratory disease (Coutsoudis et al, 2000).

Adverse effects were also documented in a study in Peru on children aged 3 months to 10 years recovering from pneumonia. The adverse effects ranged from lowered blood oxygen saturation, higher prevalence rates of retractions and auscultatory evidence of consolidation in the supplemented group thus requiring supplemental oxygen (Stephensen et al, 1998)

There have however been several studies showing beneficial effects of vitamin A supplementation in respiratory infections. These studies used large doses of vitamin A (200 000IU for children over 1 year) and showed a reduction in physiological consequences of vitamin A deficiency, such as respiratory infections. Supplementation also helped to minimise the duration of the disease (Sommer et al, 1986; Sommer 1989; Bhandari et al, 1994).

A study by Humphrey et al (1996) showed a 64% reduction in the mortality rate and a reduction in incidence of respiratory infections, when neonates were supplemented with vitamin A on the first day of life (50 000IU). Further to this another study was carried out 3 years later to assess the impact of this initial dose. It was clearly evident that the supplementation had reduced health care visits for pneumonia by > 50% and mortality by > 60% among these infants in the first 4 months of their life. There was no sign of any adverse side - effects in this group of infants (Humphrey et al, 1998). Another approach is to supplement mothers at delivery. This was found to be beneficial to the newborns as the higher concentration of vitamin A in the breast milk was maintained for 6 months and

there was a reduction in the duration of respiratory tract infections and febrile illness in these breast - fed infants (Roy *et al*, 1999b).

Supplementation of up to 200 000 IU vitamin A in a trial on children with respiratory syncytical virus infection resulted in a more rapid resolution of tachypnea (increased rate of respiration) and a shorter duration of hospitalisation. This however was only evident in children with significant hypoxemia (blood oxygen saturation $\leq 90\%$) (Dowell *et al*, 1996). This was further validated in a trial done in the United States on children with the same virus. There was a significant reduction in the duration of hospitalisation, and it was also noted that the serum retinol levels were inversely related to the severity of the illness (Bresee *et al*, 1996).

High doses of vitamin A were beneficial in a group of children in Vietnam aged between 1 - 59 months and it was found that in the undernourished supplemented group there was a shorter recovery rate especially in females > 1 year old (Si *et al*, 1997). This was further validated by a study on Mozambican children between the ages of 6 - 72 months, with acute lower respiratory tract infections. Here, the aim of the trial was to test the benefit of giving vitamin A on admission and to test the morbidity 6 weeks later. It was found that the duration of hospitalisation was shortened in supplemented group (Julien *et al*, 1999).

Another randomised, double - controlled, placebo - controlled field study also showed a reduction in incidence of acute lower respiratory infections in underweight Ecuadorian children ages 3 - 36 months receiving 10 000 IU of vitamin A weekly (Sempertegui *et al*, 1999).

The consensus has been that vitamin A supplementation has no consistent positive effect on respiratory disease. Supplementation during an infectious stage through the health services or community - based distribution programs is thought to be advantageous and useful as it is a means to improving the child's subsequent vitamin A stores, and ability to fight infection. This policy was adopted and recommended by WHO (WHO, 1997).

4.3.3 Measles

Measles is responsible for about 2 million children dying each year. There is no specific therapy and the only hope for eradication is through immunisation. Complications such as pneumonia, diarrhoea and blindness are a result of measles due to severe vitamin A deficiency.

The idea that vitamin A has a protective effect on the child during the illness was first documented by Ellison in 1932. The first study, subsequent to Ellison's original study, to test the effect of vitamin A supplementation on measles, was conducted by Barclay *et al* (1987) in Tanzania. They reported that the mortality rate in the vitamin A treated group was lower than that in the control group. This effect was significant in children < 2 years of age and the group of children suffering from croup and laryngotracheobronchitis ($P < 0.05$). No prior study had demonstrated that low vitamin A levels were associated with increased measles mortality. This was investigated by Markowitz *et al* (1989) in children (283) ≤ 5 years of age admitted to a hospital in Kinshasa. They found that low vitamin A levels increased the mortality rate in children younger than 2 years of age but not in the older children.

In a number of trials it has been shown that the course of measles is influenced by the vitamin A status of the child, and that deficiency clearly increases the severity of the measles episode.

Supplementation with vitamin A (400 000 IU), reduced morbidity and mortality by half that of the control group. In a study of 189 South African children who were hospitalised due to measles complications such as diarrhoea, pneumonia and croup (Hussey and Klein, 1990). In another study in Durban, South Africa, children with measles complications, showed quicker recovery from pneumonia, diarrhoea and fever in the vitamin A supplemented group compared to the placebo group (Coutsoudis et al, 1991).

Based on the above results from trials done by Barclay (1987), Markowitz (1989), Hussey (1990) and Coutsoudis (1991) recommendations were made that vitamin A supplements should be given to all children suffering from measles even if vitamin A deficiency was not evident. These results were also supported by a meta - analysis of vitamin A supplementation trials (Fawzi et al, 1993a) showing that vitamin A supplementation was highly protective against mortality from measles.

A later study showed beneficial effects of supplementation of children with acute measles in Kenya, where high doses of vitamin A reduced the severity of complications such as diarrhoea during measles (Ogaro et al, 1993).

An operational study testing the effect of vitamin A on measles severity was conducted at children's hospital in Cape Town, South Africa on 1720 children < 15 years of age who were hospitalised during two, two year periods (1985 - 1986 and 1989 - 1990). This study compared the children in the first group that were on

standard therapy with children receiving high - dose vitamin A therapy in the second group. The children on the high dose therapy showed improved morbidity (P < 0.001) and mortality (P < 0.001) when the two groups were compared and no adverse effects were observed in the children on this high dose. It was felt that this therapy should form part of the routine case management of all the children hospitalised with measles (Hussey and Klein, 1993).

It was recommended by UNICEF and WHO that vitamin A supplements be administered at the time of measles immunisation (100 000IU for infant aged 6 - 9 months) (UNICEF/WHO, 1993). The safety and value of linking the vitamin A supplementation with the immunisation time point was investigated in a study by Semba et al. (1995b). This study showed a reduction in seroconversion of the measles vaccine in 6 month old Indonesian children who had high levels of passively acquired maternal antibody.

However in later studies successful sero - conversion was demonstrated, as shown in a study in Guinea - Bissau where vitamin A supplementation was given in conjunction with measles immunisation. Sero - conversion was 98% in the children receiving a two - dose vaccine and 95% in the children that received a one - dose vaccine of measles (Benn et al, 1997). This was confirmed in another study in Indonesia on 9 month old infants who were given 100 000IU of vitamin A supplement and who successfully sero - converted (Semba et al, 1997). The concern over reduced sero - conversion was again investigated when 9 month old infants in a Delhi urban slum were given 30mg of vitamin A, at the same time as administration of the measles vaccine. There were no differences in sero -

conversion in the two study groups, indicating that co - administered vaccine could be safely given at public health programs (Bahl *et al*, 1999).

Measles is now accepted as being one of the situations where the development of vitamin A deficiency occurs due to vitamin A absorption being suppressed, utilisation increased and thus the stores are depleted. WHO/UNICEF (1998) recommends that during infection an oral dose of vitamin A (100 000 IU for children under 1 year and 200 000 IU for children above 1 year) should be administered to help reduce the duration and severity.

There is strong evidence that vitamin A supplementation is beneficial for all children infected with measles in communities where vitamin A deficiency is prevalent. Supplementation has been shown to successfully reduce the complications that accompany the measles attack.

4.3.4 Malaria

Malaria infections throughout the world affect over 3 million people a year and result in 2 million deaths, mainly in women and children.

Depleted vitamin A levels due to the malaria parasite have been reported in a few studies (Galan *et al*, 1990; Filteau *et al*, 1993). A significant relationship between vitamin A deficiency and malarial attacks was found in a study on preschool Congolese children and it was found that vitamin A was significantly reduced in the children suffering from malaria (P value < 0.001) (Galan *et al*, 1990).

A community based study in Ghana on 21 906 children where mortality and morbidity was investigated, found that vitamin A supplementation had no effect on malaria mortality. However there was a significant improvement in the reduction of deaths from acute gastroenteritis, chronic diarrhoea and malnutrition. This overall reduction associated with vitamin A supplementation suggests that it can only be beneficial to supplement where malaria is the major cause of child mortality (Binka *et al*, 1995). This was substantiated in a study in New Guinea on children, ages 6 - 60 months, assigned high doses of vitamin A every 3 months for 13 months. It was shown that vitamin A supplementation lowered morbidity due to Plasmodium Falciparum in these young children (Shankar *et al*, 1999). Another study showed beneficial effects on severe infections such as malaria, when supplemented with 400 000IU of retinyl palmitate intramuscularly (Davis *et al*, 2000).

Vitamin A supplementation seems to have a beneficial effect on the child during parasitic infections and indications that morbidity and mortality are lowered in supplemented groups are a positive indication that supplementation should be recommended.

4.4   Summary and Conclusion

It appears that vitamin A plays an important role in mortality and morbidity in children.   The most positive effect being on gastroenteritis and measles but not acute respiratory infections.   Further work is required in malaria before recommendations can be made.   Vitamin A may enhance the survival in older preschool children but the benefits still remain unclear for the younger children ($<$ 6 months).

In a policy statement on vitamin A, diarrhoea, and measles: The International Vitamin A Consultative Group (IVACG) - "strongly recommends that vitamin A supplements be included in all child survival programs in areas of endemic vitamin A deficiency as a prophylactic and to lessen the consequences of diarrhoea and measles" (IVACG 1996).

Duration and time of administration of vitamin A is an important factor as there are a number of factors that can affect absorption of vitamin A.   Distribution programs involved in supplementation, target the high risk groups with priority being given to pre - school children especially between the ages 6 months to 3 years and mothers within 8 weeks delivery, as the need to raise vitamin A status is important due to the extra demands of breast - feeding.   The timing of the intervention is also important as this depends on a variety of factors, such as season, logistical constraints, resources and the physical health of the child.

Dosage and timing seem to also have an effect on the absorption of vitamin A.   However what is not clear is what is the optimum timing of administration of vitamin A supplementation in order to improve acute diarrhoea.   Administration of

vitamin A during an acute or chronic diarrhoea episode may prove futile because of decreased absorption and other factors that come into play during an infection.

Therefore, the question of when to administer vitamin A during an infectious episode is asked. This study is looking at this dilemma, as it would be both beneficial to the health services and the patient if an optimum time was decided upon.

CHAPTER 5

5.0 LABORATORY METHODS

5.1 BACKGROUND AND PRINCIPLE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) arose in the late 1960's out of the experience in gas chromatography and applications in thin layer chromatography. Practical HPLC systems resulted from scientific experiments and method development enabling the separation of substances that could not be readily volatilised. These methods were also suitable for compounds with high polarities and molecular weights, thermally unstable and those that had tendencies to ionise in solution (Knox, 1980).

The principle of chromatography lies in the interaction between a mobile phase and a solid phase. The mobile phase is the liquid that is pumped through the system. This can be a variety of organic solvents or an aqueous buffer. The solid phase is the column that consists of packing material packed under pressure.

In liquid chromatography the mobile phase is pumped over the stationary - phase, which is confined to a cylindrical column. The sample is injected at the head of the column and is carried in solution in the mobile phase, through the column, which should separate the different sample components according to their relative affinity for the stationary phase. The components emerge, and their time of elution and quantity, are assessed by the detector and data analyser.

There are a number of different chromatographic techniques such as adsorption chromatography, ion - exchange chromatography and size exclusion chromatography.

In adsorption chromatography the separation is dependent on the relative adsorption of the solutes on a polar adsorbent such as silica or alumina (stationary phase), and is commonly referred to as liquid - solid chromatography. In ion - exchange chromatography the cations and anions are separated by their exchange between the mobile phase and the charged surface on the stationary phase. Size exclusion chromatography is where the stationary phase is a non - interactive porous solid such as a silica or an organic gel, and separation is effected on the basis of solute size.

In this study adsorption chromatography was used and separation here is dependent on the relative adsorption of the solute onto a stationary phase. This technique is further divided into normal phase where the stationary phase is polar or reverse phase where the stationary phase is non - polar.

Reverse phase is the more commonly used technique and is favoured above normal phase. One of the disadvantages of normal phase, are the expensive organic solvents used for the mobile phase, such as hexane and methylene chloride. These solvents also tend to be hygroscopic so careful attention has to be taken to prevent adsorption of water (molecular sieves can be used in these solvents). In normal phase chromatography the more polar solvent such as water (hexane being the least polar) results in the retention times being shortened.

In reversed phase chromatography the stationary phase can be a hydrocarbon bonded support such an octadecylsilane (ODS) - silica and the mobile phase an aqueous solution containing varying proportions of organic solvents such as methanol or acetonitrile. The chemically bonded stationary phases are made up of silica particles, either spherical or irregular in shape. However irregular packing is not so popular any more as it has been found that the spherical packing is more efficient. The silica particles are coated with a layer of silanol groups (Si - OH) that are chemically reacted to an octadecyl trichlorosilane or octadecylsilane (ODS), as in the column used in this study, resulting in a surface with hydrocarbon characteristics.

Reversed phase chromatography uses a polar mobile phase with a non - polar stationary phase and is used to separate non - polar molecules. The more polar solutes now have greater affinities for the mobile phase and so elute in reverse order of polarity that is the most polar first. Although reversed - phase chromatography is suited to the separation of non - polar molecules, polar molecules can also be successfully separated by using a mobile phase that is sufficiently rich in an organic component e.g. the methanol concentration is increased to cause the solutes to elute in a reasonable time.

There are a plethora of methods of analysis available to quantitate retinoids. However HPLC with UV detection that has been used for many years has still been found to be the method of choice. It is the most sensitive and selective and allows high sample throughput (Wyss, 1995). A flow diagram illustrating the HPLC system that was used in this study is shown in Figure. 5.1.

