# PERMEABILITY CHARACTERISTICS OF TEMOZOLOMIDE AND ITS ESTER AND AMIDE PRODUCTS THROUGH RAT AND HUMAN SKIN

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# Dedication

I would like to dedicate this thesis to my parents with everlasting love and thank for all their support, guidance and encouragement.

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The University of Aston in Birmingham

**Permeability characteristics of temozolomide and its ester and amide products through rat and human skin.**

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# Summary

Temozolomide has been shown to possess excellent activity against malignant melanoma (skin cancer) in phase II and III clinical trials. Temozolomide is administered orally. However, for the treatment of skin disorders, delivery of this agent directly to the skin would be advantageous. The permeability characteristics of temozolomide and its lipophilic derivatives, which may have greater skin permeability, were therefore investigated. New lipophilic ester and amide derivatives were synthesised using Pybrop® and DMAP as coupling agents. *In vitro* permeation studies of temozolomide and its synthesised derivatives were performed on silicone membrane, full-thickness rat skin, and whole epidermis from human skin. The cumulative amounts permeated (%) of such agents were determined using HPLC. The hexyl ester derivative was the most effective in increasing the permeation of temozolomide through rat and human skin (6-10 fold higher than parent drug). This derivative is therefore a promising product which could be used to test an *in vivo* system. This study indicates that attachment of a hydrocarbon chain to temozolomide to increase lipophilicity also increased skin permeation (up to the hexyl derivative). Skin metabolism of temozolomide esters was also found *via* viable rat and human skin. Ester prodrugs were completely hydrolysed by esterase enzymes within the skin, and generated temozolomide acids, which has been shown to exhibit similar cytotoxity to temozolomide free drug.

**Keywords:** Temozolomide, Lipophilicity, Permeability, Skin

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# Objective

Temozolomide is a newly marketed anticancer drug for treatment of brain cancer. It has also been shown to demonstrate excellent activity against malignant melanoma (skin cancer) in phase II and III clinical trials. Temozolomide is normally administered orally. However, for the treatment of skin disorders, delivery of this agent directly to the skin would be advantageous.

There is evidence that in order to enhance drug permeation *via* the skin, one conceivable strategy could be to synthesise lipophilic drug derivatives (prodrugs), which have greater affinity for the principal skin barrier (stratum corneum lipid). These prodrugs may pass more easily through the skin. It is therefore of great interest to test whether or not temozolomide can permeate the skin *versus* its derivatives and to synthesise its ester and amide lipophilic derivatives.

The purpose of this project is therefore to study the permeability characteristics of temozolomide and its lipophilic derivatives through the skin. This will be profitable fundamental data employed to develop the topical or transdermal administration of temozolomide. The new administration route could be achieved to increase the efficacy of this drug in the treatment of skin cancer.

# Chapter 1

# Introduction

## 1.1 Temozolomide as a novel anticancer agent

### 1.1.1 General background

Temozolomide is a novel anticancer agent, which was synthesised by Professor Malcolm Stevens and colleagues at the University of Aston in Birmingham (Stevens *et al.*, 1984). There has been great interest in this anticancer agent because it has shown a broad-spectrum antitumour activity against murine tumours with schedule dependency (Stevens *et al.*, 1987). Furthermore, temozolomide has been shown to demonstrate clinical anticancer activity against malignant melanoma (Newland *et al.*, 1992; Bleehen *et al.*, 1995), mycosis fungosides (Newland *et al.*, 1992), and high-grade gliomas (Newland *et al.*, 1992; Bower *et al.*, 1997) with favourable side effects, and predictable myelosuppression. On the contrary, the predecessor compound, which is mitozolomide, exhibits limited clinical activity, and elicits severe and unpredictable myelosuppression (Newland, 1985).

### 1.1.2 Antitumour activity of temozolomide

Temozolomide displayed pre-clinical antitumour activity against a broad spectrum of murine tumours *in vivo*, including leukaemia, lymphomas, and solid tumours (Stevens, 1987). This activity has been shown to be highly schedule dependent with multiple administration being more effective than a single bolus dose. Additionally, temozolomide is highly effective in the treatment of subcutaneous and intracereberal human brain

tumour xenografts, and metastatic lung carcinoma in mice (Plowman, 1994; Tentori *et al.,* 1995). *In vitro* studies of temozolomide antitumour activity have demonstrated activity against a large variety of human tumours such as brain cancer, ovarian cancer, and melanoma, including some tumours usually resistant to chemotherapy with conventional drugs (dacarbazine, carmustine, cisplatin, doxorubicin, 5-fluorouracil, etoposide, and vinblastine) (Raymond *et al.,* 1997).

According to the promising antitumour activity of temozolomide in pre-clinical studies, clinical trials of this anticancer agent have been carried out.

Temozolomide was initially tested in phase I clinical studies. Its potential activity has been seen in malignant melanoma, mycosis fungosides, and high-grade gliomas (Newland *et al.,* 1992). Moreover, therapeutic potential has also been discovered against leukemia, and breast cancer (Rathbone, 1999). Currently, phase II and III clinical studies of temozolomide are concentrating on its effectiveness in the treatment of melanoma and brain tumours (Wang and Stevens, 1997).

Temozolomide has been approved by European Medicine Authority as an anti-brain cancer drug in 1999. However, its activity against skin cancer has not yet been fully established.

### 1.1.3 Temozolomide as a new skin cancer drug

Phase II trials of temozolomide have confirmed that it has significant activity in patients with metastatic melanoma (Bleehen *et al.*, 1995). Recently, a phase III study of temozolomide in the treatment of patients with advanced metastatic malignant melanoma indicated that the efficacy of temozolomide is equal to dacarbazine, which is an oral alternative for the patients with advanced metastatic melanoma (Middleton *et al.*, 2000). However, dacarbazine presents severe cumulative bone marrow toxicity (Stevens, *et al.*, 1987).

The schedule used to administer temozolomide in clinical studies was 150 $mg/m^2$ given orally for five consecutive days in the first course. Courses were repeated every 4 weeks and if no myelosuppression (*i.e.* leukopenia, lymphopenia) was detected after the initial course, the subsequent doses were escalated to 200 $mg/m^2$ (Newland *et al.*, 1992; Bleehen *et al.*, 1995; Middleton *et al.*, 2000). The symptomatic toxicity from temozolomide was mainly nausea and vomiting. Alopecia, rash, and constipation could also be found to a lesser degree. Finally, the major toxicity reaction was leukopenia (Newland *et al.*, 1992; Bleehen *et al.*, 1995; Middleton *et al.*, 2000).

### 1.1.4 Pharmacokinetics

Pharmacokinetic studies of temozolomide in mice showed a rapid absorption phase, elimination half-lives of 1.13 h (i.p.) and 1.29 h (p.o.), and approximately 100%

bioavailability after one oral administration (Stevens, *et al.*, 1987). These studies were repeated in a phase I clinical trial, and it was found that temozolomide is rapidly absorbed, with maximum plasma concentrations being obtained in 0.7 h, a distribution half life of 1.8 h, and a good bioavailability after post dosing (i.v.) (Newland *et al.*, 1992). Like its predecessor mitozolomide, temozolomide demonstrates good tissue distribution *via* kidney, lung, liver, and can traverse the blood-brain barrier (Brindley *et al.*, 1986; Newland *et al.*, 1997).

During preliminary elimination studies performed in mice (Tsang *et al.*, 1990), it was found that renal excretion was the predominant route of elimination. The major metabolite of temozolomide found in human urine was 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-d] tetrazine-8-carboxylic acid, the carboxylic acid analogue of temozolomide (Tsang *et al.*, 1990). This metabolite exhibits cytotoxicity against lymphoma cells equal to temozolomide.

### 1.1.5 Pharmacology

Unlike dacarbazine and mitozolomide, which require metabolic activation, temozolomide spontaneously degrades in physiological fluid to generate the cytotoxic methylating species, 5(3-methyl-1-triazeno) imidazole-4-carboxamide (MTIC) (Tsang *et al.*, 1991).

The antitumour activity of temozolomide is largely attributed to the methylation of DNA

bases forming methyl addition products at $N^7$-guanine, $N^3$-adenine, and $O^6$-guanine leading to DNA strand breakage and cell death (Bull and Tisdale, 1987; Wedge *et al.*, 1997). Although a small percentage of methylation adducts at $O^6$-position, there is increasing evidence that $O^6$-alkylguanine is a major cytotoxic lesion (Baer *et al.*, 1993). This finding is supported by several experiments correlating with $O^6$-alkylguanine-DNA-alkyltransferase (ATase), which is a cytoprotective DNA repair protein, providing protection against the toxic, mutagenic, and carcinogenic effects of alkyl agents. Methyl groups adduct at $O^6$-guanine in DNA are repaired by ATase, which principally removes these groups from DNA. This reaction results in an irreversible inactivation of this repair protein. Therefore, when lesion repair has occurred, cells are depleted of ATase until the synthesis of new enzyme molecules takes place (Lacal *et al.*, 1996). Obviously, the depletion of ATase by pre-treatment with $O^6$-benzylguanine, which is a potent ATase inactivating agent, greatly improved the cytotoxicity of temozolomide (Wedge *et al.*, 1996; Dolan, 1997). In addition, it has been shown that a number of human tumours are inherently resistant to the cytotoxic effects of alkylating agents due to higher levels of ATase expression (Chinnasamy *et al.*, 1997).

Poly (ADP-ribose) polymerase (PADPRP) is also secondarily indicated in DNA-repair mechanisms. This can be explained by pre-clinical studies, which indicate that the combined treatment of leukaemia cells with temozolomide and a PADPRP inhibitor greatly potentiates the cytotoxicity of this anticancer drug (Boulton *et al.*, 1995). Moreover, Friedman and colleagues (1997) reported that the development of methylating agent resistance is caused by the deficiency of mismatch repair mechanisms. This result

suggests that the cytotoxicity of temozolomide is also dependent on a function of the DNA mismatch repair pathway.

Whilst the biological effects of temozolomide have been widely studied, the precise chemical mechanism of its activity remains less clear.

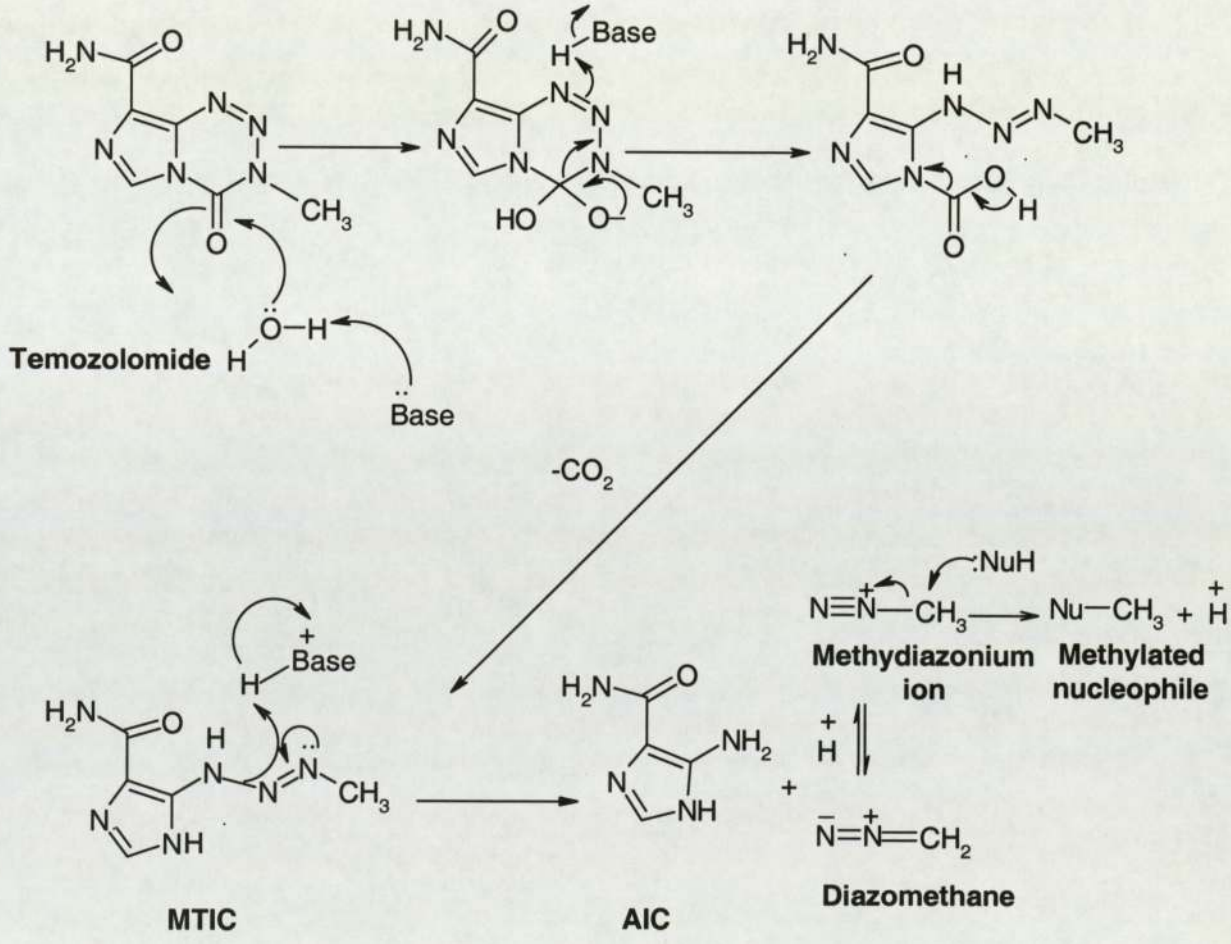
### 1.1.6 *In vitro* studies of the mechanism of action of temozolomide

*In vitro* investigations into the mechanism of action of temozolomide also confirm that temozolomide behaves as a prodrug, generating active metabolite, which transfers a methyl group to a nucleophile.

Decomposition of temozolomide was studied in an aqueous system, deuteriated phosphate buffer solution (Stevens *et al.,* 1984; Wheelhouse and Stevens, 1992; 1993). These studies indicate that the antitumour prodrug temozolomide undergoes ring opening in aqueous solutions to regenerate the reactive species (MTIC). Presumably, MTIC does not react directly with DNA, but highly reactive methyl-diazonium species, which are unimolecular fragments of MTIC, transfer the methyl group to the bionucleophile on DNA.

The mechanism of degradation of temozolomide in aqueous solution is shown in scheme 1.1. The rate-limiting step is the base-catalysed addition of water to form a tetrahedral intermediate, which collapses with breakdown of the tetrazinone ring followed by

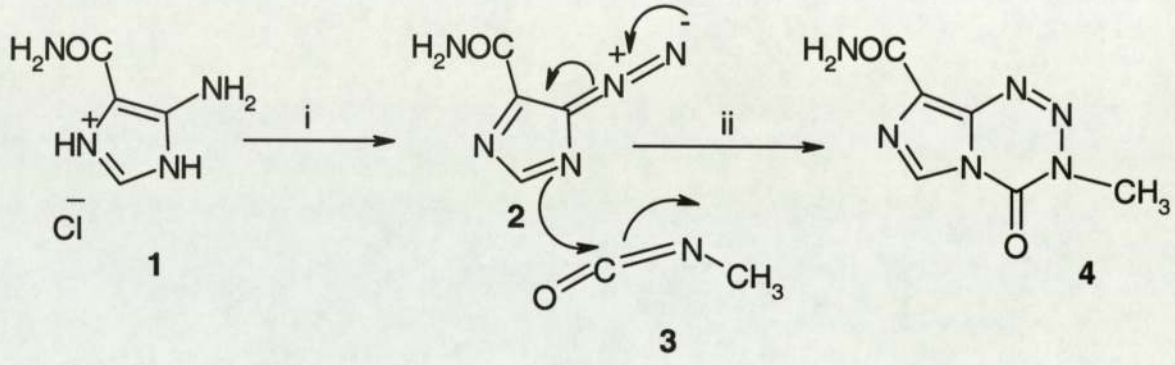
spontaneous decarboxylation. The further reaction of MTIC requires acid catalysis, and follows by the fragmentation of the triazine to form 5-aminoimidazole-4-carboxamide (AIC) and methyldiazonium ion. The final step is the reaction of methyldiazonium with a nucleophile, which may be water or the components of the buffer in the solution. The temozolomide molecule can therefore be considered, as a prodrug exquisitely developed to deliver a methylating fragment to bionucleophiles (DNA bases).




**Scheme 1.1**. Decomposition mechanism of temozolomide in aqueous solution

## 1.1.7 The synthesis of temozolomide

The original synthesis of temozolomide was from the reaction of 5-diazoimidazo-4-carboxamide with methyl isocyanate (Stevens *et al.*, 1984). This reaction is based on the general synthesis of azolotetrazinones (Ege *et al.*, 1979).




**Scheme 1.2** The original synthetic route of temozolomide

*Reagents and conditions:* **i**, excess $NaNO_2$, 0-5 °C; **ii**, EtOAc-DMSO, 25°C

Scheme 1.2 shows the original synthetic route of temozolomide. This route started with 5-aminoimidazole-4-carboxamide, commercially available as a hydrochloride salt **1**. This was converted to 5-diazoimidazole-4-carboxamide **2**, which reacted slowly with methyl isocyanate **3** at 25°C to afford the imidazotetrazine **4** in high yields.

This first synthetic route has been proven highly versatile in providing access to a wide range of analogues from the various aminoimidazoles and isocyanates.

However, isocyanate can be severely toxic, consequently, new alternative routes, which do not necessitate the use of such agents, have been developed (Wang, and Stevens, 1997)

## 1.2 Skin delivery of drugs

### 1.2.1 Introduction

Controlled release may be defined as a technique or method in which active chemicals are delivered to a specific target site at a constant rate and desirable duration designed to accomplish a therapeutic effect (Juliano, 1980; Guy, and Hadgraft, 1987). Skin delivery can, therefore, be defined as the controlled release of drug *via* intact skin.

The application of substances to the skin could be potentially for delivery to the organ itself or the body as a whole (*i.e.* for local or systemic delivery). Skin delivery systems can thus be mainly divided into two categories, which are topical, and transdermal delivery systems depending on the purpose of the application.

In topical drug delivery, the aim of this technique is to deliver and to localise molecules of the drug within the outer layers of the skin in order to treat superficial skin disease without enhancing the permeation of drug into the systemic blood circulation. For example, dermatological drugs such as corticosteroids (applied as creams or ointment) are intended to act locally within the skin with little, or minimal, systemic action. Therefore, the outermost layer of the skin may be considered as a drug reservoir for the slow release of drugs to the targeted pathological site within the skin (Chien, 1992).

Conversely, the purpose of transdermal drug delivery is to enhance the permeation of therapeutic agents from the skin through its various layers into the systemic blood circulation and finally reach the target organ. For instance, nitroglycerin is not intended to act at the skin, but at distant organs, in this case, the coronary arteries (Ranade and Hollinger, 1995).

### 1.2.2 Rationale for skin delivery

Skin delivery technology has currently been receiving increased attention due to, for example, the ineffectiveness of drugs when administered by conventional means. For example, the traditional administration route, which is oral, exhibits large fluctuations in drug concentration in the bloodstream and target tissues. In medical treatment, drugs are introduced at intervals by the ingestion of pills or liquid dosage forms (see figure1.1). Initially, drug concentration rapidly rises to high levels which can be above the minimum toxic level. This may be the cause of side effects, leading to a decrease in patient compliance, or toxic levels. Subsequently, the concentration dramatically diminishes, owing to systemic metabolism and elimination processes of the body, and the second dose is administered to prevent the concentration from dropping below minimum effective level. On the contrary, ideal controlled release systems elicit a constant concentration within the therapeutic level, and can prolong this constant delivery rate throughout the desired time.

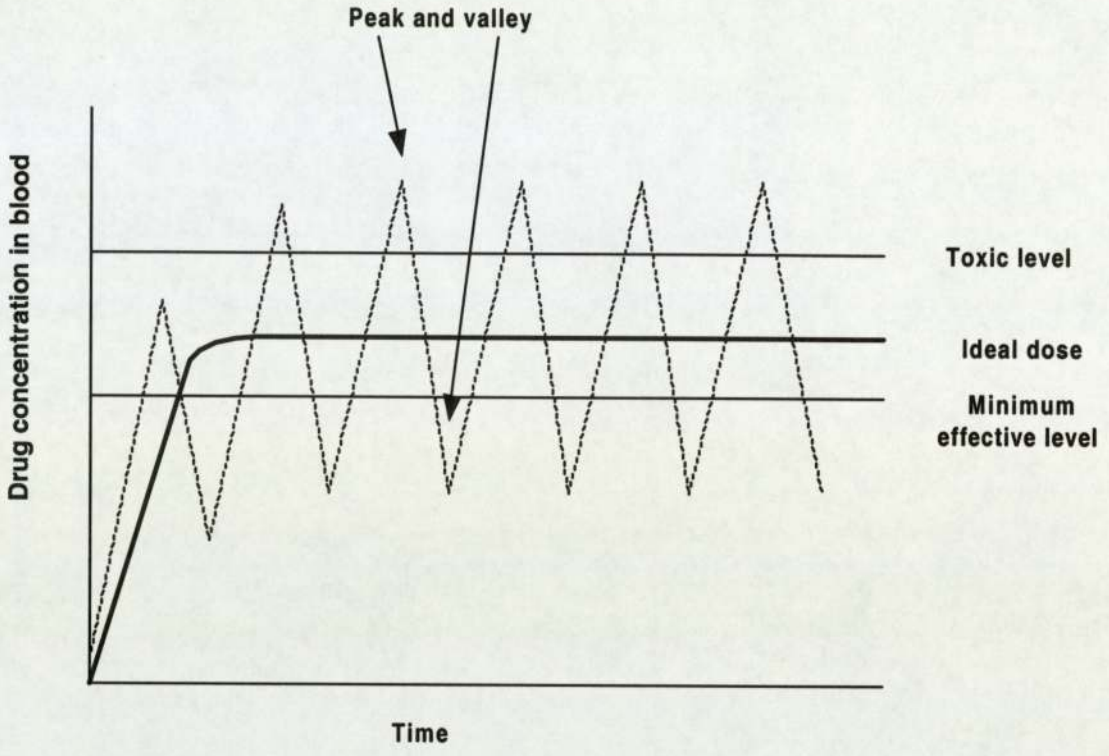



**Figure 1.1**. Hypothetical blood level pattern from a conventional multiple dosing schedule and the idealised pattern from a control release system.

In addition, intravenous administration has been recognised as an ideal mode of systemic drug delivery not only to bypass hepatic first-pass elimination, but also to maintain a constant, prolonged, and therapeutically effective drug concentration in the body. However, this administration route entails certain risks and therefore may necessitate hospitalisation of patients and close medical supervision of medication leading to increases in cost and decreases in compliance. Thus, several transdermal therapeutic systems, which enhance the drugs permeation into the bloodstream through the skin, aiming to achieve systemic medication, have been developed (*i.e.* Transiderm®, Estraderm®).

Furthermore, many drugs are used to treat dermal conditions and are still administered orally or *via* other systemic routes instead of employing topical delivery systems, which can locally sustain drugs to treat dermal abnormality within the skin. Some examples are methotrexate for psoriasis, 13-cis-retinoic acid for acne, ketokonazole for fungal infections, and steroids for dermatitis (Barry, 1983). Consequently, unfavourable patterns of efficacy and toxicity due to systemic delivery of such agents can be developed. Therefore, delivery of the topical therapeutic agent directly to the skin would be advantageous.

In this study, skin delivery deals with the concept of delivering drug both into the skin for its local effects in dermatology, and through the integument for the systemic treatment of disease states.

### 1.2.3 The advantages of topical and transdermal skin delivery

In addition to the above advantages (see section 1.2.2 for details), skin delivery systems (topical and transdermal) have several benefits compared to conventional systems.

#### 1.2.3.1 Topical skin delivery

Topical skin delivery of drugs is an extremely profitable technique to accomplish the treatment of disease, disorder, and abnormalities of the skin. Mainly, this method preferentially allows the drug to be concentrated for treatment within the skin leading to provision of the greatest activity of dermal drugs because therapeutic agents are allowed to directly contact the affected area within the skin.