Fig. 5.1 **Simplified Flow Diagram of the High Pressure Liquid Chromatography System used for analysis**

Mobile Phase

Chemstation Computer

Sample Injector

Absorbance Signal

Pump

Guard Column

Analytical Column

To Waste
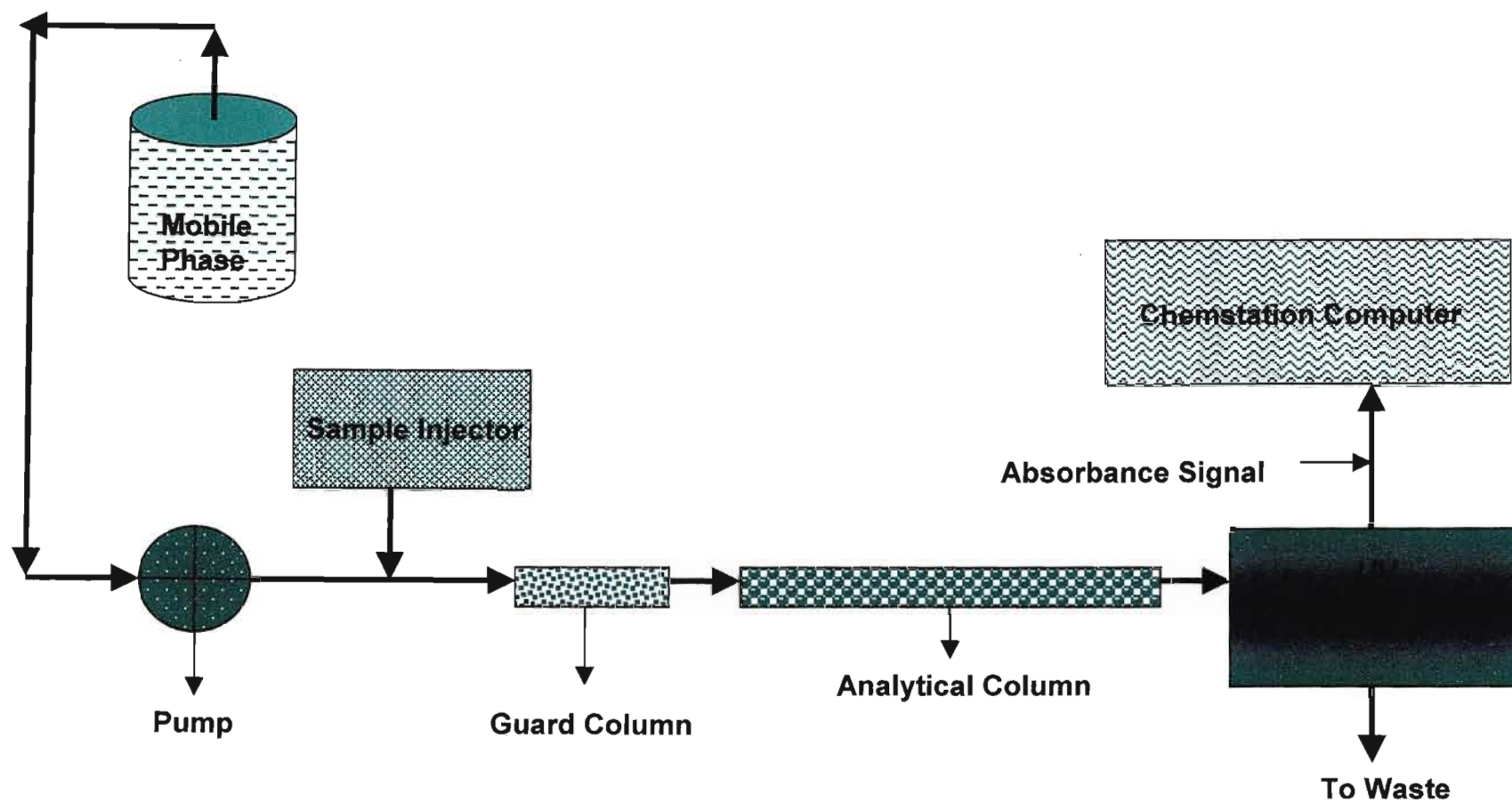
5.1.1 Background and Overview of the Method Used

Vitamin A and vitamin A2 (3,4 - didehydroretinol) were the two analytes of importance and were measured using a slightly modified method by Catignani (Catignani and Bieri, 1983). The internal standard used was retinyl acetate as it was chemically very similar to vitamin A and therefore would react similarly during the sample preparation. A small amount of the internal standard was weighed out and dissolved in ethanol. It was then measured on the spectrophotometer to determine the concentration and a small aliquot of this was added to the sample before extraction. Recovery of the internal standard was used to determine efficiency of the sample extraction, sample preparation and instrument performance.

Sample preparation consisted of a protein precipitation step, followed by a liquid, liquid extraction with hexane. The hexane phases were combined and evaporated and the precipitate reconstituted in methanol.

In this study a spherical particle packed column was used. The particle size was $5\mu m$ with a chemically bonded phase - octadecylsilane groups (C_{18}).

Once the sample has been separated by the column it passes through the detector and in this case an ultraviolet diodarray detector was used. The amount of absorption was compared to known standards that were used to quantify the unknown samples. In this study it was necessary to use a scanning ultra - violet detector i.e. diode - array detector for its ability to measure variable wavelengths simultaneously. The two analytes of interest had different λ max values viz. vitamin A λ max at 325nm and vitamin A_2 at 350nm.

The chromatograms of a typical extracted human plasma sample (Figures 5.2 and 5.3) showed good separation and resolution between A_2, vitamin A and retinyl acetate (internal standard) and a stable baseline. The retention times were approximately 4.5 minutes for 3,4 - didehydroretinol, 4.9 minutes for vitamin A and 6,5 minutes for retinyl acetate. The results were analysed using a Hewlett Packard Chemstation.
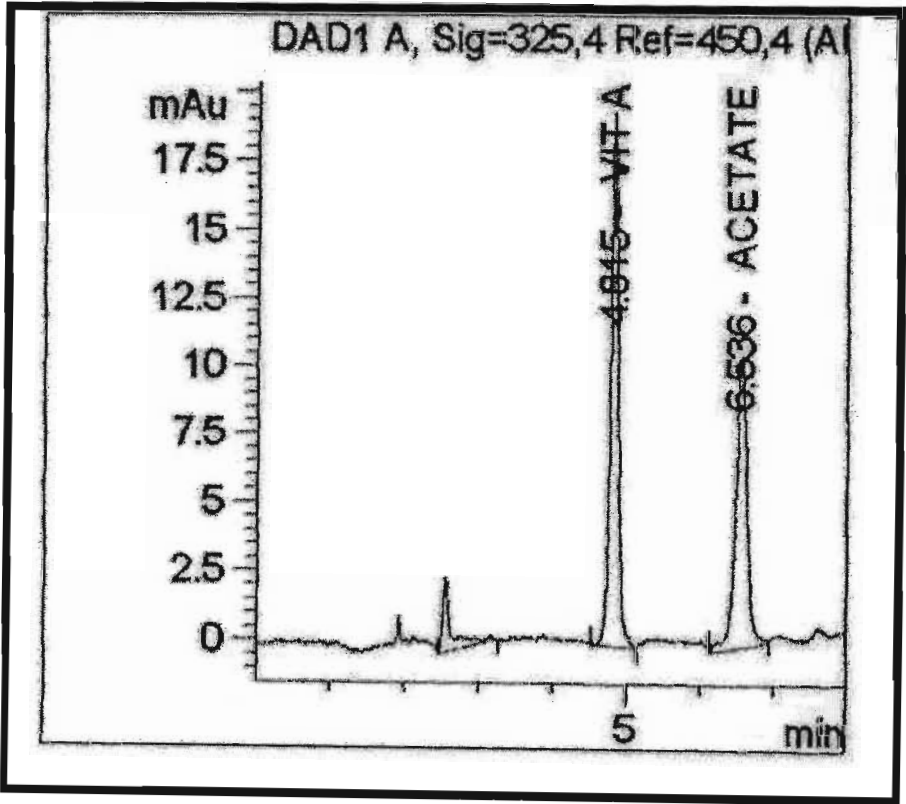
Fig. 5.2   **Actual chromatogram of vitamin A (retention time 4.815 minutes) extracted from plasma. The internal standard used was retinyl acetate (retention time 6.536 minutes).**
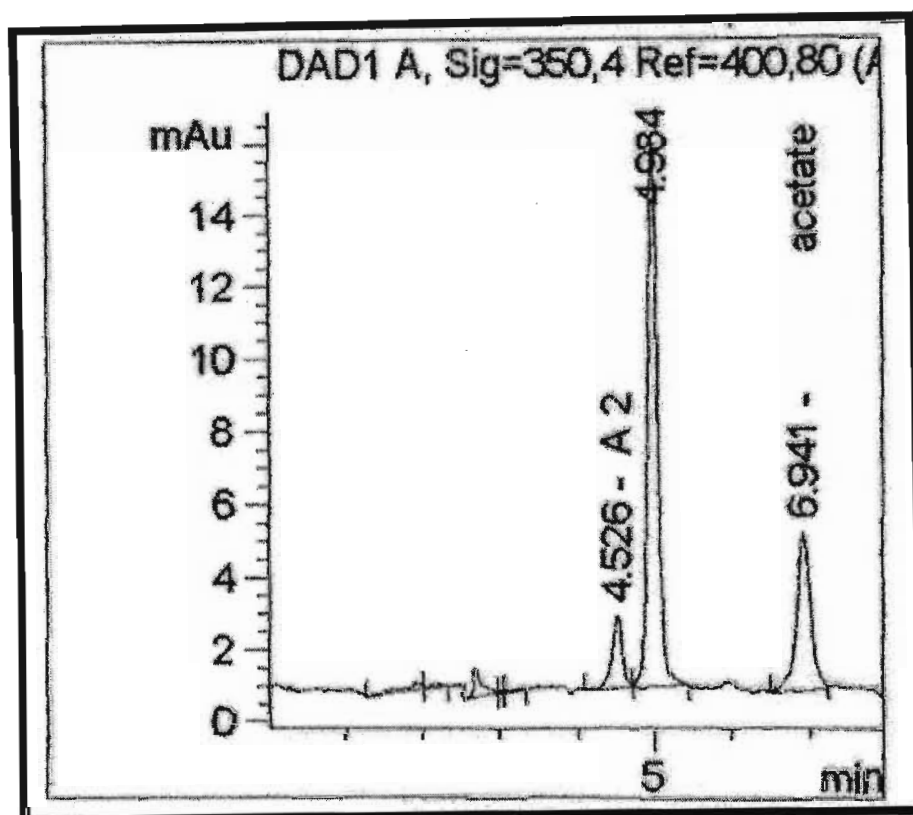
Fig. 5.3 **Actual chromatogram of 3,4 - didehydroretinol (retention time 4.526 minutes) and vitamin A (retention time 4.984 minutes) and the internal standard retinyl acetate (retention time 6.941 minutes) extracted from a plasma sample.**

5.1.2 Reagents

All - trans - retinol (Cat No. R-7632, 25mg) and *all* - trans - retinyl acetate (R-4632, 1g) were supplied by Sigma - Aldrich Chemie, Steinheim, Germany. The didehydroretinol was obtained directly from Dr. Sherry Tanumihardjo at the University of Wisconsin, United States of America. Ethanol was supplied by Scharlau Chemie SA, Barcelona, Spain, methanol and hexane from Burdick and Jackson, Musckegon, USA. The organic solvents were all HPLC grade. The water was distilled and filtered through Lida nylon filters, pore size 0.45μm, obtained from Chrom Tech, Inc.

Bovine serum albumin (cat no.   A - 8551) used in the quality control was obtained from Sigma - Aldrich Chemie, Steinheim, Germany.

5.1.3 Apparatus

HPLC analysis was performed using a Hewlett Packard 1090 (Figures 5.4, 5.5 and 5.6) fitted with an ultra - violet diode - array spectrophotometer. The samples were analysed using the Hewlett Packard Chemstation. The analytical column used was Spherisorb (S5 ODS2), 25cm x 4.6mm, and was purchased from Phase Separations Limited, Norwalk, USA. The guard column was manually packed with C_{18} packing from Whatmann Inc., Clifton USA.
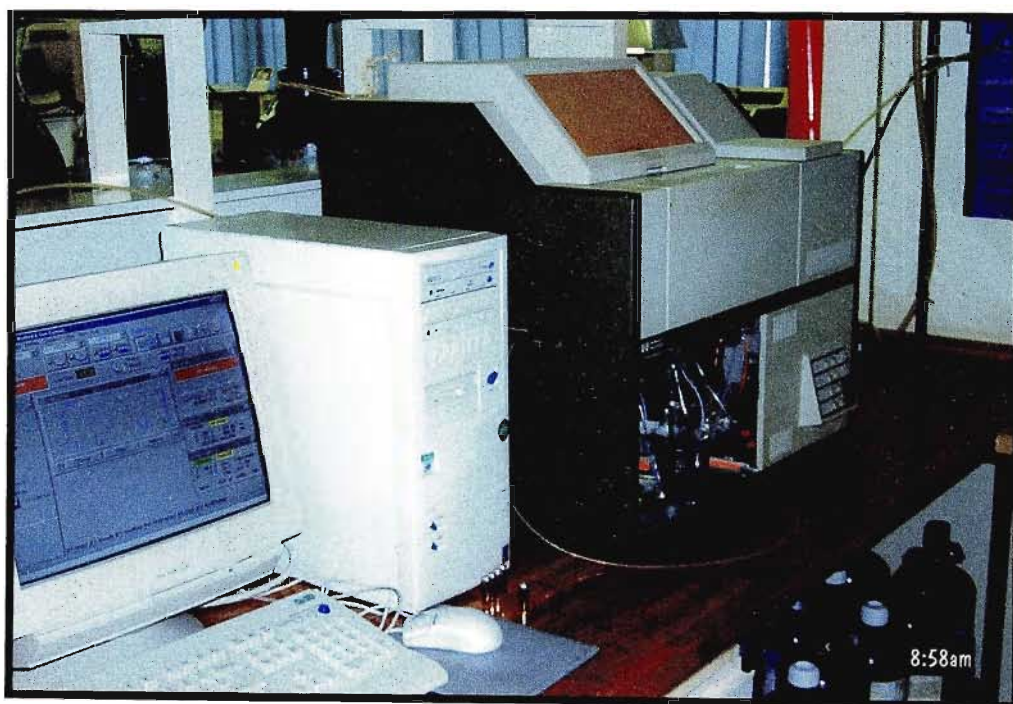
Fig. 5.4   The HPLC (Hewlett Packard 1090) used in this study to analyse vitamin A and 3,4-didehydroretinol.
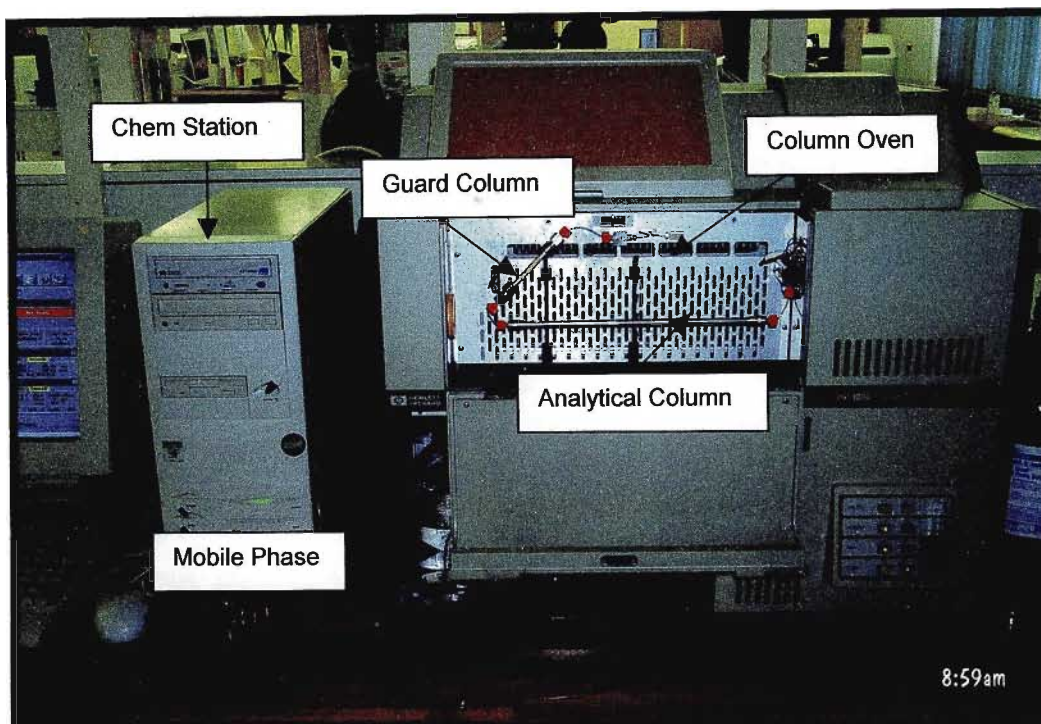
Fig. 5.5   HPLC showing the opened column oven.
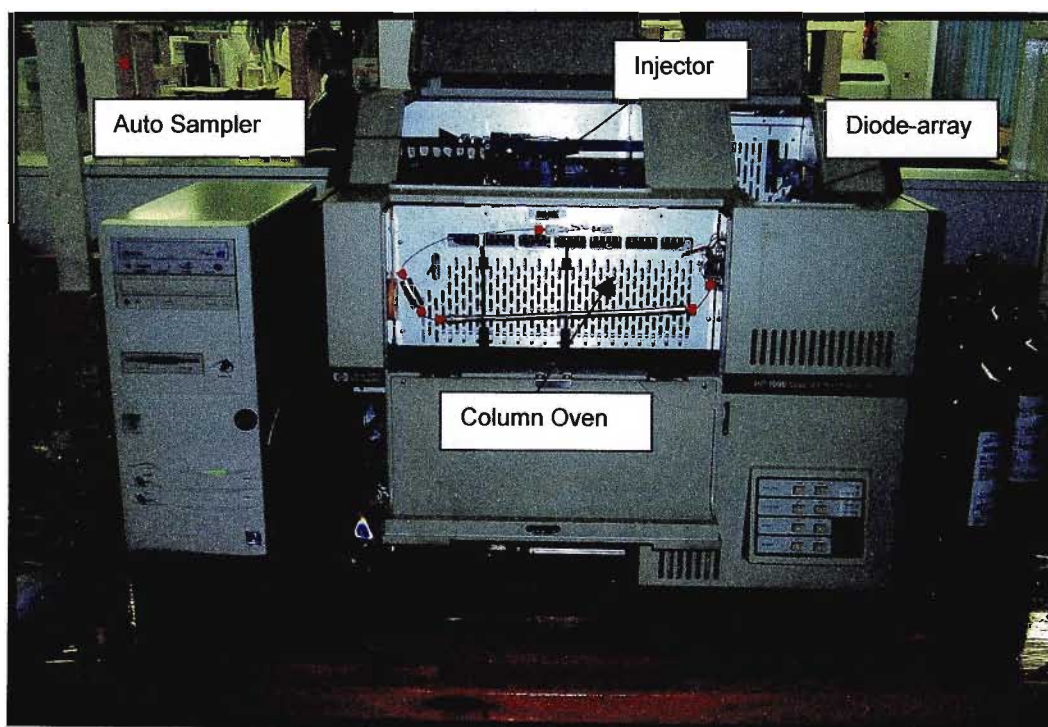
Fig. 5.6   HPLC showing the different compartments viz. autosampler, column oven and diode-array detector.