Treatment using topical preparations is also very convenient for the patient thus considerably increasing compliance.

At present, there are several topical preparations containing active agents commonly used in dermatology (see table 1.1).

| Topical preparations | Examples |
|---|---|
| Topical antibiotics | penicilllin, streptomycin, |
| Topical antifungal drugs | miconazole, clotrimazole, |
| Topical exfoliate (for acne) agents | benzoyl peroxide, tretinoin, |
| Steroid anti-inflammatory agents | triamsinolone acetonide, |
| Insect repellents | dimethylphthalate, indalone, |
| Antihistamine | diphenhydramine HCl, |
| Local anesthetics | benzocaine, lignocaine, |
| Keratolytics and Caustics | benzoic acid, salicylic acid, |
| Cytotoxic agents | 5-fluorouracil, methotrexate. |

**Table1.1** Some examples of topical drug preparation widely used in the market

Source: Adapted from Barry, (1983)

### 1.2.3.2 Transdermal delivery

Transdermal drug delivery for systemic pharmacological effects is currently recognised as a viable means to administer therapeutic agents. Transdermal medication confers the

following potential benefits (Chien, 1992; Kydonieus, 1987; Ranade and Hollinger, 1996):

1. Bypass of the variation in the absorption and metabolism associated with oral administration;

2. Provide a simplified therapeutic regime leading to better patient compliance;

3. Permit a rapid termination of medication by simple removal from the skin surface;

4. Allow effective use of drugs with short biological half-lives;

5. Allow administration of drugs with narrow therapeutic windows.

### 1.2.4 Anatomy and function of the skin

The goal of skin delivery is to optimise the permeation of a drug to pass through the skin at therapeutic levels. Consequently, understanding of the anatomy and functions of the skin is extremely important.

The skin is one of the most extensive and readily accessible organs of the human body. It covers the entire body, has a surface area of 1.5 to 2 square meters, weighs 4 to 5 kg, and accounts for about 7% of total body weight in the average adult (Marieb, 1998). The skin receives about one-third of all blood circulating through the body (Chien, 1992).

The skin is elastic, rugged, and self-regenerating, under normal physiological conditions (Montagna and Parakkal, 1976). It varies in thickness from 1.5 to 4.0 millimetres (mm) or more in different parts of the body (Marieb, 1998). The skin separates the underlying blood circulation network and viable organs from the outside environment.

#### 1.2.4.1 Functions of the skin

The skin performs a variety of functions that affect body metabolism, and protect the body from external factors such as bacteria, abrasion, temperature, and chemicals. It behaves as a site of temperature regulation, sensation, metabolic function, blood reservoir, and excretion. The main functions of the skin are briefly presented in table 1.2.

1. To contain body fluids and tissue
2. To protect the body from potentially harmful external stimuli (the protective or barrier function):(a) microorganism; (b) chemical; (c) radiation; (d) heat; (e) electrical barrier; or (f) mechanical shock
3. To receive external stimuli (the sensory function), *i.e.*, to mediate sensation: (a) tactile (pressure); (b) pain; or (c) heat
4. To regulate body temperature
5. To synthesise and to metabolise compounds
6. To dispose of chemical wastes (glandular secretions)
7. To provide identification by skin variation
8. To attract the opposite sex
9. To regulate blood pressure

**Table 1.2**. The main functions of the skin

Source: Adapted from Barry, (1983)

### 1.2.4.2 Structure of the skin

The skin is a multilayered organ composed of, anatomically, many histological layers. However it is generally described in terms of three tissue layers: the epidermis, the dermis, and the subcutaneous fat tissue (Barry, 1983; Chein, 1992) (see figure 1.2). The epidermal layer covers the external surface of the body and the underlying dermis. The

subcutaneous tissue is a layer underneath the dermis containing mostly adipose tissue. The epidermis, dermis, and subcutaneous tissue form a functional unit and are called the skin (Christopher *et al.*, 1989).

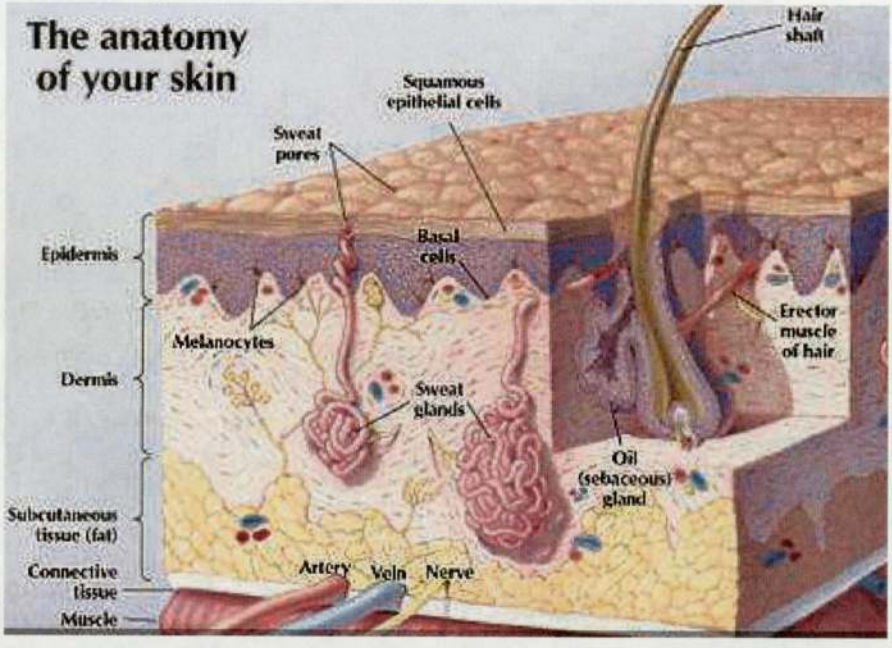



**Figure 1.2.** Three-dimensional view of the skin structure

Source: From http://www.medic.mie-u.ac.jp/derma/anatomy.html

### 1.2.4.2.1 Epidermis

The outer epidermal layer of the skin is composed of stratified squamous epithelial cells. The epithelial cells are held together mainly by highly convoluted interlocking bridges, which are responsible for the integrity of the skin (Chein, 1992). The epidermis is thickest in the area of palms and soles and becomes thinner over the ventral surface of the

trunk. Structurally, the epidermis consists of four distinct cell types and four or five distinct layers.

## I. Cells of the epidermis

The cells populating the epidermis include keratinocytes (corneocytes), melanocytes, Merkel cells, and Langerhans' cells. Most epidermal cells are keratinocytes (Marieb, 1998). (see figure 1.3)

### (a) Keratinocytes

The principal role of keratinocytes is to produce keratin, the fibrous protein that provides the protective properties of the epidermis. Intercellular junctions, in which desmosomes are the most prominent, interconnect either adjacent keratinocytes or Merkel cells and keratinocytes. The keratinocytes originate by mitotic division in the deepest layer of epidermis and migrate to the outermost layer of the epidermis. Both cell production and formation are accelerated in body areas regularly subjected to friction, such as the hand and feet.

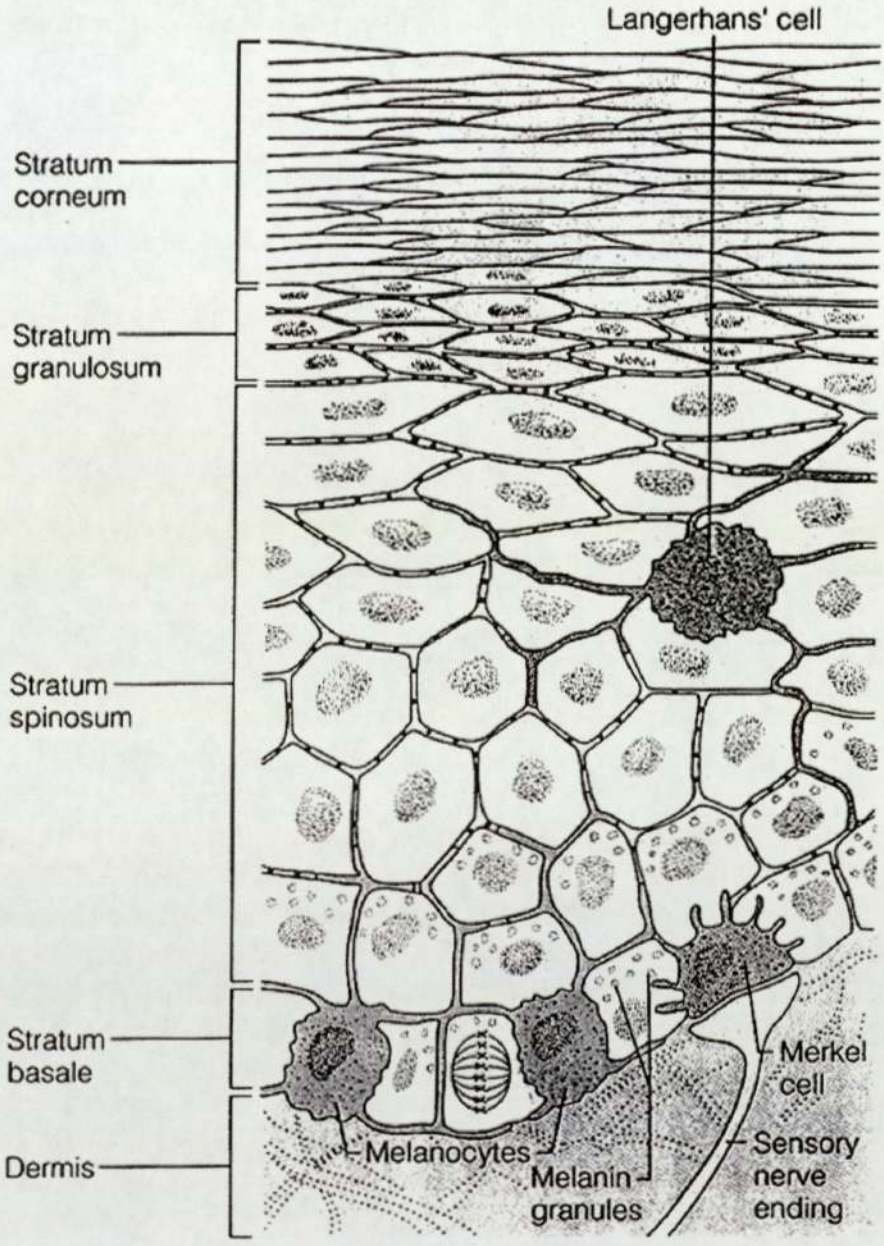



**Figure 1.3** Cells of the epidermis

(b) Melanocytes

Melanocytes are found in the deepest layer of epidermis (see figure 1.3) and are the special epidermal cells which synthesise and distribute the pigment melanin to the keratinocytes. The melanin granules accumulate on the superficial side of the keratinocyte nucleus forming a pigment shield that protects the nucleus from the damaging effects of ultraviolet (UV) radiation in sunlight.

(c) Langerhans' cells

Langerhans' cells arise from bone marrow and migrate to the epidermis. These cells are potent stimulators that help to activate the immune system when foreign substances or antigens are present (Christopher *et al.*, 1989).

(d) Merkel cells

Merkel cells are presented in small numbers at the epidermo-dermal junction. Each Merkel cell is intimately associated with a sensory nerve ending (see figure 1.3). The combination, called a merkel disc, functions as a sensory receptor (Maribe, 1998).

II. Layers of the epidermis

In thick skin, which covers the palm, fingertips, and soles of the feet, the epidermis consists of five layers (see figure 1.4). These layers are the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. However, in thin skin, which covers the rest of the body, the stratum lucidum is absent and the other four layers are thinner (Parakkal and Montagna, 1974).

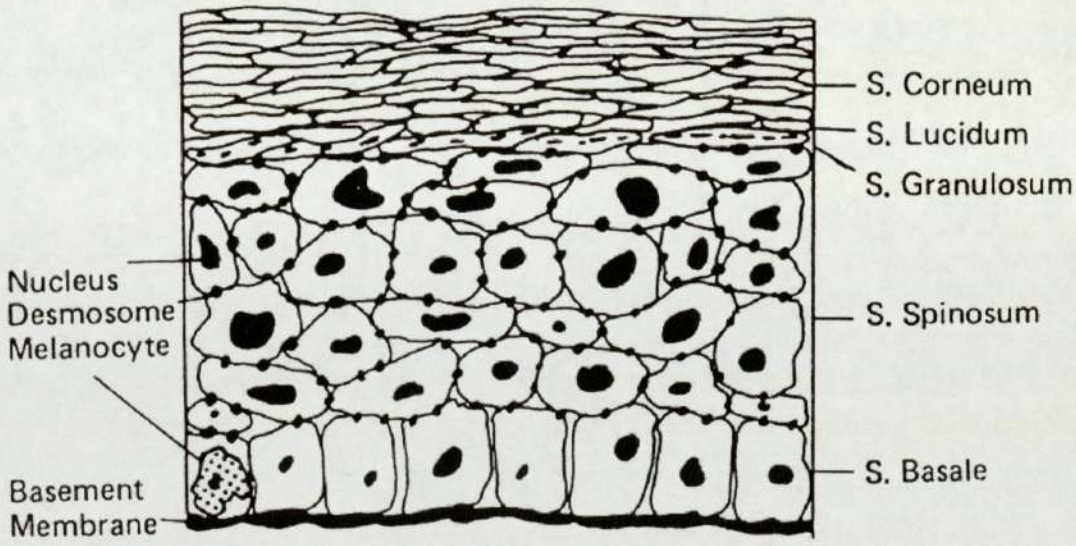



**Figure 1.4** The sublayers of epidermis

(a) Stratum basale (basal layer)

The stratum basale is the deepest epidermal layer. For the most part, it comprises a single row of cells representing the youngest keratinocytes, which are cuboidal or low columnar

in shape. Mitosis of the basal cells constantly renews the epidermis. In healthy epidermis, the rate of new cell production equals to the rate of cell loss at the skin surface, and the normal turnover time for replenishment is, on average, 28 days (Barry, 1983). The epidermis thus remains constant in thickness.

During the normal life cycle of epidermal cells, the cells progress upwards, become round (stratum spinosum), and then distinctly flattened (stratum corneum) above the basal layer.

(b) Stratum spinosum (Prickly layer)

The spinous layer of cells undergoes a rapid differentiation. The keratinocytes in this layer are round or polyhedral in shape. Langerhans' cells and melanin granules are most abundant in this epidermal layer.

(c) Stratum granulosum (Granular layer)

The thin stratum granulosum consists of three or five cell layers. The keratinocytes in this layer flatten their nuclei and organelles begin to disintegrate, and they accumulate keratohyalin granules and lamellar granules.

(d) Stratum lucidum (Clear layer)

The stratum lucidum appears as a thin translucent layer above the stratum granulosum. It comprises of a few rows of clear, flattened, dead keratinocytes with indistinct boundaries. This layer is present only in thick skin.

(e) Stratum Corneum (Horny layer)

The outermost stratum corneum is a broad zone, twenty to thirty layers thick. It consists of dead, compacted, flattened, dehydrated keratinised cells, and accounts for up to three-quarters of the epidermal thickness (Chien, 1992). It is now accepted that this layer serves as the most effective skin barrier for the penetration of most compounds (see section 1.2.5.3.2 for details).

### 1.2.4.2.2 Dermis

The dermis is a strong, flexible, connective tissue layer, which constitutes the majority of the mass of the skin (Bissett, 1987). It is made up of a dense network of fibrous protein, which are collagen, elastin, and reticular fibres (Chein, 1992; Marieb, 1998).

The dermis is richly supplied with nerve fibres, blood vessels and lymphatic vessels. The blood supply in this tissue layer is important in the systemic absorption of substances

applied to the skin. The major portions of hair follicles, as well as sweat glands, are derived from epidermal tissue but reside in the dermis.

The dermis has two major layers: the papillary and reticular layers (Barry, 1983; Marieb, 1998). The thin superficial papillary layer is heavily invested with blood vessels. The deeper reticular layer, accounting for about 80% of the dermis, is mostly made up of collagen fibre (Bissett, 1987).

### 1.2.4.2.3 Subcutaneous fat tissue

This is the sheet of fat, which is known as the superficial fascia, attaching the dermis to the underlying structure. Its thickness varies with the age, sex, and nutritional status of individual. The subcutaneous fat provides flexible linkages between the underlying structures and the superficial skin layers leading to development of its thermal barrier and mechanical cushion properties.

### 1.2.5 Fundamentals of skin permeation

The skin was generally regarded in the scientific literature as an impermeable barrier. It is now more accepted that the skin is also particularly effective as a selective barrier to the penetration of a diverse range of substances. However, the skin is normally more permeable to lipid-soluble substances than to water-soluble compounds (Chein, 1992; Barry, 1983).

#### 1.2.5.1 The stratum corneum as the primary skin permeation barrier

It is recognised that the various layers of the skin are not equally permeable. Subsequently, it was noted that the epidermis is much less permeable than the dermis. According to numerous experiments, the stratum corneum was approved as the principal barrier, which usually provides the rate-limiting step or the slowest stage in the penetration process in most conditions (Scheuplein, 1965; Williams and Barry, 1991). The best direct evidence that the stratum corneum was essentially a uniformly good permeation barrier came from studies using isotropic tracers (Chein, 1992). The largest amount of isotope was always detected in the outermost layer, and the proportionally decreased toward the dermis. Evidence from controlled stripping experiments has also demonstrated that complete removal of the horny layer by stripping enhances the absorption of many substances in contact with the skin surface (Barry, 1987). Finally, detailed pictures of the stratum corneum, gained from electron microscopy, support the idea that the barrier to penetration consists of the keratin-phospholipid complex in the

dead and relatively dry cells of stratum corneum (Idson, 1975). These above observations suggest that the horny layer of the skin greatly impedes the permeation of molecules.

A typical horny cell comprises an amorphous matrix of mainly lipid and nonfibrous protein (see figure 1.7), in which keratin filaments (60-80 A°) are distributed (Filshie and Roger, 1961). Adjacent keratinocytes are interconnected by intercellular lipid. This lipid matrix behaves as a predominant route for permeation (see section 1.2.5.3.2 for details). The composition of the stratum corneum is outlined in table 1.3.

**Table 1.3** Composition of human stratum corneum

| Components | % | Gross biochemical compositions |
|---|---|---|
| Cell membranes | 5 | Lipids and nonfibrous protein |
| Cell contents | 85 | Lipid (20%)<br>α-Protein (50%)<br>β-Protein (20%)<br>Nonfibrous protein (10%) |
| Intercellular materials | 10 | Lipids and nonfibrous proteins |

### 1.2.5.2 The phenomenon of percutaneous absorption

The phenomenon of percutaneous absorption can be simply visualised as consisting of a series of steps in sequence (see figure 1.5). Firstly, a penetrant molecule adsorbs onto the surface layers of stratum corneum. Secondly, it diffuses through stratum corneum, viable epidermis, dermis, and capillary network of blood vessels in the papillary layer of the dermis. Finally, the molecule is taken up into blood circulation for subsequent systemic distribution.

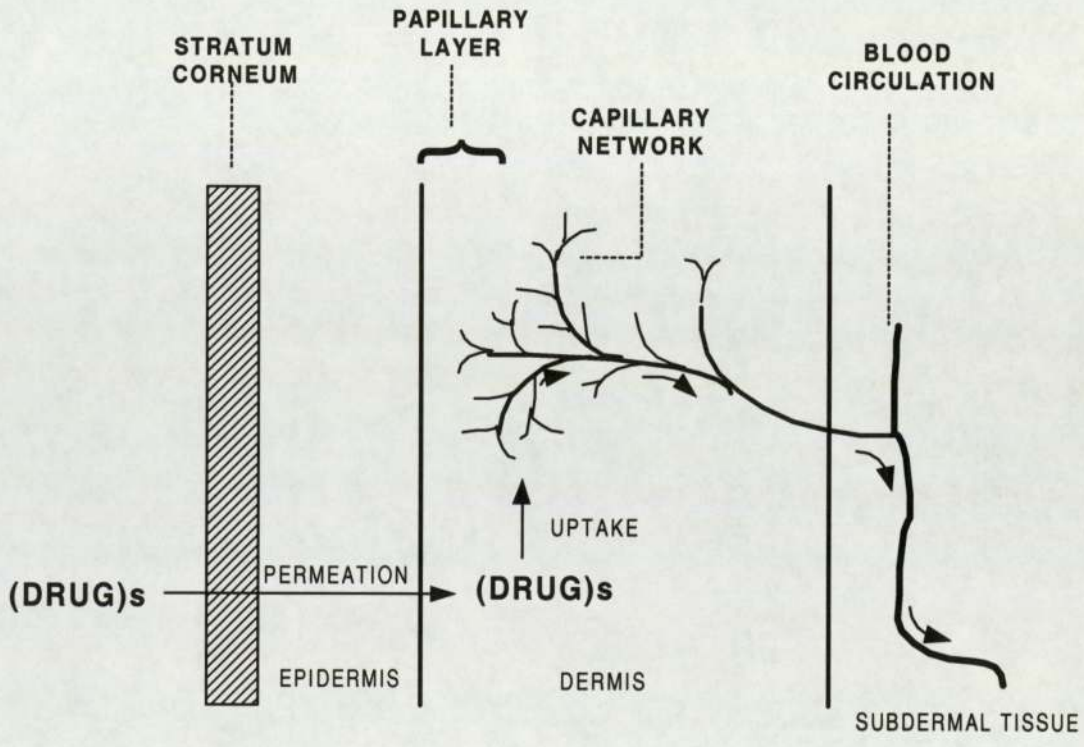



**Figure 1.5** A multilayer skin model showing the sequence of transdermal permeation of drugs for systemic delivery

### 1.2.5.3 Transport through the skin

#### 1.2.5.3.1 Pathways for transport across the skin

The transport mechanisms by which drugs permeate through intact skin are still not fully elucidated despite many years of investigation. However, it has been recognised that there are two majors transport pathways across the skin that molecules are likely to follow (Barry, 1987) (see figure 1.6). One involves transport directly through the bulk of the stratum corneum (transepidermal route). Another pathway concerns penetrating across the hair follicles and sweat glands (appendageal route). In fact, absorption through skin appendages is limited because the average human skin surface contains on average, 40-70 hair follicles and 200-250 sweat ducts *per* square centimetre of skin. These skin appendages occupy only 0.1% of the total human skin surface (Barry, 1983; Chein, 1992). Additionally, has been recently shown that the penetration of retinoic acid was greater through the skin of hairless guinea pigs than those with hair, confirming that the structure and composition of the stratum corneum is more important than follicular density for passive permeation (Hisoire and Bucks, 1997). Therefore, skin permeation of most drug molecules is considered as passive diffusion through the intact stratum corneum as a primary process. However, the appendageal route may be important at short diffusion times for ions or large polar molecules (Barry 1983; Barry, 1987).

Passive transport can be defined as the movement of a solute from a high concentration compartment to a low concentration compartment. The driving force for passive diffusion

across the membrane is the concentration gradient. The membranes itself do not actively participate in the transfer process. On the contrary, an active process involves active participation by the membrane in the transfer of molecule. Carriers, which may be enzymes or other components of membrane, interact with drug and the drug-carrier complex then move across the membrane. Drugs are finally released from the carrier to the other side of membrane. However, there is no evidence that the active transport is involved in the permeation of any drugs across human skin (Poulsen, 1973).