5.1.4 Preparation of Standards

5.1.4.1 Vitamin A (retinol)

Vitamin A is an unstable compound (see: 5.1.10), therefore it is rarely pure when purchased commercially and it is necessary to purify the standard.

The vitamin A standards were used to plot a calibration curve to enable quantitation of vitamin A (Figure 5.7). They were also used to calculate the recovery of vitamin A in the plasma samples.

This standard was purified using the following procedure:

1. The complete vial (25mg) of vitamin A standard was emptied into a 10ml volumetric flask and made up to volume with ethanol.

2. 25μl of this dilution was injected ten times onto the HPLC column, and 10 peaks were collected into a 10ml volumetric flask.

3. This solution was further diluted with ethanol to obtain an absorbance reading of less than 1 absorbance on the spectrophotometer, against ethanol, using a 325nm wavelength. The reading of this final dilution was used to calculate the concentration.

4. The concentration of the standard solution was calculated using the extinction coefficient as follows: $E^{1\%}_{1cm}$ = 1845 for vitamin A.

    i.e. Beer Lambert Law : A = KLC.
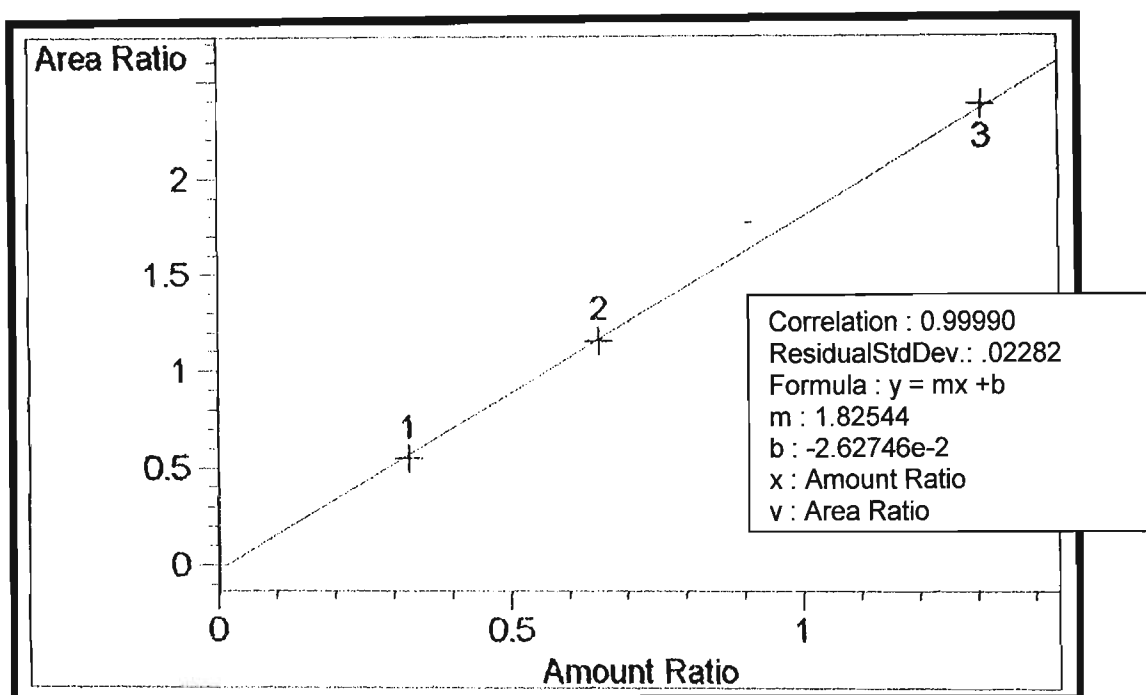
Figure 5.7   Vitamin A standard calibration curve.
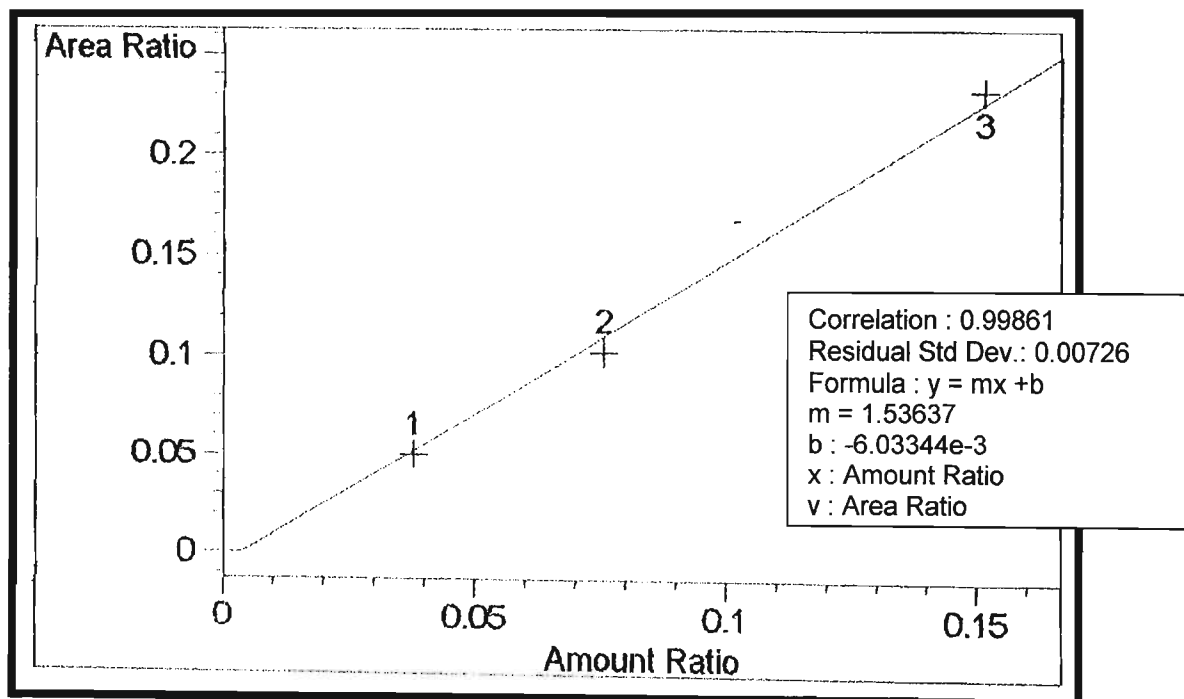
Figure 5.8   3,4 - didehydroretinol standard calibration curve.

Where: A = Absorbance

K = Absorption of a 1% solution (1845)

L = length of light path of a solution on 1 cm.

C = concentration of substance in μg/ml

5. A matched pair of quartz curvettes was used with a 1cm light path. The concentration of the solution was calculated using the following formula:

C = A/K.

6. Quantitation:

Retinol: (A_{325} x 10 000) / 1845 = concentration in ng/μl or μg/ml.

5.1.4.2   3,4 - didehydroretinol

The 3,4 - didehydroretinol standards were used to plot a calibration curve (Figure 5.8) to quantitate the didehydroretinol peaks and to calculate recoveries of didehydroretinol in the plasma samples.

The 3,4 - didehydroretinyl ester is in an oil solution and needs to be saponified to the alcohol form (3,4 - didehydroretinol), using the following method.

1. 50μl of 3,4 - didehydroretinyl acetate was pipetted into a screw cap tube.

2. To this 7.5mls of ethanol was added and vortexed.

3. 3.75mls of a 50:50 (w/v) KOH/H_2O solution was added and the sample was mixed thoroughly.

4. The tube, with a screw cap, was placed into a water-bath at 45°C for 1 hour, mixing periodically.

5. After the reaction had taken place, 5mls of H_2O and 7.5mls of hexane was added. It was then necessary to mix thoroughly and centrifuge, to separate the two layers. The upper layer (hexane) was transferred into a clean tube. The lower layer (aqueous) was extracted once more with 7.5mls of hexane and the two layers of hexane combined and evaporated under nitrogen.

6. The residue was reconstituted in mobile phase (98% methanol and 2% water) and 25µl was injected onto the HPLC column to determine where the 3,4 - didehydroretinol would elute. The wavelength used for detection was 350mn.

7. These peaks were collected into a 10ml volumetric flask as they eluted from the analytical column (± 5 peaks are collected).

8. This stock solution was further diluted until a reading of < 1 was obtained on the spectrophotometer. The concentration of the standard was calculated as for vitamin A using the extinction coefficient for A_2 of 1455 ($E^{1\%}_{1cm}$=1455).

9. Quantitation:

   Didehydroretinol: (A_{350} x 10 000) / 1455 = concentration in ng/µl or µg/ml

5.1.4.3 Internal Standard (*All - trans* - retinyl acetate)

Commercially available retinyl acetate was used as the internal standard (Sigma - Aldrich Chemie). It is not necessary to purify this standard. Retinyl acetate is used as an internal standard because it is chemically very similar to vitamin A and will therefore react similiarly during sample preparation and under the selected chromatographic conditions for vitamin A. The acetate was well

resolved from the vitamin A and A_2 peaks. Retinyl acetate is not present in any concentration in human plasma eliminating any interference during sample analysis, making it a good choice as internal standard. It was also found to be relatively stable when exposed to light and air.

A stock dilution was made directly from the vial by weighing out accurately $\pm 200mg$. This was dissolved in ethanol in a 10ml volumetric flask. The stock solution was further diluted until a reading of less than 1 absorbance was obtained on the UV spectrophotometer. The concentration of the standard was calculated as for Vitamin A using the extinction coefficient for retinol acetate of 1560 ($E^{1\%}_{1cm}$ = 1560). An accurate amount of the calculated dilution of retinyl acetate was added to each sample just before deproteinisation and extraction. The internal standard recovery was used to indicate the efficiency, accuracy and preparation of sample extraction, and instrument performance.

5.1.5 Standard Curves and Calibrations

The extraction procedure used for the standard curves was the same as for sample extractions described further on in this chapter. The only difference is that the plasma was replaced by bovine serum albumin that was an analyte - free matrix, thus eliminating interference and having to use standard addition methods. The bovine serum albumin was made up of 100 mls of phosphate buffered saline to which is added 3,5g of bovine serum albumin.

A multi - point calibration was used in this study for both vitamin A and didehydroretinol. The standard graphs were linear when comparing the peak area

and varying concentrations over a working range of 5.0 µg/dl to 40 µg/dl for retinol and 0.5 µg/dl to 4.0 µg/dl for didehydroretinol. These calibration curves for both vitamin A and 3,4 - didehydroretinol are illustrated in Figures 5.7 and 5.8.

The correlation coefficient for retinol was 0.9999 (Figure 5.7) and for didehydroretinol was 0.99861 (Figure 5.8) showing that both compounds were linear over the specified range. A perfect positive correlation relationship is 1.00. In the specified range there were three concentrations for the vitamin A curve (5, 10 and 40 µg/dl) and three concentrations for the didehydroretinol curve (0.5, 1.0, 4.0 µg/dl). For each concentration 3 extractions were carried out and the mean peak area ratio was plotted for each point. The relationship between the peak area ratio and the concentration was defined by the equation: $y = mx + b$ where y was the peak area ratio, m was the slope of the line, x is the concentration, and b is the y - intercept of the line.

The concentrations were calculated as follows: Vitamin A / Didehydroretinol Concentration in µg/dl

$$= \frac{\text{Area of Vit A / A}_2}{\text{Area of Internal Standard}} \quad \text{x} \quad \frac{\text{Concentration of internal standard}}{\text{Response Factor}} \quad \text{x} \quad \frac{1}{250} \quad \text{x} \quad 100$$

The response factor used in the calculation is the ratio between vitamin A or A_2 and the internal standard each of the same concentration i.e. the area of the vitamin A or A_2 divided by the area of the internal standard. This response factor was used as an indicator of any changes and deterioration that might occur in the instrument and standards.

5.1.6 Sample Preparation

Vitamin A is soluble in most organic solvents but not water. And is extracted from the serum using an ethanol extraction and then a liquid/liquid extraction with hexane. Vitamin A is easily oxidised and degenerates when exposed to light. It is therefore advisable to store vitamin A serum samples under argon or nitrogen at a temperature of -70°C. (Stability 5.1.10)

1. 500µl of serum (sample), 50µl of internal standard (all - *trans* retinyl acetate, made up in ethanol) and 450µl of ethanol was pipetted into a 12 X 75mm disposable borosilicate glass tube.

2. The above was vortexed for approximately 30 seconds. This step deproteinises the sample.

3. 500µl of hexane was added and vortexed for exactly 1 minute.

4. The sample was centrifuged for 10 minutes at 4000U/min to expedite phase separation.

5. The organic layer (top) was taken off into a clean test tube.

6. The hexane step was repeated and the organic layers pooled.

7. These combined hexane layers were evaporated down under a flow of nitrogen in a fume cupboard at room temperature.

8. The residue was reconstituted using 50µl of methanol.

9. 25µl was injected onto the analytical column of the HPLC.

10. All procedures were conducted away from direct sunlight and there was no artificial lighting. All the samples were placed into a micro - vial sleeve, which was contained in an amber vial. The samples were placed into an autosampler, which was completely protected from direct light.

5.1.7 Chromatographic Conditions

The performance of the instrument was monitored each day using quality control samples, the internal standard and the response factor ratio. Each day the HPLC system, including the injector was flushed out with mobile phase for approximately 15 minutes before injecting the quality control samples and the response factor samples (external standards). The system was fitted with a guard column ahead of the analytical column. This is used to protect the analytical column from any contamination due to a precipitant or particulate matter. The condition of this guard column was monitored using column pressure as an indicator. If the pressure went above 200 bar it was necessary to repack the guard column and to replace the column frits.

The mobile phase was continually degassed using high purity helium. The water used in the mobile phase was filtered through membrane filters. Filtering the mobile phase removes particulates and simultaneously degasses the solution.

The HPLC used in this study had the facility for variable wavelength settings, which were set at 325nm for vitamin A, and 350nm for 3,4 - didehydroretinol.

The following chromatographic conditions were used:

Mobile Phase	= Methanol 98% and Water 2%
Flow Rate	= 1ml/min
Oven Temperature	= 40° C
Injection Volume	= 25ul
Maximum Pressure	= 400 bar
Stop Time	= 8 minutes

5.1.8 Quality Control

The precision and recoveries for vitamin A and 3,4 - didehydroretinol were analysed using bovine serum albumin which simulates human plasma. Vitamin A is not present in bovine serum albumin. This approach was used to eliminate the problem of using serum or plasma that already contained Vitamin A in various concentrations. The bovine serum albumin was made up as follows:

1. 100 mls of phosphate buffered saline (PBS) was made up using the same concentrations as used for the ELISA plates (see: 5.2.6). 1 ml of this concentrated phosphate buffered saline was diluted with 10mls of distilled water.

2. 3.5grams of bovine serum albumin was added to the diluted PBS (precautions were taken when mixing as the bovine serum albumin tends to clump).

3. To this mixture, vitamin A, at a concentration of 29.57 µg/dl and 3,4 - didehydroretinol, at a concentration of 3.94 µg/dl was added. This was mixed for 4 hours on a rotating mixer.

4. Aliquots of 1 ml of this plasma were pipetted into eppendorf tubes and frozen at -4°C.

5. These samples were then extracted daily using the sample preparation method for vitamin A samples.

6. Six samples were cleaned up on the same day and analysed and one sample was done each day during the analysis of the plasma samples, giving both intra - day and inter - day values.

Table 5.1   Precision and Recovery for 3,4 - didehydroretinol.

Concentration of 3,4 - didehydroretinol: 3.94 µg/dl.

INTERDAY : DATE	VALUE µg/dl	RECOVERY IN %
30.7.99	3.77	95.70
31.7.99	3.60	91.37
05.8.99	3.78	95.95
06.8.99	3.73	94.67
07.8.99	3.94	100.00
11.8.99	3.78	95.94
INTRADAY : DATE	VALUE µg/dl	RECOVERY IN %
07.8.99	3.94	100.00
07.8.99	3.97	101.00
07.8.99	3.91	99.24
07.8.99	3.91	99.24
07.8.99	3.87	98.22
07.8.99	3.98	101.00

Table 5.2   Precision and Recovery for Vitamin A

Concentration of Vitamin A: 29,57 µg/dl.

INTERDAY : DATE	VALUE µg/dl	RECOVERY IN %
30.7.99	29.95	101.29
31.7.99	30.28	102.4
05.8.99	29.21	98.78
06.8.99	31.31	105.88
07.8.99	27.97	94.59
11.8.99	29.06	98.28
INTRADAY : DATE	VALUE µg/dl	RECOVERY IN %
07.8.99	27.97	94.59
07.8.99	28.51	92.27
07.8.99	29.50	99.76
07.8.99	28.73	97.16
07.8.99	30.57	103.38
07.8.99	30.35	102.60

The mean for the 3,4 - didehydroretinol (A_2) quality control interday samples (Table 5.1) was 3.77, and the coefficient of variation % was 2.89%. The intraday samples (Table 5.1) mean was 3.93 µg/dl and the coefficient of variation % was 1.06%. Vitamin A quality control samples interday mean (Table 5.2) was 29.63 µg/dl and the coefficient of variation % was 3.88%. The intraday samples mean (Table 5.2) was 29.27 µg/dl and the coefficient of variation % was 3.57%.

Standards were also obtained from the National Institute of Standards and Technology (NIST). These standards were prepared in the same way as the samples and quality control samples (Table 5.3).

Table 5.3   NIST standards.

	OUR RESULT	NIST STANDARD	RECOVERY IN %
LEVEL 1	82.8 µg/dl	84.1 µg/dl	98.1%
LEVEL 2	48.35 µg/dl	48.4 µg/dl	99.9%

A proficiency test for selected laboratories, especially in Africa, measuring retinol and carotenoids in serum was carried out at the end of 1999 by myself in the analytical unit, Chemical Pathology Department, Medical School, University of Natal.

The test was co - ordinated by the Division of Human Nutrition and Epidemiology, Wageningen University, Netherlands. Sixteen laboratories were included in this study, 12 within the African region (4 South African) and 4 outside Africa. Only seven laboratories performed well in this proficiency test, this included our laboratory (Brouwer *et al*, 2001).

5.1.9 Problems Encountered when Setting up the Method

The MRDR test requires good analytical precision and any baseline instability during HPLC runs will markedly affect the reproducibility of the result, as the A_2 peak is very small. The sensitivity of this method for 3,4 - didehydroretinol (A_2) was found to be 0.2 µg/dl based on the signal to noise ratio of 2:1. Over the range of 0.5 µg/dl - 4.0 µg/dl the coefficient of variation % was 4.2%. The

didehydroretinol (A_2) peak was initially not completely resolved from the vitamin A peak. It was also necessary to be able to determine very low levels of A_2. To separate the two peaks the methanol concentration was increased to 98%, which resulted in a well - resolved baseline between the two peaks.

Sensitivity was compromised by the variable wavelength diode array detector (DAD). The fixed wavelength detectors are generally known to be more sensitive, however the variable wavelength facility available in this instrument, was essential to this study to enable detection of both the vitamin A and didehydroretinol simultaneously. Quantification limits in the lower levels that were required in this study were however met by the Hewlett Packark 1090 diode - array UV spectrophotometer.

To increase the sensitivity a larger sample volume in the sample preparation step (500µl instead of 250 or 200µl) was necessary. The minimum blood sample of 1,5 mls was therefore required. An additional way to increase sensitivity was to concentrate the final step viz. sample was reconstituted with only 50µl of methanol. It was necessary to mix the sample in the final step using a lid as evaporation was a problem because of the large surface area in the tube and then to transfer the sample quickly into a sealed amber microvial.

Both Vitamin A and A_2 oxidise when exposed to light and air. To compensate for this problem the working area where all the analyses were carried out was kept free of artificial light and the standards were stored under nitrogen at -20^0C in sealed amber glass containers.

5.1.10   Stability of 3,4 - Didehydroretinol, Vitamin A and Retinyl Acetate

Retinoids are sensitive to light, oxygen and heat, all of which must be considered when collecting and storing samples.   Vitamin A and 3,4 - didehydroretinol are very sensitive to light.   The reference standards and the biological samples should all be protected from natural light and fluorescent light. Yellow light or subdued light should be used in this case (Wyss, 1995). However a study on stability of retinoids showed that retinol samples kept on the bench under fluorescent lights for up to 72 hours showed no signs of deterioration (Su *et al* 1999). The standards used in this study were kept at -20°C, under nitrogen, in sealed amber vials.

Special precautions should also be taken when collecting the samples. The samples should be separated immediately to prevent haemolysing and the plasma stored at either -20°C or -70°C.   Retinol was also found to stable for 18 hours at room temperature, in the autosampler in ethanol (Wyss, 1995). This was further validated in a study showing that retinol samples were stable, whether they were kept at -20°C, -4°C or room temperature for up to 48 hours (Su *et al,* 1999).

In this study we found that the reconstituted samples were stable overnight in the freezer at -20°C. In another study it was found that lyophilized serum stored at -25°C and -80°C was stable for 10 years and serum frozen at -80°C was found stable for 5 year (Brown Thomas *et al,* 1998).   In this study all the samples were stored at -70°C. The most obvious negative effects that are seen concerning stability are when the sample is thawed and frozen repeatedly (Brown Thomas *et al,* 1998).

5.2 ELISA METHOD FOR DETECTING RETINOL BINDING PROTEIN

Enzyme - linked immunosorbent assays (ELISA) are used to detect antibody or antigen levels. The antibody or antigen is captured and detected using a conjugate with a suitable enzyme such as alkaline phosphatase, peroxidase or beta - galactosidase. This enzymatic activity is measured colourimetrically or fluorimetrically after the completion of the antigen/antibody reaction and the addition of the substrate.

5.2.1 Principle of the ELISA method used to detect Retinol Binding Protein

There are a variety of techniques in ELISA testing viz. direct and competitive. In the direct ELISA the patient's serum is added to the solid phase containing the specific antibody and is incubated for a specific time, at a specific temperature. The direct ELISA produces more colour as the unknown antigen concentration increases in the sample. Conversely with low concentrations of antigen smaller amounts of conjugate will bind resulting in less substrate cleaved and less colour produced and lower spectrophotometer readings. The antigen is therefore directly proportional to the colour produced. The direct ELISA was used in this study to measure retinol - binding protein in plasma.

Competitive ELISA's differ in that the antigen in the sample competes with the conjugate for the active sites on the bound antibody. Here the sample and the conjugate are added at the same time to the solid phase. If the antigen concentration is very high there will be little conjugate bound to the immobilised antibody. Therefore there will be little colour development as there will not be

enough enzyme to cleave the substrate. Hence the amount of unknown antigen in the sample is inversely proportional to the colour produced.

In this study the retinol binding protein (RBP) antibody (anti human RBP antibody) was attached to wells of a plastic microtiter plate. After the antibody had bound to the plate, the unbound excess was washed away using blocking buffer containing bovine serum albumin (BSA) that is added in excess to prevent non - specific binding of the antigen onto the plastic plate.

The antibody captured the RBP antigen that was present in the plasma and controls. The RBP antibodies recognise the RBP antigen and are specific to this antigen. Any non - bound material was removed by washing and this was followed by the addition of anti - human RBP horseradish peroxidase (HRP)(conjugate), which binds with the antibody/antigen complex. Conjugates are antibodies directed against a human antigen (in the indirect assays) or the antibody (in the competitive assays) on the solid support. In both cases these antibodies are coupled (conjugated) to an enzyme such as alkaline phosphatase or horseradish peroxidase (HRP) (the latter being used in this study).

After removal of the non - bound HRP conjugate by washing, a substrate solution was added to the wells. The enzymes converted the substrate in the presence of a chromogen (colour producing compound) to produce a coloured product that could be detected spectrophotometrically. This colour - reaction was in proportion to the amount of antigen in the samples and controls. Adding a stop reagent terminated the reaction and the absorbance was measured using a microtiter plate reader. A diagram showing this ELISA reaction is shown in Figure 5.9.