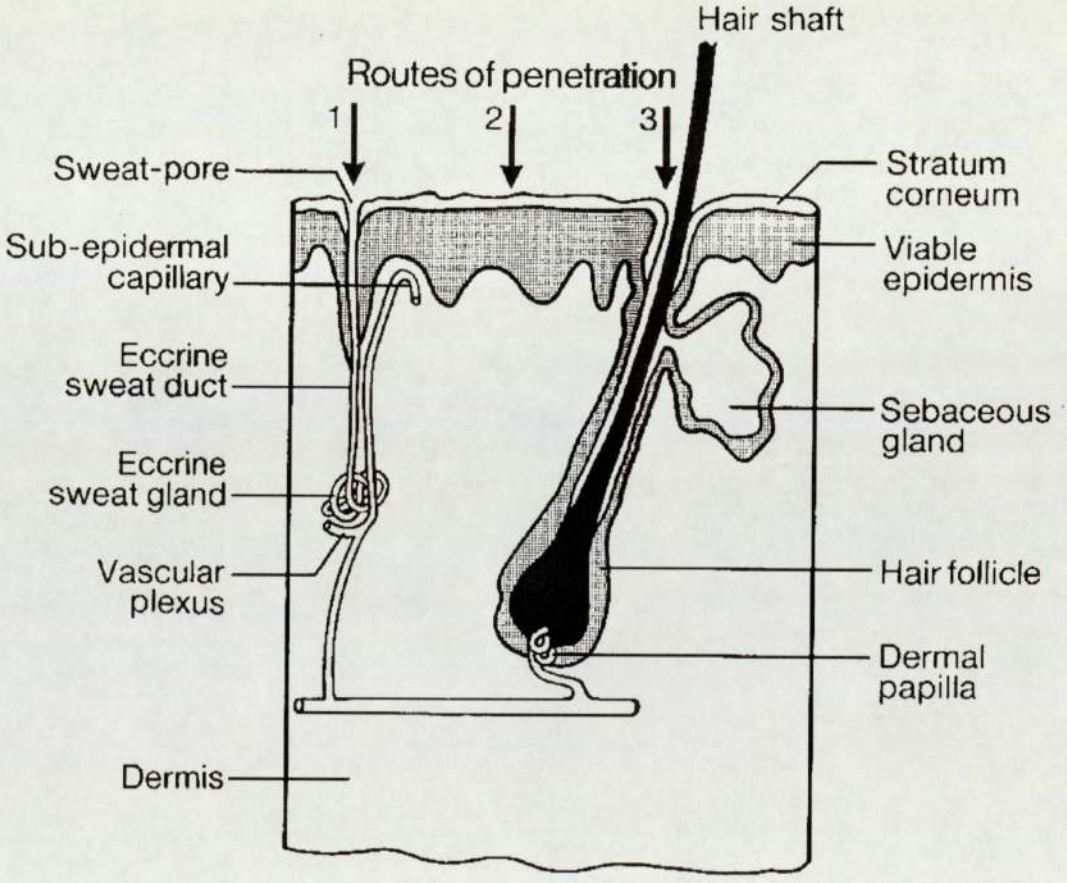



**Figure 1.6** The possible pathways of molecules transport through the skin-across the intact stratum corneum (2) or *via* the hair follicles (3) and sweat glands (1).

### 1.2.5.3.2 Pathways for transport across the stratum corneum

As previously described, application of topical or transdermal delivery is limited largely by the stratum corneum barrier properties, which prevent the permeation of most compounds at therapeutic levels. The stratum corneum is a muticellular membrane, in which intercellular regions are filled with a lipid-rich amorphous material (Parakkal and Matoltsy, 1965) (see figure 1.7). The intercellular lipid is a layered structure, in which the polar head groups of lipids are gathered in layers with the non-polar chains pointed in the opposite direction (Friberg, 1990). There are two conceivable routes for the drug permeation through this outermost horny layer (Moghimi *et al.*, 1999): the transcellular (*via* the protein-filled cells) and the intercellular (*via* lipid matrix between cells) pathways (see figure 1.7). The relative contribution of these routes depends on the solubility, partition coefficient and diffusivity of the drug within these protein or lipid phases (Banga, 1998). It was believed that, in hydrated tissue, in which stratum corneum contains more than 40% water, polar molecules may partition and diffuse *via* the hydrated keratin of keratinocytes (Scheuplein, 1972). However, it is now seems more probable that the dominant pathway is the polar region of intercellular lipid, with the lipid chains providing the non-polar route (Katz, 1973; Higuchi, 1977; Barry 1987). Recently, there has been one direct piece of evidence in support of the intercellular pathway as a significant route (Bodde'*et al.*, 1991). A vapour fixation technique with electron microscopy was used to visualise $Hg^{2+}$permeating through the stratum corneum. It was shown that corneocytes also took up the small amount of $Hg^{2+}$, and during the washing process, most of the intercellular material was washed away, while the small

amount of $Hg^{2+}$present in the cell remained. This observation suggests that the intercellular lipid pathway is the main route of drug permeation.

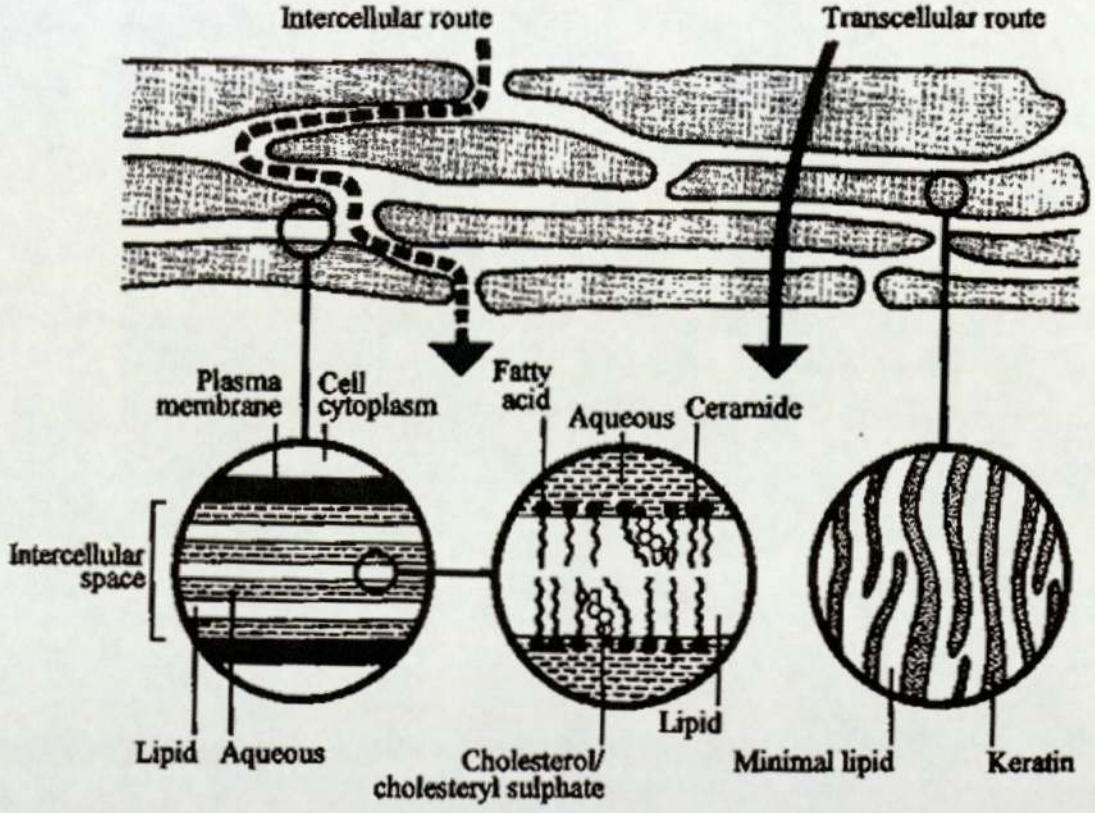



**Figure 1.7.** The possible routes for drug transport through the stratum corneum (transcellular or intercellular). Details are shown of the suggested structure of the intercellular lipid and the intracellular protein fibrils with minimal lipid.

Permeation into the skin, which is *via* the transepidermal or transfollicular pathway, also depends on numerous factors that may influence the permeability characteristics of each compound. For example, the time scale of permeation, the physicochemical properties of the penetrant (*e.g.*, $pK_a$, molecular size, stability, binding affinity, solubility, partition

coefficient), integrity and thickness of the stratum corneum, density of sweat glands and follicles, skin hydration, and skin metabolism. Indeed, in order to specify the route of penetration, other existent conditions should be considered. It is probable that the penetrants permeate through a variety of routes mainly depending on time of diffusion, physicochemical properties of the penetrants and skin condition.

### 1.2.5.4 Factors influencing in percutaneous absorption

The transport of drugs through the skin is complicated, since there are many factors that influence their permeation. However, the concept of percutaneous absorption may be explained by considering a simple diagram, which represents the difficulties and complexity in the permeation process (see figure 1.8).

The drug particles must first dissolve in the solvent so that the molecules of the drug diffuse within the vehicle to reach the vehicle-stratum corneum interface. The penetrant must partition into the stratum corneum and diffuse within this greatly impermeable barrier. Some drug molecules may interact with a so-called depot site possibly forming a reservoir, demonstrating therapeutic activity for days or even weeks. Free drug eventually diffuses through the horny layer meeting the interfacial barrier of the stratum corneum and viable epidermis, and partitions into viable epidermis. There is a potential problem in that a drug or prodrug designed to partition from a vehicle into the horny layer may have difficulty leaving the stratum corneum to enter the water-rich epidermis. Therefore, the substances which have high affinity for the horny layer and very low water solubility,

may not be absorbed percutaneously (Barry, 1987). For highly lipophilic drugs, clearance from viable tissue may replace diffusion through the stratum corneum as the rate-limiting step. Viable epidermis is a living tissue, which may have enzyme activities of 80-90% of those in the liver (Wester and Noonan, 1980; Bronaugh, 1999). Hydrolytic, oxidative, reductive and conjugation reactions all take place. Metabolism in living skin may alter the permeability characteristics of the drugs. For instance, drugs may be activated by enzymes within the skin, generating active or inactive metabolites resulting in altered pharmacological or toxicological activity. The permeation characteristics of these metabolites may be different from parent compounds. It is also probable that the drug may interact with pharmacological receptors in this viable tissue. After the penetrant partitions into the dermis, additional receptor, metabolic and depot sites may intervene in the progress of drug permeation to blood capillaries, partitioning into the wall, and finally removal by the blood circulation. A portion of the penetrant may even partition into the subcutaneous fat and the underlying muscle to form a further depot.