Principle of ELISA reaction
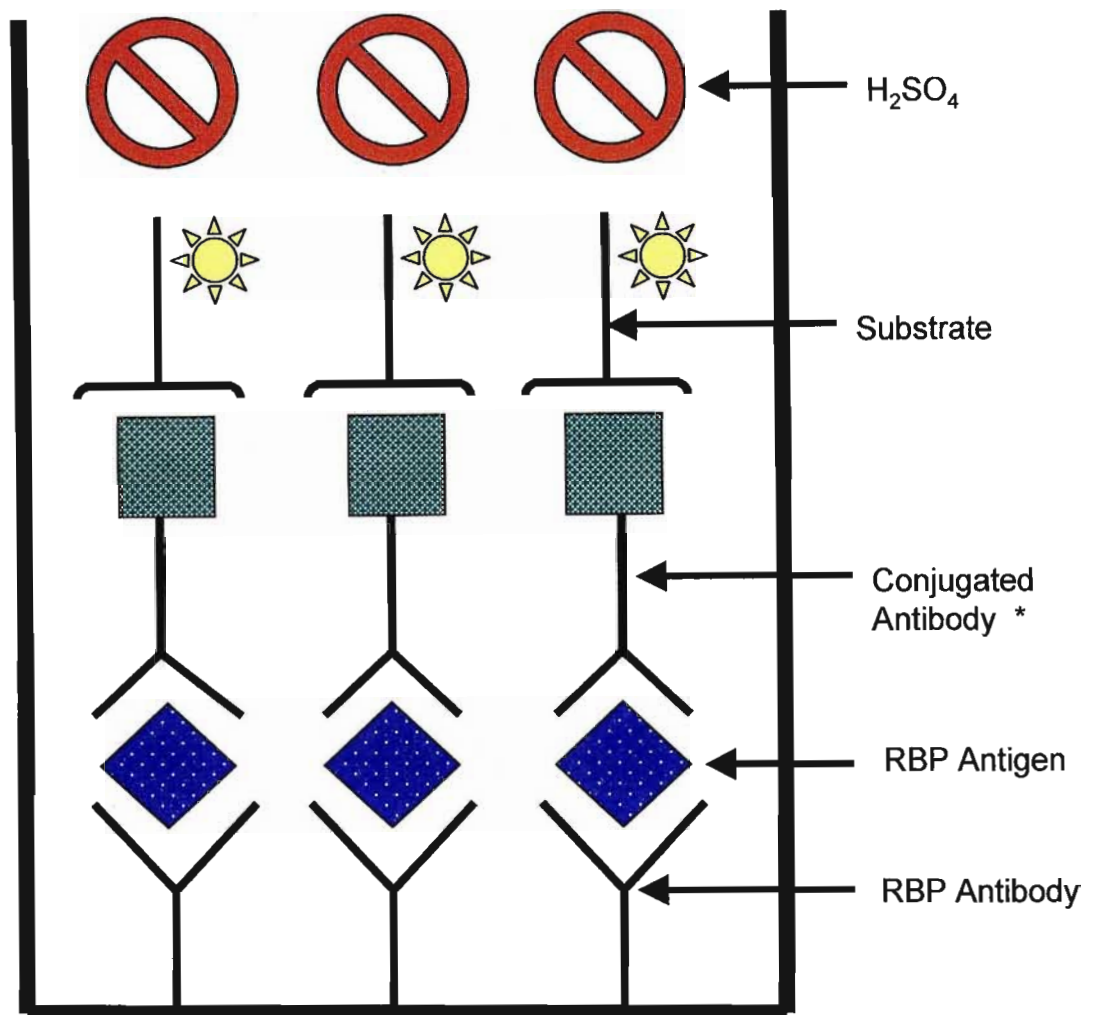
Figure 5.9   Illustration showing the reaction taking place during the RBP ELISA test. (* This antibody is anti human RBP antibody conjugated to the enzyme horseradish peroxidase)

5.2.2 Reagents

Chemicals used to make up the coating and washing buffers were obtained from Riedel-de-Haen, Germany. Bovine serum albumin and the tetramethylbenzidine dihydrochloride tablets for the substrate solution was obtained from Sigma Chemical Co., St Louis, USA. Retinol Binding Protein antibody (Code. A0040) and the antibody conjugated to horse - radish - peroxidase (Code. P0304) were purchased from Dako, A/S, Denmark. The RBP standard serum (OWXK 13 N/T) was from Behring, Milton Keynes, UK.

5.2.3 Apparatus

The plate reader used was a Bio – Rad, Microplate Reader, Model: Benchmark (Figure 5.10). The microtiter plates used in the test were from Polychem, South Africa.
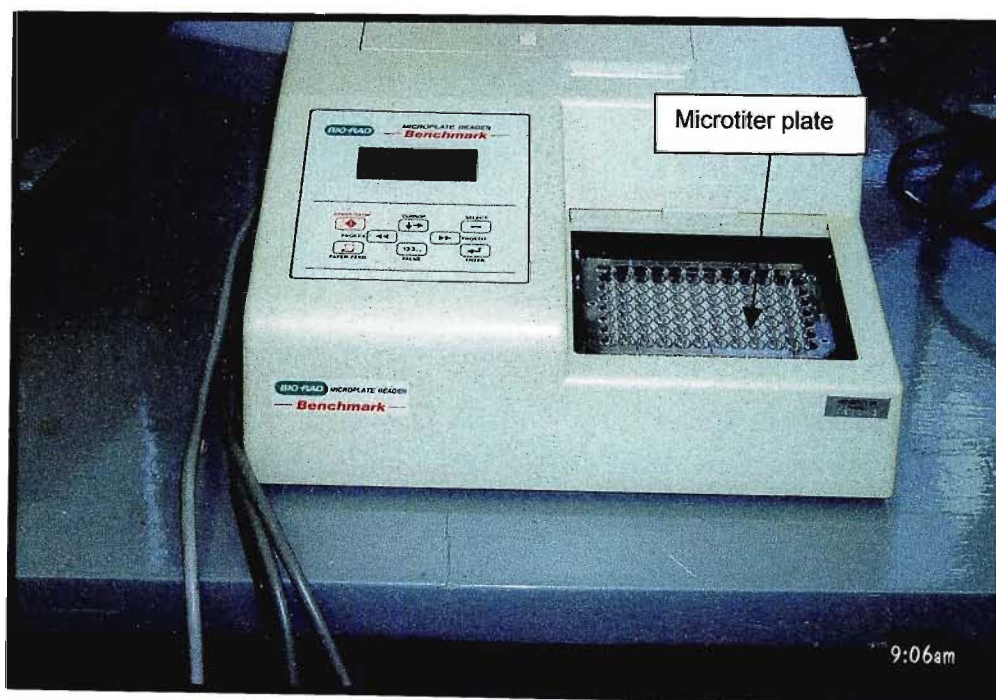
Fig. 5.10 The ELISA plate reader used in the study to quantitate retinol binding protein.

5.2.4 Standard Curve and Calibration

The standard mentioned above (Behring OWXK 13 N/T) was used in serial dilutions to calculate the concentration of the samples. Dilutions were expressed in μg/ L as follows: 50.0, 25.0, 12.5, 6.25, 3.13, and 1.56. A graph was plotted using the absorbance and concentration values of these dilutions. The curve was a linear log fit and the correlation coefficient was calculated for each graph with the values usually around 0.99 for each plot. The samples were then read off this graph of absorbance against concentration (Figure 5.11).

Calculations for retinol binding protein was as follows:

1.   RBP in μg/L x 5000(dilution factor) = μg/L x 1000 = RBP in mg/L

2.   Need the RBP value to be in μmol/L therefore:

$$\frac{RBP\ mg/L}{21000\ (molecular\ weight\ of\ RBP)} = mmol/L \times 1000 = \mu mol/L$$

3.   The ratio between the retinol and RBP is calculated as follows:

Retinol μM divided by RBP μM/L

A normal ratio value should be around 1.
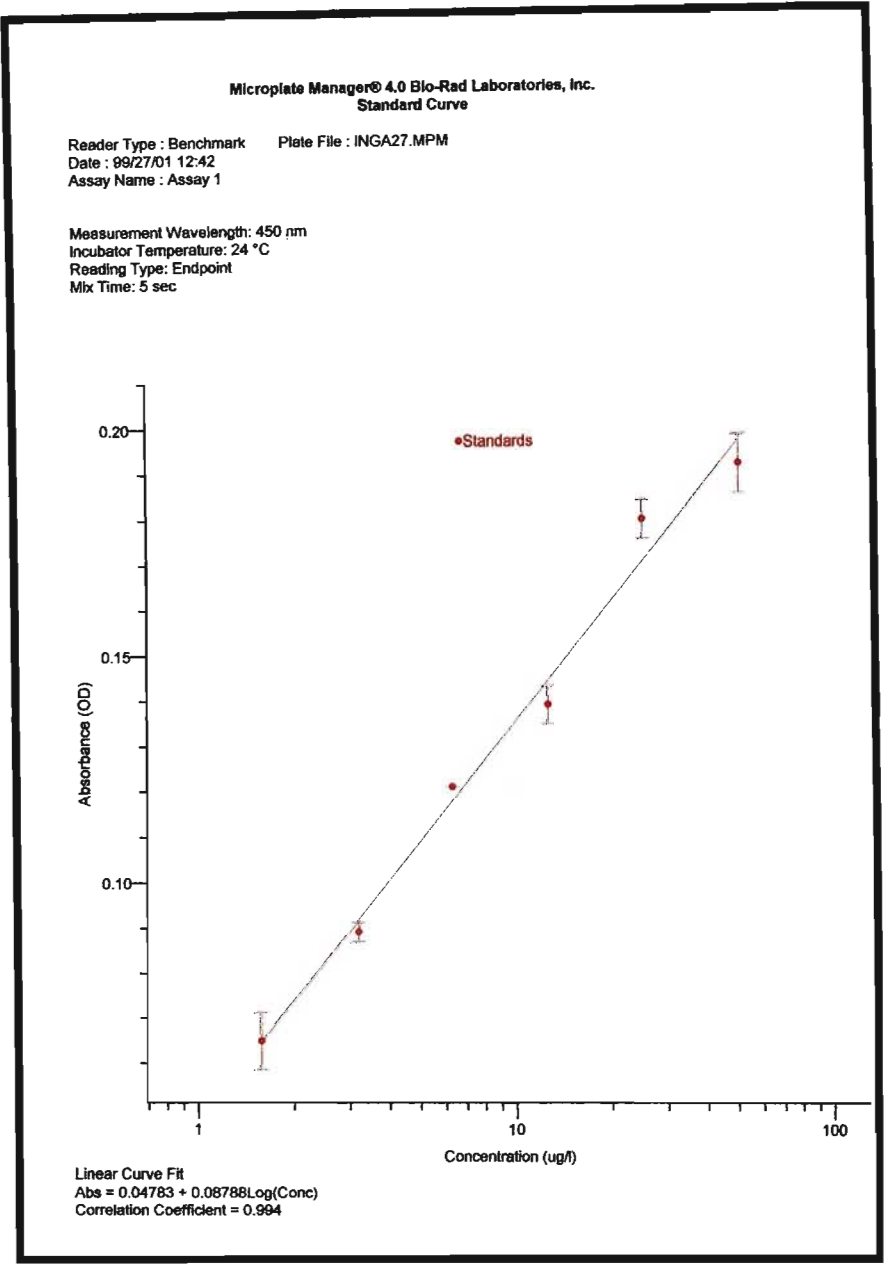
Fig. 5.11 The ELISA test standard curve plotted using the absorbance reading against concentration of the specified standards. This graph was used to quantitate RBP concentrations in µg/L. (The above graph is a scan of the actual print out from the ELISA plate reader)

5.2.5 Quality Control

These samples were prepared using a blood bank sample (Natal Blood Transfusion Services, Durban, South Africa). The blood was separated and 1 ml of serum was aliquotted into Eppendorf tubes and frozen at -70^{0}C. These samples were used as quality control samples to monitor inter and intra plate. The absolute value of the RBP in the blood bank serum was calculated using the standard curve for that day.

Two quality control samples were analysed each day on each ELISA plate. The mean RBP value for the quality control samples was 44.00mg/L. The mean intraday % CV was 11.95% and the mean interday % CV was 15.26%. These values were slightly higher than normal as the ELISA test that was used in this study was developed in a laboratory using purchased reagents and not a kit method. The method was developed by Dr S. Filteau and Dr J. Willumsen at the Institute of Child Health, London.

5.2.6 Preparation of Buffers and Solutions for ELISA Test

- *Coating Buffer* - A 0.1 M Na_2CO_3 and a 0.1M $NaHCO_3$ were made up and equal volumes of each were mixed together and adjusted to pH 9.6.

- *Phosphate Buffered Saline (PBS) (Washing Buffer)* - A stock solution was made at 10X the concentration of the working solution and was diluted when required. The stock solution was made up as follows:-

160g   NaCl

23g Na_2HPO_4 (anhydrous)

4g   KCl

4g KH_2PO_4 (anhydrous)

The above solution was made up in 2 litres of distilled water and the pH adjusted to 7.25. This solution was further diluted by taking 200ml of concentrate and adding 1.8 litres of distilled water and 0.05% of Tween20. Tween 20 is a surfactant and prevents non - specific binding viz. anything binding to the antibody on the microtiter plates other than the antigen.

- *Blocking Buffer* - A 1 % dilution of bovine serum albumin was made up in PBS.

- *Diluting Buffer* - A 0.2% dilution of bovine serum albumin was made up in PBS (containing 0.05% Tween).

- *Substrate* - The substrate solution was made up 5 minutes before use. To 10 mls of a 0.11M Sodium Acetate, one 3,3', 5,5' - tetramethyl - benzidine dehydrochloride tablet and 10ul of H_2O_2 (to start the reaction) was added.

- *Stop Solution* - The reaction was stopped with a solution of 2M H_2SO_4.

5.2.7 Preparation of ELISA Plates

1. The ELISA plates were coated with 50 μl per well of a 1:1000 dilution of anti - RBP antibody (Dako A040) diluted in coating buffer, leaving 4 wells uncoated as a negative control. This was incubated overnight at 4°C.

2. The plates were washed twice with washing buffer and dried by blotting vigorously on a pile of tissue paper.

3. 100μl of blocking buffer was added to each well and incubated at 37°C for 1 hour.

4. The plate was washed twice with washing buffer and dried.

5. The RBP standard was diluted to a concentration of 100 μg/L (Concentration of the standard was 46 mg/L). Serial dilutions of a 1:2 were made on the plate to give 6 standards dilutions of 50ul per well (standard dilutions: 50, 25, 12.5, 6.25, 3.13, 1.56 μg/L). Samples were diluted 1:5000 and 50 μl of this was added to each well. The plate was incubated for 1 hour at 37°C.

6. The plate was washed 3 times with washing buffer and dried vigorously.

7. To each well 50 μl of peroxidase - RBP conjugate (Dako P0304) was added which was diluted 1:1000 with diluting buffer. The plate was incubated at 37°C for 1 hour.

8. The plate was washed 3 times with washing buffer.

9. To each well 50 μl of the substrate was added and the plate was then incubated in the dark for exactly 10minutes at room temperature. The reaction was stopped by adding to each well 25 μl of 2M H_2SO_4.

10. The plates were read at an absorbance of 450nm (reference 550nm) on the microtiter plate reader and the absorbances were quantitated using the standard curve as shown in Figure 5.11. The standard and sample concentrations of an actual serum sample are shown in Figure 5.12

Microplate Manager® 4.0 Bio-Rad Laboratories, Inc.
Unknown Concentration

Reader Type : Benchmark   Plate File : INGA27.MPM
Date : 99/27/01 12:42
Assay Name : Assay 1

Measurement Wavelength: 450 nm
Incubator Temperature: 24 °C
Reading Type: Endpoint
Mix Time: 5 sec

Linear Curve Fit
Abs = 0.04783 + 0.08788Log(Conc)
Correlation Coefficient = 0.9937
[1] Extrapolating above highest standard.
[2] Extrapolating below lowest standard.