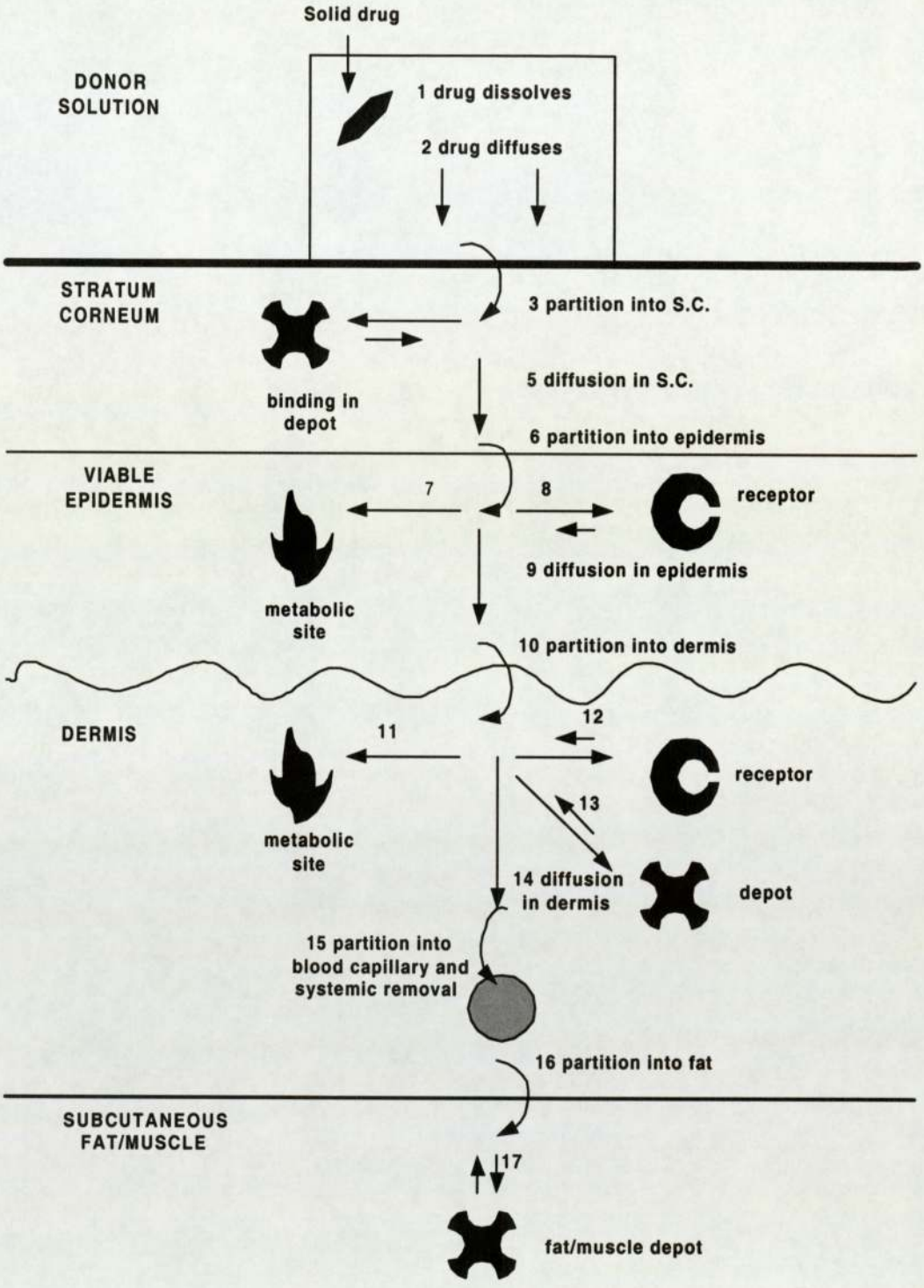



**Figure 1.8** The percutaneous absorption process of drug from solution and some factors which influence the process

### 1.2.5.5 Theoretical basis of percutaneous absorption

The process by which a solute (penetrant) moves from a region of high chemical potential (the vehicle) to a region of low chemical potential (the skin) can be referred to passive diffusion (Poulsen, 1973). A typical permeation profile of a drug through the skin is presented in figure 1.9. There is an initial lag phase, which is the period of time that penetrant flux is attaining equilibrium. This period is followed by a linear or steady state phase, which occurs when there is a balance between drug diffusion in and out of the skin membrane.

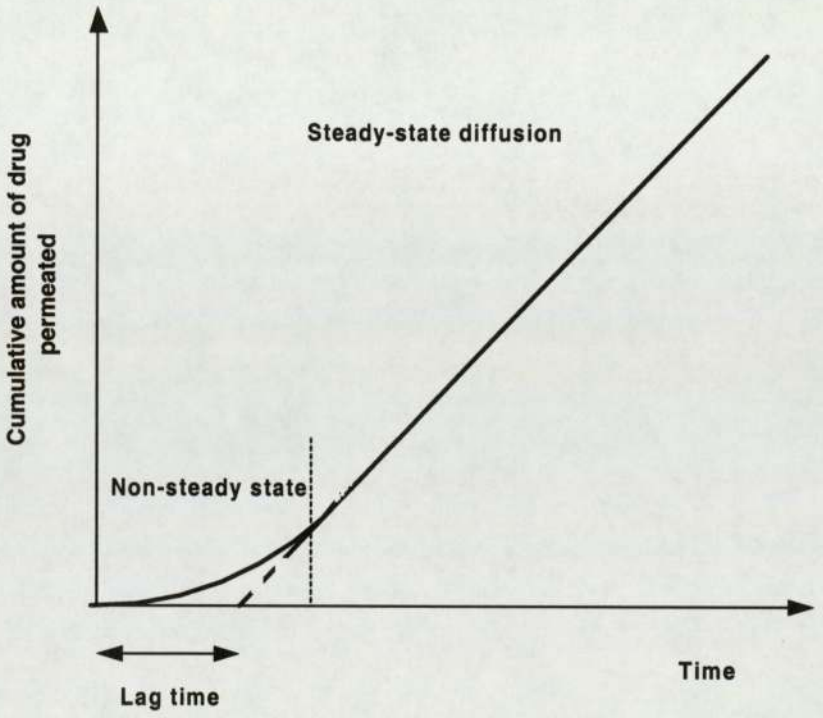



**Figure 1.9** Typical profile for percutaneous drug absorption

At steady state, in which the permeation rate of a substance is directly proportional to the concentration gradient, the passive diffusion of molecules can be expressed by Fick's first law of diffusion as:

$$J = -D\frac{dC}{dx} \tag{1.1}$$

Where $J$ is the amount of drug penetrating through a unit area of the skin *per* unit time (the flux), $D$ is the diffusion coefficient of drug in the skin, and $dC/dx$ is the concentration gradient across the skin, where $C$ is the concentration and $x$ is the distance.

However, the concentration gradient across the skin cannot be exactly measured under certain conditions. For example, the membrane may exhibit an affinity for the permeant. As a consequence, the drug concentration at the skin surface is not identical to that in the donor solution but is related to the membrane-vehicle partition coefficient. The permeation rate through the skin can thus be approximated by the product of the partition coefficient and the concentration difference across the membrane. The steady state flux, $J_s$, through the skin barrier is thus given as:

$$J_s = \frac{K.D}{h}.\Delta C_s \tag{1.2}$$

Where $K$ is the partition coefficient, $\Delta C_s$ is the concentration difference, and $h$ is the thickness of the skin. $K$, $D$, and $h$ can be combined into a single constant ($P_s$):

$$J_s = P_s \Delta C_s \tag{1.3}$$

where $P_s$ is termed the permeability coefficient. Experimentally, if the drug dose in the donor compartment is infinitely large (*i.e.* sink conditions apply) compared to the amount permeating to the receptor chamber, then $\Delta C_s$ can be replaced with $C_s$, which is the drug concentration in donor compartment. The cumulative amount of drug permeating through the skin ($Q_t$) is given by:

$$Q_t = \frac{K.D.C_s}{h(t - h^2/6D)} \tag{1.4}$$

Where $C_s$ is the saturated concentration in donor phase when sink conditions are maintained in the receptor solution. When the steady state line is extrapolated to the time axis (see figure 1.9), the value of lag time, $t_L$ is obtained by the intercept at $Q = 0$

$$t_L = \frac{h^2}{6D} \tag{1.5}$$

The intercept, $t_L$, is the measure of the time that the penetrant takes to achieve a constant concentration gradient across the skin.

However, the equations listed in 1.1 to 1.5 may not be applicable under conditions that are influenced by other factors. For example, the evaporation of donor solution will

increase the drug concentration resulting in an increase in the rate of diffusion. Some vehicles such as water or ethanol can also enhance drug delivery (Barry, 1983).

## 1.2.6 Optimisation of percutaneous absorption

### 1.2.6.1 Strategies employed to enhance drug permeation through the skin

It has been increasingly recognised that not every drug can be delivered through the skin at therapeutic rates. This may be attributed to the stratum corneum lipid limiting transport of most compounds as well as some of physicochemical properties of penetrants such as low lipophilicity and high molecular mass which may greatly impede (Bronaugh and Maibach, 1986) their ability to permeate the skin.

There are two main strategies employed to enhance drug permeation *via* the skin (Barry, 1987). The first scheme involves utilising a vehicle or device to maximise drug partition into the skin without significantly affecting the physicochemical properties of the stratum corneum.

Transdermal drug delivery (TDD) systems are topical devices designed to deliver drug through the skin at a controlled rate. A TDD system is generally composed of a drug reservoir and adhesive. In this approach, the device instead of stratum corneum controls the rate at which the drug diffuses through the epidermis and dermis and passes into the systemic blood circulation *via* the capillaries. In other words, the TDD device is the rate-limiting step for the diffusion of drug, while, the skin can be regarded as a perfect sink.

The initial commercial success of the nitroglycerin patch (Gardiner, 1987) was an impetus to further develop transdermal drug delivery systems. There have been now numerous TDD systems launched on the world-wide prescription drug market (see table 1.4). Furthermore, several TDD systems have been submitted for regulatory review and approval.

**Table 1.4** Transdermal controlled-release products

| Drug | Trade name | Indication |
|---|---|---|
| Scopolamine | Scopoderm<br>Kimite Patch | Motion sickness |
| Nitroglycerine[a] | Transiderm-Nitro<br>Deponit<br>Nitro-Dur<br>Nitrodisc<br>NTS | Angina |
| Isosorbide-dinitrate | Frandol Tape | Angina |
| Clonidine | Catapress-TTS | Hypertension |
| Estradiol | Estraderm | Hormone treatment |
| Estradiol ester | [b] | Hormone treatment |
| Testosterone | TheraDerm-LRS | Hormone treatment |
| Timolol | [b] | Cardiovascular |
| Propanolol | [b] | Cardiovascular |
| Fentanyl | Duragesic | Opioid analgesic |
| Glycol salicylate | [b] | Analgesic |
| Methyl salicylate | [b] | Analgesic |
| Chlorpheniramine | [b] | Antihistamine |
| Diphendydramine | Zenol | Antihistamine |
| Physostigmine | [b] | Cholinergic |
| Insulin | [b] | Diabetes |
| Albuterol | [b] | Bronchodilator |
| Piroxicam | [b] | Arthritis |
| Ketorolac (Toradol) | [b] | Non-narcotic analgesic |
| Flubiprofen | Zepolas | Anti-inflammatory |
| Indomethacin | Indomethin | Anti-inflammatory |
| Bufuralol | [b] | Angina, hypertension |
| Bupranolol | [b] | Angina, hypertension |
| Nicotine | Habitrol, Nicoderm | Aid to smoking cessation |

[a] Other trade names are Diafusor, Minitran, Nitriderm, Nitrol Patch, Nitrocine, Deponit, Millistrol Tape, and Herzer.
[b] In research and development.

**Table. 1.4** Transdermal controlled-release products and devicesSource: Adapted from Ranade and Hollinger, (1995).

In addition to TDD devices employed to enhance drug permeation, one possible method, which avoids disturbing structure of the skin, is to synthesise lipophilic derivatives of the drugs (see section 1.2.6.2 for details). These lipophilic analogues may easily permeate through stratum corneum lipid (*e.g.* steroids).

The alternative strategy incorporates into the formulation materials known as penetration enhancers. These are chemical substances which enter and reversibly alter the skin to promote the penetration of the drugs. The desirable attributes of such enhancers include the following:

- they should be pharmacologically inert, interacting with no receptors in the skin or in the body
- the enhancer should be neither toxic, irritating nor allergic
- the onset of enhancer activity and the duration of effect should be predictable, controllable and suitable
- the skin should immediately and fully recover its normal barrier property when the enhancer leaves the tissue
- the enhancer should promote penetration into the skin without developing significant problems of loss of body fluids, electrolytes or other endogenous materials
- the chemical should be compatible with a wide range of drugs and pharmaceutical adjuvants
- the substance should be a suitable solvent for the drugs

- for traditional formulations, the material should spread well on the skin and it should have a suitable skin 'feel'
- the chemical should be capable of formulation into creams, ointments, gels, lotions, suspensions, aerosols, skin adhesives and delivery devices
- it should be odourless, tasteless, colourless and relatively inexpensive.

Some examples of effective skin enhancers include dimethyl sulfoxide (DMSO), azone (1-dodecylazacycloheptan-2-one), unsaturated fatty acid (e.g., oleic acid, linoleic acid), and surfactants (*e.g.*, sodium dodecyl sulphate) (Barry, 1983). The mechanism of action of those various skin enhancers may be attributed to their activity on intercellular lipid and/or hydrophilic protein in the stratum corneum (Chien, 1992). However, these skin enhancers reversible alter skin structure to promote the penetration of the drugs. This can thus be the cause of skin irritation or toxicity.

In this study, the strategy employed to enhance drug across the skin is the synthesis of lipophilic derivatives.

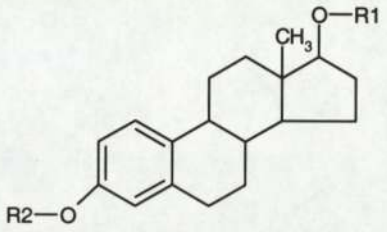
### 1.2.6.2 The enhancement of drug permeation by chemical modification to form a lipophilic prodrug

The application of the prodrug approach in topical or transdermal drug delivery can be considered as the alteration of skin permeability *via* the physical or chemical modification of the penetrating molecule to enhance its rate of percutaneous absorption

(Valia *et al.*, 1985). This approach appears to offer an advantage over traditional enhancers in that the enhancement is specific to the compound optimised, there is no disturbance of skin barrier function and the absorption of other compounds is not enhanced.

A drug with poor skin permeability may be chemically modified to form a lipophilic prodrug, which has greater affinity for the stratum corneum lipid. These prodrugs may pass more easily through the skin, where they can be transformed by the metabolic processes within the skin tissue to regenerate the active drugs. Therefore, the transport of the drug through the skin is substantially enhanced (Chien, 1992).

A typical example of such an approach is the esterification of less-skin-permeable oestradiol to form lipophilic oestradiol ester (Valia *et al.*, 1985) (see table 1.5). This study suggests that all the oestradiol ester derivatives investigated are extensively metabolised by the esterase in viable skin during the course of skin permeation to regenerate the biologically active oestradiol. Some oestradiol esters achieved permeation rates two-to-four fold greater than the permeation rate of free oestradiol drug.




| R1 | R2 |
|---|---|
| —H | —H |
| —C(=O)—CH3 | —H |
| —C(=O)—CH3 | —C(=O)—CH3 |
| —C(=O)—(CH2)3—CH3 | —H |
| —C(=O)—(CH2)5—CH3 | —H |
| —C(=O)—CH2—CH2 | —H |

**Table. 1.5** Chemical structure of oestradiol (R1=H, R2=H) and its ester prodrug

Source: Adapted from Chien, 1992

Several studies, which may be considered as the foundation for prodrug development, have been conducted on a homologous series of compounds. They are exemplified by the *in vitro* percutaneous absorption studies of straight-chain low-molecular-weight alkanols $C_1$-$C_8$ (Scheuplein, 1965) and steroid homologues (Scheuplein *et al.*, 1969). It is likely that less polar molecules can readily permeate through the skin.

Bodor *et al.*, (1980) studied extensively the transdermal delivery of prodrugs of steroids, theophylline and cromolyn. Hydrocortisone-21-diethylsuccinamate was reported to almost double the rate of delivery of hydrocortisone through hairless mouse skin, while it caused significantly less local toxicity than hydrocortisone. Acyloxymethyl as well as 7-(hydroxymethyl) derivatives of theophylline were found to be effective in increasing the amount of this drug permeating through the skin by 3.5 to 5 times. Finally, cromolyn lipophilic prodrugs (hexanoyloxyethylidene, hexanoyloxymethyl and pivalyloxymethyl-nitrate esters) significantly penetrated through the skin compared to the parent compound. It was concluded that the prodrug approach seems to be promising for the topical delivery of such highly polar compounds as cromolyn.

The feasibility of employing a prodrug approach in the dermal delivery of 5-fluorouracil to humans was also studied (Mollgaard *et al.*, 1982). The 1-buytryloxymethyl derivative of 5-fluorouracil was reported to permeate more readily than 5-fluorouracil.

According to the improvements in drug permeation reported using a prodrug approach in numerous experiments, this method may be one successful strategy to enhance permeation and to achieve controlled release of drug through the skin.

## 1.2.7 Skin metabolism

### 1.2.7.1 Introduction

The skin was formerly thought to be an inert organ, composed of only dead skin cells. Many preparations are placed on the skin on the assumption that the skin is biologically inert. Currently, it is abundantly clear that the skin comprises living tissue, which is metabolically active. Hence, the skin contains enzymes that are able to catalyse and metabolise both endogenous and foreign ('xenobiotics') chemical compounds (Hotchkiss, 1992). The first implication of cutaneous xenobiotic metabolism was observed as early as 1775 (Hotchkiss, 1998). The English doctor, Sir Percivall Pott, reported an increase incidence of scrotal cancer in chimney sweeps, due to skin contact with the polycyclic aromatic hydrocarbons (PAHs) in soot. Several studies have proved that the PAHs themselves are relatively harmless. They are activated by enzymes in the skin to regenerate active metabolites that bind cellular DNA and thereby can cause cancer. Apparently, topically applied compounds may be metabolised in the skin resulting in altered pharmacologic or toxicologic activity.

### 1.2.7.2 Enzymes and metabolic pathways in the skin

The skin contains many drug metabolising enzymes, which can also be found in the liver (Hotchkiss, 1992). The specific activities of cutaneous xenobiotic metabolising enzymes are approximately 0.1 to 28 percent for phase I reactions and 0.6 to 50 percent for phase

II reactions lower than their hepatic counterparts (Hotchkiss, 1998). However, when the large surface area of the skin is considered, it is apparent that the skin is an efficient drug-metabolising organ, which is likely to make a significant contribution to the overall metabolic disposition of topically applied compounds.

Xenobiotic-metabolising enzymes participate in the metabolism of foreign compounds. They metabolise substrates, which generally are predominantly lipophilic, into the substrates that are hydrophilic and less active. The hydrophilic metabolites can be easily excreted in the urine *via* the kidney.

There are two metabolic steps concerning dermal metabolism (Bashir and Maibach, 1999). The first step is known as phase I reactions. The function of this process is to introduce a polar reactive group into the molecule, which renders the molecule suitable for further metabolism as part of phase II reactions.

Phase I reactions involve metabolism by various enzymes including cytochrome P-450 mixed function oxidase enzymes, which are the most important oxidation enzymes. Cytochrome P-450 enzymes add a single oxygen atom from a molecule of $O_2$ to a carbon atom resulting in the formation of an –OH group on the substrate (hydroxylation) and one molecule of water.

Subsequently, these metabolites formed by phase I reaction may undergo further metabolism, phase II reactions. This step involves conjugation, which confers

hydrophilicity to the substrates, allowing renal excretion. Metabolites can be conjugated with substances such as glucuronic acid, sulphur, and gluthathione, resulting in the production of easily excretable products.

Enzymes and reactions involved in skin metabolism can be summarised in table 1.6

| Reaction (Phase I / II) | Enzymes involved |
|---|---|
| **Oxidation (Phase I)** | |
| Aliphatic C atoms | mixed function oxidase |
| Alicyclic C atoms | mixed function oxidases |
| Alcohol | dehydrogenase |
| **Reduction (Phase I)** | |
| Carbonyl groups | keto-reductases |
| C=C double bond | 5(∝) reductase |
| **Hydrolysis (Phase I)** | |
| Esters | esterases |
| Epoxides | epoxide hydrolases |
| **Conjugation (PhaseII)** | |
| Glucuronidation | UDPG-transferases |
| Sulphation | sulpho-transferases |
| Methylation | catechol-O-methyl transferase(COMT) |
| Gluthathione | gluthathione S-transferases |

**Table 1.6**. Enzymes and reactions involved in skin metabolismSource: Adapted from Hotchkiss, 1998.

# Chapter 2

# Synthesis of temozolomide ester and amide derivatives

## 2.1 Introduction

As stated (in section 1.2.6.2), attaching alkyl groups (-R) to parent drugs in order to form lipophilic derivatives may be employed to attempt to enhance the absorption of drugs across the skin.

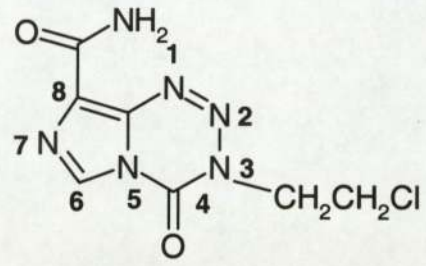
The functional groups of parent molecules modified to form lipophilic prodrugs are carboxyl, hydroxyl, and amino groups (Higuchi and Yu, 1987). The carboxylic acid group (-COOH) can be modified into esters (-COOR) or amides (-NHR); the hydroxyl group (-OH) can be modified into esters (-COOR) and ethers (-COR); the amino group ($-NH_2$) can be modified into amides (-NHR), and peptides (-NHCOOR).

Percutaneous absorption studies for many drugs (*i.e.* steroids, oestradiol) (Higuchi and Yu, 1987; Hotchkiss, 1998) indicate that ester formation for such compounds is a successful approach to enhance the skin permeation in prodrug design.

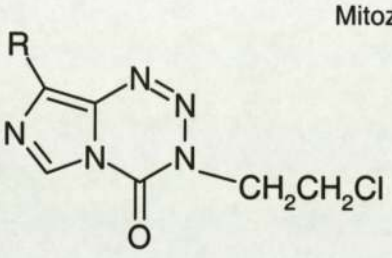
Synthesis of temozolomide ester lipophilic derivatives in order to enhance its permeability has been therefore employed. It is suggested that the ester products may pass more easily through the skin, where in they can be hydrolysed by esterase enzymes within the skin to generate biologically active temozolomide carboxylic acids, which have been shown to exhibit similar cytotoxicity to free temozolomide (Tsang *et al.*, 1990).

8-substitution derivatives (*e.g.* 8-carbamoyl, and 8-sulphamoyl) of mitozolomide (see figure 2.1) are extremely potent compounds against leukemia and lymphoma *in vitro* (Lunt *et al.*, 1987). Therefore, it was envisaged that the lipopholic derivatives of temozolomide to be investigated should bear alkyl groups at the 8-carbamoyl position.

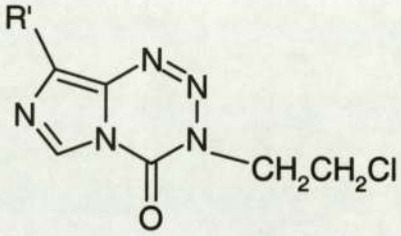
Additionally, mitozolomide amide derivatives were also shown to exhibit high cytotoxicity against tumour cells (Lunt *et al.*, 1987) (see figure 2.1). Consequently, temozolomide amide derivatives were also synthesised. This modification could be expected either to enhance the permeation of temozolomide free drug through the skin, or to promote its bioactivity against skin cancer cells.




Mitozolomide




Mitozolomide carbamoyl compounds




Mitozolomide sulphamoyl compounds

**R=**

$CONMe_2$

CONHMe

$CON(CH_2)_4CH_2$

CONHPh

$CON(CH_3)Ph$

$CONHCH_2Ph$

$CON(CH_2C_6H_4OMe)Ph$

$CON(CH_2C_6H_4OMe)CH_2Ph$

**R'=**

$SO_2Me$

$SO_2NHMe$

$SO_2NMe_2$

$SO_2N(CH_2C_6H_4OMe)H$

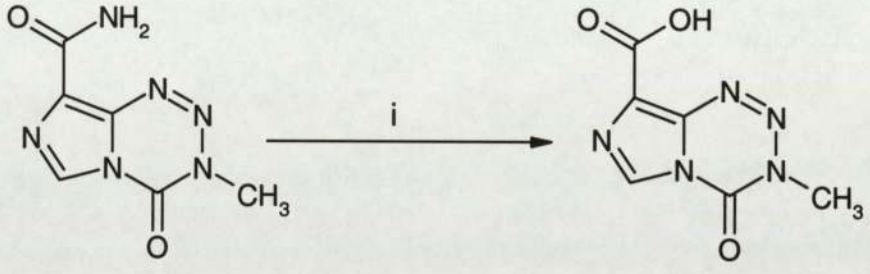
$SO_2NH_2$

**Figure 2.1** The structure of mitozolomide and its highly active derivatives

Source: Adapted from Lunt, 1987

Similarly to mitozolomide, the key intermediate to successfully synthesise ester or amide derivatives, is *via* the 8-carboxylic acid derivative, which provides a ready handle for the synthesis of a wide range of chemical analogues (Horspool *et al.*, 1990). To synthesise the temozolomide carboxylic acid the starting compound is temozolomide itself, and concentrated sulphuric acid is used for hydrolysis of the carbamoyl group to carboxylic acid (see scheme 2.1). The resulting temozolomide acid from this reaction can then serve as a precursor to synthesise temozolomide ester and amide derivatives.