Label	Mean Absorb. (OD)	Std. Dev.	%CV	Concentration ug/l	Dilution
Standards					
S1	0.192	6.364e-003	3.32	50.000	
S2	0.180	4.242e-003	2.36	25.000	
S3	0.139	4.243e-003	3.05	12.500	
S4	0.121	0.000e+000	0.00	6.250	
S5	0.089	2.121e-003	2.40	3.130	
S6	0.065	6.364e-003	9.72	1.560	
Unknowns					
X1	0.065	7.064e-004	1.10	1.568	1.000
X2	0.093	2.829e-003	3.04	3.266	1.000
X3	0.056	2.121e-003	3.75	1.239 [2]	1.000
X4	0.101	6.364e-003	6.33	4.027	1.000
X5	0.069	3.536e-003	5.09	1.741	1.000
X6	0.062	4.243e-003	6.84	1.449 [2]	1.000
X7	0.073	2.829e-003	3.87	1.934	1.000
X8	0.065	2.121e-003	3.29	1.568	1.000
X9	0.061	2.828e-003	4.64	1.412 [2]	1.000
X10	0.120	1.414e-003	1.18	6.625	1.000
X11	0.068	2.828e-003	4.16	1.696	1.000
X12	0.078	4.243e-003	5.44	2.204	1.000
X13	0.065	8.485e-003	13.05	1.568	1.000
X14	0.045	4.950e-003	11.12	0.928 [2]	1.000
X15	0.055	1.414e-003	2.57	1.207 [2]	1.000
X16	0.088	2.121e-003	2.42	2.865	1.000
X17	0.059	7.071e-003	11.98	1.340 [2]	1.000
X18	0.082	4.243e-003	5.17	2.448	1.000
X19	0.064	2.121e-003	3.34	1.527 [2]	1.000
X20	0.080	4.243e-003	5.30	2.323	1.000
X21	0.095	4.950e-003	5.18	3.441	1.000
X22	0.063	2.828e-003	4.49	1.488 [2]	1.000
X23	0.124	7.071e-004	0.57	7.358	1.000
X24	0.119	6.364e-003	5.33	6.454	1.000
Blanks					
Blank	0.051	3.304e-003	6.51		

Fig. 5.12 **A scan of the actual printout from the ELISA reader showing sample and standard concentrations.**

5.2.8 Special Precautions taken when Setting up the ELISA Technique

The ELISA test is very sensitive to temperature and enviroment changes. It is important to work accurately and to avoid any form of contamination as this will result in false results. All glassware must be clean and it is necessary to be careful not to contaminate the ELISA microtiter plates. Incubation temperatures must be accurate and the timing of incubation is important. These variables can influence the colour development in the final stages and cause falsely high or low results.

Washing in between stages is essential to eliminate any carry over of the previous antibody, conjugate, or antigen. When washing it is necessary to blot the plates on blotting tissue vigorously so that the PBST is drained off completely, preventing dilution of the next step.

Blocking buffer is a dilution of bovine serum albumin in PBS. This however is not readily soluble in the PBS. It was found necessary to carefully place the bovine serum albumin on top of the PBS and allow it to dissolve gradually as it tends to clump. The blocking buffer is added to prevent non - specific binding that can result in higher readings.

The dilution of the standards was carried out in the wells, however care must be taken to avoid bubbles at this stage, as it will affect the accuracy. The last stage, which is the colour development, must be incubated for exactly 10 minutes as this can cause variables if it is taken out before or after the allotted time.

CHAPTER 6

6.0 STUDY METHODS

6.1 STUDY DESIGN AND BACKGROUND

Vitamin A has well - recognised benefits for prevention of diarrhoea and improved recovery from diarrhoea but the impact of supplementation given during diarrhoea on the biochemical and clinical outcomes due to metabolic changes that occur during the infection are less clear. In this study we investigated the potential therapeutic benefits of supplementation at different time points. The idea of delayed supplementation may be a more effective way of improving vitamin A status of the children at risk and thus a more rational and cost - effective use of vitamin A supplements. Improved protocols for promoting vitamin A status will have additional benefit of decreasing childhood illness and demand on the health care resources in low - income countries.

This study was designed to determine both the best time during diarrhoeal illness in which to give vitamin A in order to improve vitamin A stores in the longer term and the acute effects of high dose vitamin A supplementation on recovery from diarrhoea.

6.1.1 Aim

The aim of the study was to determine the optimum timing for vitamin A supplementation in pre-school children with severe diarrhoea.

6.1.2 Objectives

1.   To determine baseline (on admission) Vitamin A status in pre - school children with diarrhoea.

2.   To determine the acute effect of vitamin A supplementation in these children.

3.   To determine the 8 week vitamin A stores in pre - school children with diarrhoea after supplementation with vitamin A at either day 1 or day 3 - 7.

4.   To determine the effect of vitamin A supplementation, when given on day 1 versus day 3 - 7, on weight and morbidity after 4 and 8 weeks.

6.1.3 Study Design and Subjects

This study is a randomised; double - blind, placebo - controlled clinical trial. Children from the ages $\geq$ 3 months and $<$ 60 months were enrolled in ward N1A, at King Edward Hospital, Durban, South Africa. These children presented with diarrhoea from the surrounding townships and rural areas of Durban with the majority (over 50%) from Umlazi, Kwa Mashu, Inanda, and Clermont.

Children were not eligible for entry into the study if they had any of the following: -

- dysentery (defined as visible blood and mucus in the stool);
- clinical instability (at the discretion of the clinician);
- significant metabolic acidosis (at the discretion of the clinician);
- circulatory impairment or severe electrolyte disturbances, or those who could not take oral fluids;
- vitamin A supplementation in the last month or during the trial.

Eligible children (174 patients) were randomised to receive a single dose of 60mg retinol as retinyl palimate in soybean oil (30mg for children under 12 months old) either on day of admission or after acute diarrhoeal symptoms had resolved, usually between day 3 and day 7. At the other respective time point each child received an identical placebo capsule. Therefore from entry until day 3 - 7 this was effectively a randomised, double blind, placebo - controlled study of the effect of vitamin A treatment on severe diarrhoea. These children were assigned on admission to a numbered pair of capsules, one containing the retinol and the other the identical placebo capsule. The placebo contained soybean oil without vitamin A. One capsule was designated for giving at admission and the other later, after resolution of diarrhoea.

Data forms were completed on admission and at the 4 and 8 week follow - up (Appendix 1,2 and 3). The questionnaires collected maternal and child information, basic medical history relating to the presenting case of diarrhoea and basic socio - economic data. The questions were based on information needed for the study and the previous experience of the researchers involved in the study.

The decision to give the second vitamin A dose between day 3 - 7 was based on the results of a previous study in Tanzanian children hospitalised for diarrhoea. This study showed that depending on diet manipulation, improvement in gut permeability may be seen as early as day 3 (Willumsen JF et al, 1997).

Based on this study we therefore hypothesised that the best time to give vitamin A would be between 3 and 7 days post admission, providing that diarrhoea had resolved, as the gut would probably be restored sufficiently to enable adequate vitamin A absorption.

Diarrhoea resolution was defined as < 3 loose or watery stools per day and < 6 stools total per day. Once diarrhoea had resolved and normal hydration was adequately maintained the second vitamin A capsule or placebo was given at the discretion of the study physician (3 - 7 days). If the diarrhoea was not resolved within the 7 days the child was given the capsule at this time anyway in order to reduce the variability between supplementation time and admission which might confound measurement of subsequent vitamin A status at 8 weeks post admission. The number of days at which the second vitamin A or placebo was administered was similar in both groups; 5.94 ±3.45 in the first group and 5.65 ±2.61 in the group receiving vitamin A supplementation later.

On admission and at day 3, bloods were drawn for serum retinol and RBP measurements. In addition haemoglobin (Hgb), white cell counts (WCC), total protein and albumin were measured. These measurements were necessary for clinical assessment and management of the patient.

The day 3 bloods were drawn before the second dose of vitamin A was given. The reason for measuring serum retinol and RBP at this time point was to test the effect of the initial vitamin A dose (given on admission) on serum retinol levels.

In addition the baseline socio - economic status of these children was examined as it was felt that this could impact on the diarrhoeal disease. This included the living conditions, sanitation and age of the mother.

Children's temperatures were monitored, and they were given antibiotics and antipyretics where necessary. Children were asked to return for clinical follow

- up assessment and morbidity history at 4 weeks and 8 weeks after discharge. At this 4 - week follow up the child's general state of health (morbidity) and weight was documented. At the 8 week - visit a venous blood sample was taken for the modified relative dose response test to measure the vitamin A liver stores. The 8 week time point was chosen to monitor longer term changes and it was felt that it was the longest time at which a reasonable rate of return for follow - up could be expected. In an attempt to maximise the follow - up rate, accurate address information was obtained at discharge and the transport costs were paid for both the children and parents to return to the hospital for the 4 weeks and 8 weeks follow - up visits. Another strategy employed in an attempt to increase follow up was to send a field worker to the homes of those who did not return in order to encourage them to do so.

Initially HIV testing was planned, however it was felt that HIV testing might deter mothers from giving consent to partake in the study and was therefore not included in the study.

A summary of the investigations performed (X) in this study are shown in Table 6.1.

Table 6.1   Study Design and Time-line.

	Enrollment Day 1	Day 3	4 - Weeks Follow - up	8 - Weeks Follow - up
Socio - economic status	X			
Morbidity	X	X	X	X
Weight Measurements	X		X	X
Serum Retinol	X	X		X
Serum RBP	X	X		
Total Protein & Albumin	X			
Hgb and white cell count	X			
MRDR Test				X

6.1.4 Ethics

The ethical review committees of the University of Natal approved the study protocol on the 23rd July 1998 (REF. 005/97).

Written informed consent was obtained from parents and guardians on entry to the study.   Parents were able to withdraw from the study at any time point with no loss of clinical care.   Publications or any other presentations of these results maintained individual confidentiality.

The study was designed such that no group received *only* placebos since this was considered unethical given the current knowledge of the importance of taking all the opportunities available to the child to improving vitamin A status of children from vitamin A deficient areas such as Southern Africa.

6.1.5 Laboratory Analysis and Sampling

On the day of admission and on day 3, venous blood samples were collected in plain tubes for the serum retinol and retinol binding protein measurements.   These samples were centrifuged and serum was separated as soon as possible to prevent any deterioration in the vitamin A levels.   Various papers look at the stability of vitamin A and RBP in serum.   However a study investigating the stability of vitamin A and RBP in serum after the blood had been separated, found that the vitamin A and RBP were stable even after 24 hours stored at 4°C or at room temperature (26 - 28°C) (Mejia *et al,* 1983) (see: 5.1.10).

The serum samples were aliquoted into eppendorf tubes and frozen at - 70^{0}C.   The serum retinols were analysed using a method that requires protein -

precipitation followed by an organic solvent extraction. The prepared samples were then analysed using HPLC. The RBP's were analysed using an ELISA method.

At 8 weeks after admission, samples were taken for analysis of didehydroretinol to assess the liver stores. This sample was taken in a plain tube, centrifuged and the serum stored at -70^{0}C. HPLC was used to separate the didehydroretinol (350nm) that was analysed at the same time as the vitamin A (325nm) using two different wavelengths on the diode - array detector. Both these methods are described in detail in the chapter on laboratory methods (Chapter 5).

6.1.6 Statistics

Data was analysed by standard methods using SPSS 9.0 (Statistical Package for Social Sciences; Version 9.0 for Windows). Descriptive data are presented as arithmetic means ± standard deviation (SD). Comparisons were by *t* test for continuous variables and chi square for categoric variables with the use of a 5% level of significance.

The sample size calculation was based on compared proportions. This sample size was designed to permit detection of 20% differences in MRDR at 8 weeks at a 5% level of statistical significance and 80% power. With these assumptions it was calculated that the number required in each arm of the trial would be 84 per group. This was further increased by 10% to allow for drop - out so that the sample size was set at 186.

CHAPTER 7

7.0 RESULTS

The recruitment and follow - up status of the study are shown in the trial profile in Figure 7.1

Fig. 7.1   **Vitamin A Trial Profile**

```
                    ┌─────────────────────────┐
                    │ 186 children recruited  │
                    └─────────────────────────┘
                              │
                              │        ┌─────────────────────────┐
                              ├───────▶│ 12 not eligible as       │
                              │        │ received multivitamins   │
                              │        │ in hospital              │
                              ▼        └─────────────────────────┘
                    ┌─────────────────────────┐
                    │     174 children        │
                    └─────────────────────────┘
```

Group 1	Group 2
Vitamin A on admission	*Placebo on admission*
Placebo on day 3-7	*Vitamin A on day 3-7*
86 children	**88** children

47 children drop-out	41 children drop-out

4-week follow-up visit	*4- week follow-up visit*
39 children	**47** children

3 children drop-out	13 children drop-out

8-week follow-up visit	*8-week follow-up visit*
36 children	**34** children

Table 7.1 shows descriptive and admission clinical and biochemical data for the 174 children included in the study. There were no differences between the two groups on initial clinical presentation or basic descriptive results. The children in the group given vitamin A later, 3 - 7 days after symptom resolution, had higher blood leukocytes and lower retinol and RBP (Table 7.1) than the children given vitamin A at entry. These differences were not significant.

Stool type was watery (71%), loose (13%) or unspecified (16%). Stool type on admission had no significant effects on biochemical or other measures. The most common systemic complications were pneumonia (27%) and urinary tract infection (11%) while 48% of children had no complications.

For each child, a Z - score (i.e. the number of standard deviations from the reference population mean) for weight for age was calculated. The reference data used was that of the National Centre of Health Statistics of USA (National Centre for Health Statistics, 1977) and the weight/age Z scores were calculated using Epi Info Version 6.02 (Dean et al, 1994).

In all tables mean results are presented as arithmetric means with standard deviation. Numbers for each of the parameters investigated were not always the same due to circumstances and conditions discussed in detail in the Discussion (Chapter 8). The exact numbers for each parameter investigated are presented in parenthesis.

Table 7.1   Baseline descriptive and biochemical data on the 174 eligible children admitted into the study. *

	Group receiving vitamin A on admission	Group receiving vitamin A later (3-7 days)	P Value
Sex (#F/#M)	35/51 (n=86)	37/51 (n=88)	0.98
Age (months)	12.6 (n=86)	12.3 (n=88)	0.8
Weight/age (Z)	-1.46 (SD 1.64) (n=86)	-1.67(SD 1.32) (n=88)	0.36
Stool/day 24 hours prior to admission	5.79 (SD 3.39) (n=86)	5.79 (SD 3.56) (n=88)	0.99
Days diarrhoea pre-admission	7.28 (SD 9.8) (n=86)	7.08 (SD 6.96) (n=88)	0.88
Haemoglobin g/dl	10.22 (SD 1.33) (n=75)	10.21 (SD 1.66) (n=81)	0.98
Leucocytes $(X10^9/L)$	16.31 (SD 9.26) (n=75)	17.31 (SD 10.32) (n= 81)	0.53
Total Protein (g/L)	68 (SD 15.3) (n=78)	73 (SD 48.7) (n=75)	0.35
Albumin (g/L)	31.2 (SD8.3) (n=78)	30.7 (SD 8.4) (n=75)	0.69
Retinol (μmol/L)	0.568 (SD 0.38) (n=58)	0.493 (SD 0.32) (n=65)	0.24
RBP (mg/L)	21.28 (SD 15.96) (n=58)	17.05 (SD 12.62) (n=65)	0.096

*Numbers for each of the parameters investigated were not always the same. The exact numbers for each parameter investigated are presented in parenthesis.

The socio - economic status of the children was similar in both groups (Table 7.2).

Table 7.2   **Socio - economic data according to treatment group.**

	Group receiving vitamin A on admission	Group receiving vitamin A later (3-7 days)
Age of mother	25.9 (SD 6.26) (n=86)	25.9 (SD 5.75) (n=88)
% Children that had enemas	16.7%	16.7%
% Informal housing	25%	31%
% Untreated water supply	5.8%	5.2%
% No water-borne sewage	27%	25%

Most children (67%) had no complications by the time they were discharged from hospital although 14% had respiratory signs and 6% had urinary tract symptoms that became apparent during hospitalisation; there were no differences in complications between treatment groups.

Correlation analysis showed that greater duration of diarrhoea before admission and greater number of stools per day on admission - contributed to longer time to resolution of diarrhoea and to discharge from hospital. Hospital morbidity (Table 7.3) however indicated no differences between the two groups showing that the different time points for supplementation had no effect on this criteria.

Table 7.3   Hospital morbidity data according to treatment group.

	Group receiving vitamin on admission	Group receiving vitamin A later (3-7 days)	P Value
Days till no diarrhoea	5.4 (SD 3.2) (n=86)	5.5 (SD 4.2) (n=88)	0.86
Days till discharge	7.8 (SD 6.8) (n=86)	7.8(SD 9.4) (n=88)	0.96

The retinol and RBP measurements from the day 3 sample collection are shown in Table 7.4. The blood samples were collected before the later supplementation group had received the vitamin A supplementation dosage. Difficulty in being able to draw sufficient blood from all the children resulted in the retinol and RBP measurements only being done on 58 of the 86 in the first group and 65 of the 88 in the second group.

Table 7.4   Effect of vitamin A treatment/placebo on acute (day 3) retinol and RBP results and later supplementation

	Vitamin A on admission	Vitamin A later (placebo on admission)	P Value
Retinol day 3 (μmol/L)	0.97 (SD 0.5) (n= 58)	0.90 (SD 0.45) (n= 65)	0.43
RBP day 3 (mg/L)	31.06(SD 23.09) (n= 58)	24.80 (SD 11.79) (n= 65)	0.06

RBP is a surrogate measurement for vitamin A status as discussed in Chapter 3. The vitamin A/RBP ratio (Table 7.5) was calculated in this study as an additional indicator of vitamin A status. This value indicates the ability of retinol to bind to the RBP when it is released from the liver. There was no significant difference between the two groups.

Table 7.5   Ratios of vitamin A and RBP.

	Vitamin A on admission	Vitamin A given later (placebo on admission)	P Value
Vitamin A/RBP Ratio Day 0	0.60 (SD 0.26) (n=58)	0.69 (SD 0.37) (n=65)	0.11
Vitamin A/RBP Ratio Day 3	0.78 (SD 0.36) (n=58)	0.85 (SD 0.36) (n=65)	0.24

Of the 174 children enrolled in the study only 86 returned at 4 weeks and 70 at 8 weeks. The children who returned at 8 weeks were largely representative of the initial 174 and did not differ significantly from those lost to follow - up in terms of any descriptive parameters or admission clinical and biochemical parameters (Table 7.6).   A possible discharge variable which may have encouraged return for follow - up was significantly more complications, especially respiratory signs, becoming apparent during hospitalisation in those who returned; 13 of 70 patients who returned had respiratory signs compared with 6 of 104 who did not return (P=0.03).

Table 7.6   Baseline data of children that returned for the 8 - week follow - up investigations. *

	Group receiving vitamin A on admission	Group receiving vitamin A later (3-7 days later)	P value
Sex (F/M)	14/22 (n=36)	14/20 (n=34)	0.96
Age (months)	12.2 (SD 5.4) (n=36)	13.2 (SD 6.5) (n=34)	0.55
Weight/Age (Z)	-1.42 (SD 1.82) (n=36)	-1.30 (SD 1.22) (n=34)	0.74
Stools/day 24 hours prior to admission	6.67 (SD 4.13) (n=36)	6.21 (SD 2.79) (n= 34)	0.60
Days diarrhoea pre-admission	6.19 (SD 4.8) (n=36)	5.97 (SD 6.23) (n= 34)	0.87
Haemoglobin (g/L) % < 11g/L	10.4 (SD 1.56) (n=32) 68.8%	10.7 (SD 1.90) (n=32) 59.4%	0.35
Leucocytes (x10^9/L) % > 11x10^9/L	17.5 (SD 8.2) (n=32) 84.4%	18.4 (SD 13.2) (n=32) 78.1%	0.76
Total Protein (g/L) % < 60 / > 80(g/L)	71 (SD 13.2) (n=32) 18.8%/21.9%	65 (SD 11.1) (n=32) 21.9%/12.5%	0.06
Albumin (g/L) % < 32 / > 50(g/L)	34.7 (SD 7.6) (n=32) 31.3%/0.0%	31.1 (SD 8.9) (n=32) 50.0%/3.1%	0.10
Retinol (μmol/L) % < 0.70μmol/L	0.65 (SD 0.39) (n=26) 61.5%	0.49 (SD 0.35) (n=24) 83.3%	0.13
RBP (mg/L) % < 24 mg/L	24.83 (SD 14.65) (n= 26) 61.5%	17.12 (SD 14.7) (n=24) 79.2%	0.07

* Numbers for each parameter were not always the same and exact numbers for each parameter are presented in parenthesis.

Table 7.7 records the weight gain from birth to 8 weeks, showing a gradual improvement of weight over the eight week period of treatment, but there was no significant difference between the two treatment groups. Birth weight data that was missing was reported as unknown as the mother of the child did not know the birth weight.

Table 7.7   Weight changes according to treatment groups. *

	Vitamin A on Admission	Vitamin A later	P
Weight at birth	2.95 (SD 0.88) (n=64)	3.06 (SD 0.58) (n=62)	0.95
Weight on Admission	8.17 (SD 1.80) (n=86)	7.88 (SD 1.58) (n=88)	0.27
Weight at 4 weeks	9.25 (SD 1.76) (n=39)	8.89 (SD 1.61) (n=47)	0.33
Weight at 8 weeks	9.46 (SD 1.86) (n=36)	9.66 (SD 1.72) (n=34)	0.65

* Numbers for each parameter were not always the same and exact numbers for each parameter are presented in parenthesis.

The 4 and 8 - week morbidity results (Table 7.8 and 7.9) indicate the morbidity rate at these follow - up visits. The classification of morbidity is as follows:

- **No illness** (no reported symptoms).

- **Mild symptoms** (mild self-limiting diarrhoea; feeding well; mild urinary tract infections; mild occasional fever).

- **Moderate symptoms** (persistent diarrhoea; feeding well; unwell intermittently; significant recurring fever; reported respiratory distress/cough).

- **Severe symptoms** (persistent diarrhoea; feeding poorly; reported respiratory distress/cough, unscheduled clinic visit, admission to hospital).

Table 7.8   Morbidity at the 4 - week follow - up.

	Vitamin A on Admission (n=39)	Vitamin A later 3-7 days (n=47)	P Value
No. (%) with no illness	24 (61.5%)	23 (48.9%)	0.24
No. (%) with mild illness	15 (38.5%)	20 (42.6%)	0.70
No. (%) with severe illness requiring treatment	None	3 (6.38%)	0.25
No. (%) with severe illness requiring hospitalisation	None	1 (2.13%)	1.00

Table 7.9   Morbidity at 8 - week follow - up.

	Vitamin A on Admission (n=36)	Vitamin A later 3-7 days (n=34)	P Value
No. (%) with no illness	22 (61.1%)	24 (70.6%)	0.56
No. (%) with mild illness	13 (36.1%)	8 (23.5%)	0.37
No. (%) with severe illness requiring treatment	1 (2.8%)	2 (5.9%)	0.61
No. (%) with severe illness requiring hospitalisation	None	None	-

Very few specific morbidities were reported at either 4 (Table 7.8) or 8 weeks (Table 7.9) after admission and most were mild. There were no significant differences between treatment groups in reported morbidity or in weight gain at either the 4 or 8 - week follow - up. However it must be noted that the two treatment groups both had improved morbidity with < 4; showing severe illness and only 1 patient requiring hospitalisation at 4 weeks.

Plasma retinol at 8 weeks (Table 7.10) did not differ significantly (P=0.74) between children given vitamin A on admission (0.69 μmol/L) compared with those given vitamin A later (0.73 μmol/L).

As can be seen from the 8 week MRDR values in Table 7.10 the timing of vitamin A administration in hospital did not affect vitamin A stores 8 weeks later. The majority of children had normal stores as indicated by the mean MRDR results of 0.036 in the group given vitamin A on admission compared with 0.049 in the group treated later (P = 0.2). The percentage of children with normal stores as indicated by MRDR < 0.06 was 81% (29/36) in the early treatment group and 68% (23/34) in the later treated group (P = 0.34). This represented a 13% difference in the MRDR values when comparing the two groups and was not significant.

Table 7.10   Vitamin A status at 8 week follow-up.

	Vitamin A on admission (n=36)	Vitamin A later 3-7 days (n=34)	P Value
Retinol (μmol/L)	0.69 (SD 0.27)	0.73 (SD 0.26)	0.74
MRDR	0.036 (SD 0.024)	0.049 (SD 0.051)	0.2
MRDR ≥ 0.06	7/36	11/34	0.34

In order to account for any possible baseline differences in retinol and retinol binding protein the differences between the various time points (day 1, day 3 and 8 weeks) was calculated and the mean differences compared between groups as shown in Table 7.11 for retinol differences and Table 7.12 for the retinol binding protein differences. Note RBP was only measured at day 1 and day 3 as it was not thought to be useful in terms of the hypothesis of the study (RBP restores itself at resolution of the infection and in this study it was hypothesised that the infectious stage would be resolved between 3 and 7 days).

Table 7.11   Differences in retinol between the different time points. *

	Vitamin A on Admission	Vitamin A later	P Value
Increase in retinol; d3-d1 (µmol/L)	0.41(SD 0.49) (n=58)	0.42 (SD 0.38) (n=65)	0.92
Increase in retinol; d3-8week (µmol/L)	0.34 (SD 0.35) (n=36)	0.32 (SD 0.48) (n=34)	0.86
Increase in retinol; 8week-d1 (µmol/L)	0.02 (SD 0.33) (n=36)	0.15 (SD 0.28) (n=34)	0.14

* Numbers for each parameter were not always the same and exact numbers for each parameter are presented in parenthesis.

Table 7.12   Differences in retinol binding protein between the different time points.

	Vitamin A on admission	Vitamin A later	P Value
Increase in RBP; d3-d1 (mg/L)	9.49 (SD23.88) (n=58)	7.09 (SD 14.33) (n=65)	0.52

There was no significant difference in the changes with time in either retinol or RBP between the two treatment groups.

CHAPTER 8

8.0 DISCUSSION

The hypothesis for this study was that the children who received vitamin A on resolution of the diarrhoea rather than receiving vitamin A on admission would have better vitamin A stores at the 8 - week follow - up visit as measured by the MRDR test.

The mechanism on which this hypothesis is based is that the gastrointestinal epithelial integrity is affected by diarrhoeal associated inflammation and that this would result in the vitamin A not being absorbed and transported efficiently during this period. Vitamin A supplementation given to children on admission to hospital with diarrhoea may therefore not be beneficial and it was felt that the optimum time for supplementation may rather be after resolution of the diarrhoea, when the epithelial integrity had been restored.

Diarrhoea is a common illness resulting in frequent visits to the health services and therefore it was felt that this presents a window of opportunity for intervention with vitamin A supplementation. However the timing is of utmost importance so as to ensure cost - effectiveness and a clinically beneficial supplementation regime. This information would make it possible to formulate a policy that could then be implemented in clinics and hospitals in areas that are categorised as vitamin A deficient problem areas.

When the study protocol and design were being drawn up there were approximately 100 children being admitted to King Edward hospital every month

with severe diarrhoea. Of these approximately half had persistent diarrhoea with or without malnutrition, 5 - 10% with dysentery and the remaining 30 - 40% having acute diarrhoea usually with moderate to severe dehydration. With these figures and information, recruitment of adequate numbers did not appear to be a problem and the sample size was seen to be easily attainable.

However recruitment was very problematic in this study (Figure 7.1). The initial patient recruitment started in March 1997 and the last patient was recruited 2 years later in January 1999. In the first year only 34 patients had been recruited, the reason for this was that there was another study running concurrently with this study on patients with shigellosis and consequently these patients were not eligible for this study. For unexplained reasons there was also a decrease in diarrhoea patients being admitted into King Edward Hospital during the period of the study.

Although 186 children were initially recruited for this study, 12 of these children were deemed ineligible for the trial from the onset because they had received multivitamins in hospital, without the knowledge of the research staff. Therefore the sample size on which this study was based was 174 children. Due to funding and time constraints we were not able to recruit a further 12 children to compensate for these 12 who were deemed ineligible (Figure 7.1).

Follow - up also proved problematic and the drop - out at the 4 week follow-up visits was 50,6% (n=88). The drop - out for the eight - week follow-up visit, where weight, morbidity, vitamin A and MRDR measurements were collected was 59.8% (n=104) (Figure 7.1). This drop - out was thought to be ascribed to

worsening conditions in the townships; difficulty with obtaining transport from the outlying areas; and difficulty in tracing the patients as they had no fixed abode.

The baseline descriptive and biochemical results (Table 7.1) of these 174 children enrolled in the study indicated that these children were probably malnourished and in an infectious stage with complications such as dehydration and weight loss. This was shown in the mean weight/age Z scores of -1.46 and -1.67 in the two treatment groups. It was difficult to ascertain to what extent this poor weight was due to pre - existing malnutrition or to the current diarrhoea episode.

The leucocyte counts in the baseline samples were above normal and are an indication of an infectious episode (normal values 4 - 11 x 10^9/L). The haemoglobin levels were below normal (< 11 g/dl = classification of anaemia) which could be indicative of a current infection and possibly iron deficiency anaemia due to malnutrition. The total protein and albumin results were within the normal range (total protein 60 - 80g/L and albumin 32 - 50 g/L) indicating that these children's liver synthetic function was intact. The baseline results (Table 7.1) showed that both groups had poor vitamin A status at entry to the study as indicated by the low retinol and RBP levels (normal values for retinol are > .70 μmol/L/ 20μg/dl and for RBP are 24 - 30 mg/L).

Although HIV testing was not carried out in this study, previously known HIV status was documented in 16 of the children viz. 16 out of the 174 were positive before the trial. At the time of the trial anonymous blinded testing of HIV status of all the children in the ward was undertaken. This investigation showed that 32.3% of the children were HIV positive.

Data missing from the baseline results (Table 7.1) for the haemoglobin, white cell counts, retinol and RBP, total protein and albumin were either due to insufficient blood being drawn or difficulty when bleeding these children as they were very ill; the majority being dehydrated on entry to the study. The baseline data for both the treatment groups did not differ from each other in any of the clinical or biochemical parameters.

The socio - economic status (Table 7.2) of these children was determined as it may impact on the diarrhoeal episode. There was no significant difference between the two groups. However the percentage living in informal housing and without water - borne sewage and treated water was 56%, 52% and 14% respectively. These living conditions may be conducive to viral and bacterial infections and may increase the risk of transmitting diseases.

Hospital morbidity (Table 7.3) showed no significant difference between the two treatment groups and the mean day for resolution of diarrhoea and infection was 5.5 days for both groups. The mean number of days on which the second vitamin A supplement / placebo was given was 5.7 ±2.6 days and 5.9 ±3.5 days respectively. Children took between 7 and 8 days to be discharged from hospital.

When examining the changes (Table 7.4) in the vitamin A status from baseline to day 3 it was noted that the day 3 vitamin A levels improved as the infection was resolved possibly due to the antibiotic treatment or natural immune defence mechanism. Our results also indicated that the retinol and RBP values had increased by the same amount in both of the groups. The retinol values in the group receiving supplementation on admission increased from 0.57 to 0.97µmol/L and in the group receiving supplementation later, increased from 0.49

to 0.90µmol/L. It must be noted that the group supplemented later (3 - 7 days later) only received supplementation after the day 3 bloods had been taken, so when comparing the two groups it is important to remember that the supplementation in the earlier group seemed to have no effect on the changes that occurred in the retinol and RBP values. This is also the case in the time it took for the diarrhoea to resolve itself to enable administration of the second dose of vitamin A. This indicates that these children may not have actually had low vitamin A status due to inadequate liver stores, and that the deficiency could have instead been part of the acute phase response that comes into play during an infectious episode. These initial low serum retinol levels therefore may be a result of the infection and the body's acute phase response to this infectious state however when the infection is resolving the existing vitamin A liver stores restore the circulating vitamin A levels.

The results of the ratios between vitamin A and RBP show an indication that the acute phase response affects the circulating RBP levels in the blood thus resulting in the decrease of the ratio of vitamin A to RBP (Table 7.5). However, there is a trend towards an increased ratio in both groups indicating an improvement in the retinol:RBP ratio due to the resolution of the infectious stage. As with the retinol there was no significant difference in retinol/RBP ratios between the two groups.

The acute phase response is the body's response to an infectious episode. These acute phase proteins (APP) affect the vitamin A concentration in patients suffering from infections. The retinol concentration in plasma is affected as the RBP and transthyretin synthesis is reduced resulting in decreased mobilisation of

retinol from the liver stores. During an infectious episode, C - reactive protein (CRP) and α 1 - antichymotrypsin (ACT) are increased and reach a maximum in approximately 24 hours after the initial infection occurs, with CRP reducing quickly after the acute stage is resolved and ACT remaining elevated for longer. Between 2 - 5 days the α 1 - acid glycoprotein (AGP) achieves its maximum and remains elevated for weeks.

This acute phase response effect on retinol levels during infectious episodes was investigated in a recent meta analysis presented at the IVACG Conference in Vietnam (Thurnham *et al*, 2001). It was found that the APP affected the retinol concentrations in serum by varying degrees and the analysis enabled an estimation to be made allowing adjustment for this loss according to the varying degrees of illness and infection. The degree of change in the acute phase protein concentration depends on the severity and time of exposure to an infection or trauma. In recently ill patients the depression of retinol was found to be approximately 16%, and in currently ill patients by 32%. Serum retinol levels in chronically ill patients were found to be depressed by 13%. This indicates that many children with very low serum retinol levels are not really deficient and could be considered as only mildly deficient (Thurnham *et al*, 2001). This could have been the case with the children in this study as at day 3, both groups (supplemented on admission and supplemented later) showed improved vitamin A status as the infection improved.

The group of children that returned at 8 weeks (n=70) were not different in terms of baseline data (Table 7.6) compared with the initial group of 174 children

recruited (Table 7.1). They were, therefore, a good representation of the children enrolled for the study even though it only represented 40% of this initial group.

Examining the weights at 4 and 8 week weight follow - up visits showed an increase in weight from the admission weight (Table 7.7) This could be due to the resolution of the diarrhoea and/or nourishment during the hospital stay. The weight seemed to stabilise at the 8 week follow - up and was a definite improvement from the weight on admission indicating that these children were generally healthier. Some of the birth weights of these children were very low (1-1.5kg) which could present a problem as these children take longer to catch up to the normal weight/age Z score than children born with normal weights.

The morbidity data at 4 and 8 weeks (Table 7.8 and 7.9) show that vitamin A supplementation given at the two different time points viz. at admission or on resolution of the diarrhoeal episode usually 3 - 7 days; had no effect on the MRDR/vitamin A liver stores. However both the groups had improved morbidity that was not affected by the different timing of supplementation. There was a marked improvement in morbidity at 4 weeks where over 55% of the children showed no signs of illness and only 40% presented with mild illness which ranged from mild self limiting diarrhoea, to mild urinary tract infection and occasional fever. Only 5% of these children at four weeks presented with moderate to severe illness that either required treatment or hospitalisation.

The 8 week morbidity (Table 7.9) follow - up presented with very similar data, with 66% showing no signs of illness and 30% presenting with mild symptoms of illness as mentioned above. Only 4% presented with severe illness requiring treatment and there were no children needing further hospitalisation in

this 8 week follow - up indicating that there was an improvement in the general health of the children in both the groups at this follow - up time point.

At the eight week follow - up the vitamin A levels (Table 7.10) had dropped when compared to the day 3 retinol results. This could be attributed to the fact that some of these children returned with sub-clinical illness. It might have been useful to have also measured RBP at 8 weeks and to have examined whether this also had decreased relative to the day 3 RBP results, to ascertain whether these children did in fact have sub - clinical illness. These day 3 retinol levels (0.69 and 0.73μmol/L in the 2 groups respectively) are similar to the levels seen in Kwa Zulu Natal children, many of whom live in poor socio-economic conditions where sub-clinical infections and worm infestations are common. The mean vitamin A levels for children (3 - 6years) in informal settlements in Kwa Zulu Natal are 0.68μmol/L (19.6μg/dl) (Coutsoudis et al, 1993). In a national survey the mean serum retinol levels in children (6 - 71 months) in Kwa Zulu Natal were found to be 0.81μmol/L (23.3μg/dl) (South African Vitamin A Consultative Group, 1995).

The MRDR results at 8 weeks (Table 7.10) confirmed the probability that the liver retinol stores of the children in this study were not actually depleted, although these children had presented with low serum retinol levels at entry to the study. At day three the serum retinol levels showed an improvement in both treatment groups. There was no significant difference in serum retinol levels in the two supplementation groups at 8 weeks indicating that the time at which supplementation was given during a diarrhoeal episode made no difference to the vitamin A status at 8 weeks.

The results showed only a small percentage of children presenting with high MRDR values (≥ 0.06) indicative of low liver stores of vitamin A (Table 7.10). In the group that were supplemented on admission there were 7 out of the 36 (19%) showing low liver vitamin A stores and in the group that were supplemented later 11 out of the 34 (32%) showed low liver vitamin A stores.

This difference was not statistically significant. It was considered whether the lack of significance was due to the sample size being too small to show a significant change and that if we had a larger sample size as originally planned this might have in fact shown a significant difference. However when recalculating the significance level with the same proportions (currently found) and with the planned sample size of 186 instead of 70 (as in current study) the difference was still not significant. This would suggest that even with this increase in sample size there was no benefit to delaying vitamin A supplementation until resolution of the diarrhoeal episode.

Regardless of supplementation there remained 18/70 (26%) of children with inadequate vitamin A stores, as estimated by MRDR tests. A limitation of this study is that we could not compare the improvement in the MRDR levels, as we did not calculate MRDR levels at admission.

There has been one study that investigated the effect of vitamin A supplementation given at different times on gut integrity during diarrhoeal disease by McCullough et al (1999). However this study did not look at the vitamin A status of these children and whether the difference in the timing of the supplementation had an effect on the vitamin A status. It merely investigated the effect that vitamin A (given at different time points) had on gut integrity that could

be informative as to the usefulness of delaying supplementation during a diarrhoeal episode. The effect of vitamin A supplementation on gut integrity in 100 infants (4 - 18 months) hospitalised with acute respiratory infections and diarrhoeal disease was examined. There were two treatment groups; one receiving high dose vitamin A on admission, the other at 5 days post admission and a placebo group. Gut integrity was measured using the lactose:mannitol ratios. In all the groups there was no change between baseline and day 5 on gut integrity. There was a significant improvement in gut integrity in both treatment groups after 10 days (p<0.05) when compared to the placebo group. However there was no difference between the two treatment groups showing that there was no apparent advantage for giving vitamin A on admission or later.

This study might help to understand why we found no significant effect in delaying supplementation. Based on the above study the ineffectiveness of the supplementation which was given at day 3 - 7 may have been a result of too early a time point to presume normal absorption of vitamin A in the gut and to see the benefits of this delayed supplementation.

A suggestion for a further study would be a later time point for the second dose of vitamin A and this time point could probably be as long as 2 weeks especially if the children in the particular study are very ill. This extended time would allow for the gut integrity to be fully restored to normal function.

To further improve the study design recommendations would be to have two treatment groups, one receiving vitamin A on day 5 and the other at 14 days (Table 8.1). This would help to understand more clearly the mechanism of the

absorption of the vitamin A in the gut and the timing involved during resolution of an infectious episode.

As mentioned previously RBP measurements should be taken at the 8 week time point as well as the MRDR measurement at admission. Because there may be ethical problems in withholding vitamin A from the one group until day 14 it is advisable to give both groups vitamin A on admission.

Table 8.1   Proposal for an improved study design.

	Admission	Day 5	Day 14
Group 1	Vitamin A supplementation	Vitamin A supplementation	Placebo
Group 2	Vitamin A supplementation	Placebo	Vitamin A supplementation

Another recommendation is that when calculating sample size a larger percentage for drop - out should be factored in, as in the present climate in South African hospitals the patient drop - out is very large due to numerous circumstances out of the control of the researchers involved in the studies. Unfortunately even though there is strict documentation of all information during the study, numerous patients do not return for follow - up and are very difficult to trace.

A limitation of the present study was that we did not collect breast - feeding information and pattern (exclusive or mixed breast - feeding) and it would be

useful for any further study to collect this information as breast - feeding may have a separate effect on the diarrhoeal recovery and vitamin A status which should ideally be controlled for (Bhandari *et al*, 1997).

With some design modification as suggested, this study may have proved significant enabling exact dosage times to be pinpointed and supplementation to be more efficiently and effectively administered. Taking these suggestions into consideration further research is, therefore, warranted.

In conclusion, although this study did not find any differences the results do not negate international policy to give vitamin A supplements to children presenting at health services in areas where vitamin A deficiency is a public health problem. Such supplements are likely to improve morbidity even in the face of the major changes in vitamin A metabolism, which occur during illness.

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VITAMIN A STUDY

In-patient Data Form

BASELINE / ADMISSION DATA

DATE:

1	Name	First		Surname
2	Study number			
3	Hospital I/P no.			
4	Sex	M	F	
5	Date of Birth	-	-	
6	Age		mths	
7	Mothers name			
8	Other contact			
9	Address			
10	Alternative contact			
11	Phone number			
12	Date of admission			
13	Weight on admission		kg	
14	Birth weight		kg	
15	Age of mother		yrs	
16	Mothers employment / school			
17	Total number of children			
18	Type of housing	U - Formal	U - Informal	Rural
19	Water supply	In - House	Tap	River
20	Sanitation supply	Water-borne	Pit	None
21	Main Caregiver			
22	Retroviral status (pre-admission)	Pos	Neg	Unknown
23	Vitamin A/multivitamins in the past 4 weeks	Yes	No	
24	Medicine (antibiotics) in past 4 weeks	Yes	No	
25	Which medicine – if known			
26	Enema? (nil or days pre- admission)		Days	
27	Number of stools in past 24 hours			
28	Predominant type of stool	Watery	Loose	Soft	Mucoid

29	Duration of diarrhoea pre-admission			days					
30	Temperature on admission				.	°C			
31	Associated complications	Pneumonia	UTI	Septicaemia	Skin sepsis	OT.media	URTI	Other
32	Antibiotics given in hospital	Yes		No				
33	Type of Antibiotic in hospital							
34	Supplement A (day 0) given on							
35	Supplement B given on							

Tick if completed   #A   #B

36	Serum retinol / RBP		37	

Discharge Information

38	Date of discharge								
39	No of stools in 24 hours prior to Supp B								
40	Predominant type of stools	Watery		Loose		Soft		Mucoid	
41	Associated complications	Pneumonia	UTI	Septicaemia	Skin sepsis	OT.media	URTI	Other

42	Retro status checked during hospitalisation	Yes	No		
43	Retrovirus results	ELISA -ve	ELISA +ve	P24 +ve	IgG3 +ve

Admission results

44	Haemoglobin			
45	MCV			
46	WCC			
47	Platelets			.
48	Total Protein			
49	Albumin			

Date of 4 week review: _____

Date of 8 week review / MRDR: _____

Appendix 2.

<div align="center">

VITAMIN A STUDY

4 - Week Review Data Form

</div>

PERSONAL DETAILS

DATE:

1	Name	First	Surname
2	Study Number		
3	Hospital I/P no.		
4	Sex	M F	
5	Date of birth dd/mm/yy	- -	
6	Age	mths	
7	Mothers Name	First	Surname
8	Other Contact	First	Surname
9	Address		
10	Alternative contact		
11	Phone number	( )	
12	Weight no napkin	kg	

Morbidity History

13   General

Well	No reported symptoms	
Mild symptoms	Mild self-limiting diarrhoea, Feeding well, Mild URTI occasional fever	
Moderate symptoms	Persisting diarrhoea, Feeding well/unwell intermittently, Significant recurring fever, Reported respiratory distress / cough	
Severe Symptons	Persisting diarrhoea, Feeding respiratory distress / cough **and** Earlier review than appointed or admission	

Medical History over Past 3 days

14	Diarrhoea	3 or more loose stools per day	Yes	No
15	Fever		Yes	No
16	Fast Breathing		Yes	No
17	Other		Yes	No

Date of 8 week review / MRDR: ——————————————

Appendix 3.

<div align="center">

VITAMIN A STUDY

8 - Week Review Data Form

</div>

PERSONAL DETAILS

DATE:

1	Name	First	Surname
2	Study Number		
3	Hospital I/P no.		
4	Sex	M F	
5	Date of birth dd/mm/yy	- -	
6	Age	mths	
7	Mothers Name	First	Surname
8	Other Contact	First	Surname
9	Address		
10	Alternative contact		
11	Phone number	( )	
12	Weight no napkin	kg	

Morbidity History

13   General

Well	No reported symptoms	
Mild symptoms	Mild self-limiting diarrhoea, Feeding well, Mild URTI occasional fever	
Moderate symptoms	Persisting diarrhoea, Feeding well/unwell intermittently, Significant recurring fever, Reported respiratory distress / cough	
Severe Symptons	Persisting diarrhoea, Feeding respiratory distress / cough **and** Earlier review than appointed or admission	

Medical History over Past 3 days

15	Diarrhoea	3 or more loose stools per day	Yes	No
16	Fever		Yes	No
17	Fast Breathing		Yes	No
18	Other	Specify	Yes	No

MRDR: _____