**Scheme 2.1.** The synthesis of temozolomide acid from temozolomide free drug




Temozolomide Temozolomide acid

*Reagents and conditions*: i $NaNO_2$, $H_2SO_4$ (conc.), distilled water, T below 15°C

## 2.2 Experimental

### 2.2.1 Materials and instrumentation

#### 2.2.1.1 Materials

Temozolomide free drug (synthesised)

Sodium nitrite (AnalaR BDH Chemicals Ltd. Poole England)

Concentrated sulphuric acid (Fisher Scientific Ltd. UK)

Anhydrous methanol ethanol, propanol, butanol, hexanol, octanol (Sigma-Aldrich Co. Ltd. UK)

Isopropylamine (BDH Chemicals Ltd. Poole England)

n-butylamine (BDH Chemicals Ltd. Poole England)

sec-butylamine (BDH Chemicals Ltd. Poole England)

t-butylamine (Sigma-Aldrich Co. Ltd. UK)

Pybrop® (Calbiochem-Novabiochem Corp. UK)

4-dimethylaminopyridine, DMAP, (Avocado, Research chemicals Ltd. UK)

Dried dimethylformamide, DMF, (Fisher Scientific Ltd. UK)

Dried tetrahydrofuran, THF, (Fisher Scientific Ltd. UK)

## 2.1.2 Instrumentation

Bruker NMR AC 250 spectrometer

IR MATTSON 3000 FTIR spectrometer

HP G1034C MS chemstation spectrometer

Rotary evaporator

### 2.2.2 Preparation of temozolomide acid

Temozolomide (2.577mmol, 0.5g) was stirred in 4ml of conc. $H_2SO_4$. Sodium nitrite (9.4mmol, 0.65g) was dissolved in 2.6ml distilled water. The sodium nitrite solution was gradually added dropwise into the liquid mixture of temozolomide and concentrated acid. This reaction was carried out on an ice bath at temperatures below 15°C and then the mixture was kept stirring overnight at room temperature. After completion of the reaction, ice was added into the aqueous suspension, and the white solid emerging from this reaction was filtered and washed with distilled water. The product was dried under vacuum. Temozolomide acid as a white powder was obtained (0.493g, 98.6%yield).

### 2.2.3 Synthesis of temozolomide ester and amide products

#### 2.2.3.1 Experimental methods

Temozolomide acid (1mmol, 0.195g), and Pybrop® (1mmol, 0.466g) were added into a flask with a stirring bar. Dried DMF (2ml), and THF (3ml) were injected into the flask using a 5ml syringe, then the mixture was stirred until the solids were completely dissolved. The solution mixture was held stirring on an ice bath and then a dried alcohol (2.2mmol) or an amine solution (1.1mmol) was injected into it followed by the addition of DMAP (2mmol, 0.244g) into the suspension mixture. The reaction was allowed to stir on an ice bath for half an hour. After this time the mixture was kept stirring overnight at room temperature.

### 2.2.3.2 Separation and purification

#### 2.2.3.2.1 Separation

After completion of the reaction, the suspension was filtered through an Ace Buchner funnel. The obtained clear solution was evaporated under a normal rotary evaporator and rotary high vacuum pump to remove THF and DMF respectively. Ice was added to the residue and the product was extracted from the suspension using 10 ml of ethyl acetate in a separating funnel. The extraction was repeated three times. The ethyl acetate layers were collected, and a drying agent, magnesium sulphate, was added. The clear solution was separated and then it was evaporated using normal rotary evaporator.

#### 2.2.3.2.2 Purification

The purification of each ester or amide product was different because of their varying physicochemical properties. The purification procedure therefore is not identical for these compounds, however the methodology can be divided into two major processes.

1. For highly lipophilic products (butyl ester, hexyl ester, and octyl ester), a small amount of ethyl acetate was added to dissolve the residue and then the concentrated liquid mixture was passed through a chromatographic column using the appropriate eluent (see table 2.1).
2. In addition to ethyl acetate as a solvent, acetronitrile was also introduced as a co-solvent for the products which had less lipophilic character (methyl ester, ethyl ester, propyl ester, isopropyl amide, n-butyl amide, s-butyl amide, and t-butyl amide) to completely dissolve the residue. After the residue was entirely dissolved, silica gel was added to the solution, which was then evaporated. The solid was packed into the chromatographic column and then it was flushed with suitable eluent (see table 2.1).

The eluate in each case was evaporated, and the pure solid product was obtained (yields see table 2.2).

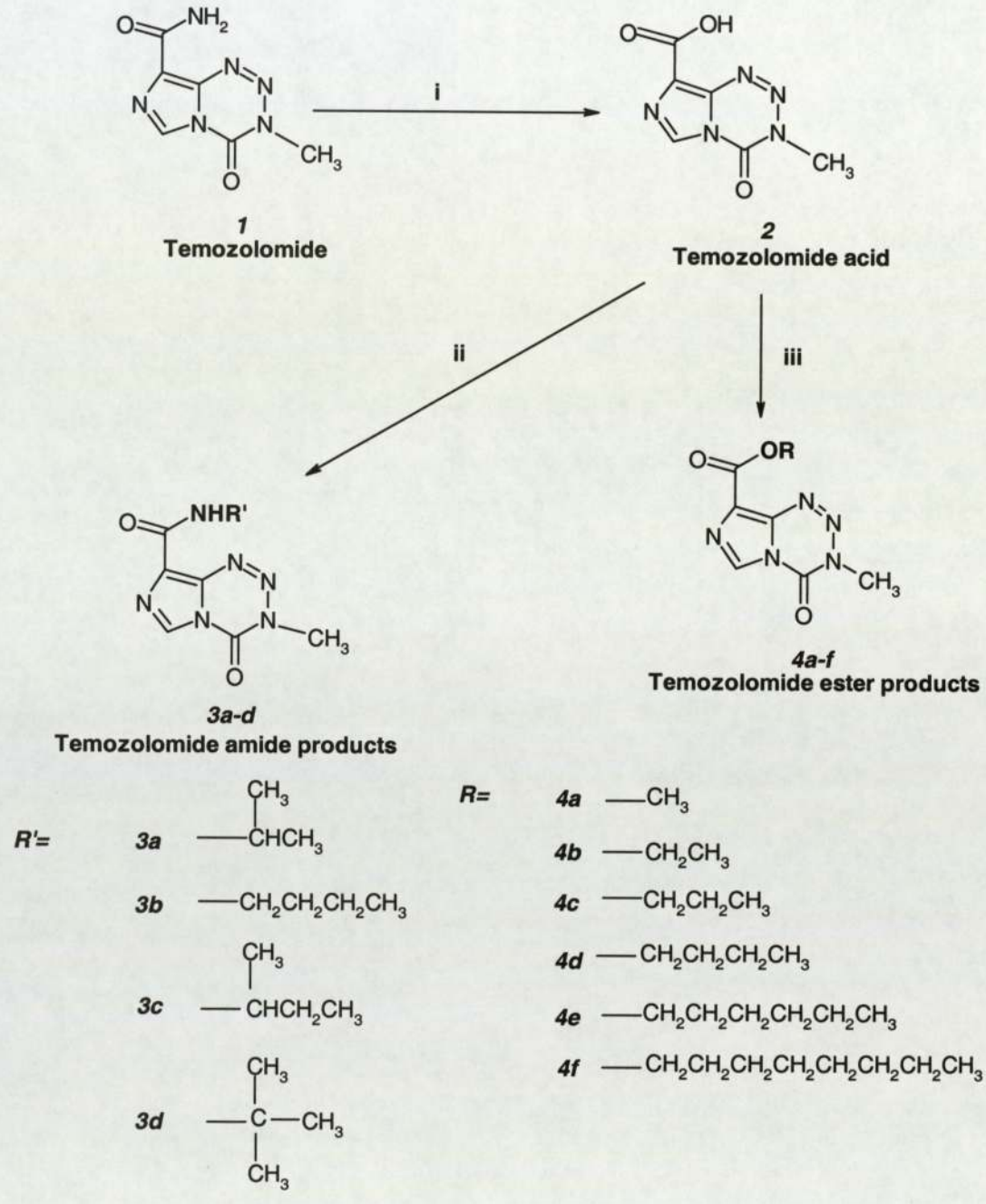
Each ester and amide product was characterised using NMR, IR, and MS spectroscopy. Finally, melting point determination was performed using a microscope.

| Product | eluent | ratio (V/V) |
|---|---|---|
| Methyl ester | petroleum spirit 60-80 / ethyl acetate | 70/30 |
| Ethyl ester | petroleum spirit 60-80 / ethyl acetate | 70/30 |
| Propyl ester | petroleum spirit 60-80 / ethyl acetate | 50/50 |
| Butyl ester | ethyl acetate | - |
| Hexyl ester | ethyl acetate | - |
| Octyl ester | ethyl acetate | - |
| Isopropyl amide | petroleum spirit 60-80 / ethyl acetate | 70/30 |
| N-butyl amide | hexane / ethyl acetate | 50/50 |
| S-butyl amide | petroleum spirit 60-80 / ethyl acetate | 50/50 |
| T-butyl amide | petroleum spirit 60-80 / ethyl acetate | 50/50 |

**Table 2.1** The eluents used for purifying temozolomide products

## 2.3 Results and discussion

### 2.3.1 Synthetic scheme




**Scheme 2.2** Synthetic scheme of temozolomide derivatives **(i)** $NaNO_2$, $H_2SO_4$(conc.), distilled water; **(ii)** and **(iii)** Pybrop®, DMAP, DMF, THF

To successfully synthesise temozolomide ester and amide products, varying coupling agents and ratios of starting materials were investigated to optimise the conditions. Both Pybrop® and DMAP were used as the coupling agents which allowed the reaction to proceed to successfully yield the products. The optimum conditions were found to be a ratio of alcohol: drug: Pybrop®: DMAP of 2.2: 1: 1: 2 for ester products and a ratio of amine: drug: Pybrop®: DMAP of 1.1: 1: 1: 2 for amide products. The reaction time for each was around 12 hours at room temperature. The percentage yield of each product was obtained around 40-60 % (see table 2.2).

| Products | yield % (w/w) | melting points (M.P, °C) |
|---|---|---|
| Methyl ester | 44 | 205 |
| Ethyl ester | 43 | 204 |
| Propyl ester | 51 | 109 |
| Butyl ester | 57 | 145 |
| Hexyl ester | 55 | 113 |
| Octyl ester | 58 | 99 |
| Isopropyl amide | 49 | 272 |
| N-butyl amide | 52 | 99 |
| S-butyl amide | 48 | 208 |
| T-butyl amide | 44 | 181 |

**Table 2.2** Yields (%) and melting points (°C) of temozolomide product

## 2.3.2 Nuclear magnetic resonance (NMR) analysis

### 2.3.2.1 Introduction

NMR spectroscopy is widely used to determine the molecular structure of a compound. It is frequently employed to examine the hydrogen in organic compounds. This is based on the magnetic properties of nucleus of elements that result from their nuclear spin properties. The hydrogen nucleus, $^{1}H$, that is the proton, can reside in either + ½ or – ½ spin states. In the absence of a magnetic field, the two spin states have the same energy. When the magnetic field is applied, the two spin states have different energies. The + ½ spin state has lower energy than – ½ spin state. Therefore, when the magnetic field is applied to the hydrogen nuclei in a chemical sample, more protons have spin + ½ than - ½ because they favour the lower energy state. Thus, there is an energy difference ($\Delta E$) between two states. If the energy of the electromagnetic radiation applied is exactly equal to $\Delta E$, this energy will be absorbed by some of nuclei in + ½ spin state, and invert to – ½ spin state. The energy absorbed by nuclei in a magnetic field is termed 'nuclear magnetic resonance'. The magnetic field strength required for NMR absorption of protons is different depending on the chemical environment of the proton. For example, for protons on aromatic rings and aliphatic chains, their NMR signals will appear at different position in a NMR spectrum.

For nuclei to absorb energy, they must have a nuclear spin and must be situated in a magnetic field. NMR can thus be detected for other nuclei that have nuclear spin such as

$^{19}F$, $^{31}P$, and $^{13}C$. However, some nuclei, which have no nuclear spin (*i.e.* $^{12}C$) will not provide an NMR signal.

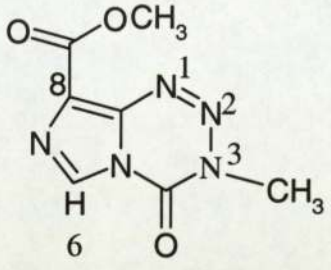
### 2.3.2.2 Results

$^{1}H$ NMR and $^{13}C$ NMR spectra were obtained on a Bruker NMR AC 250 Spectrometer, using appropriate solvents.

A typical NMR ($^{1}H$ NMR and $^{13}C$ NMR) spectrum of each temozolomide derivative is shown in appendices 1-20.

## A. Temozolomide ester products

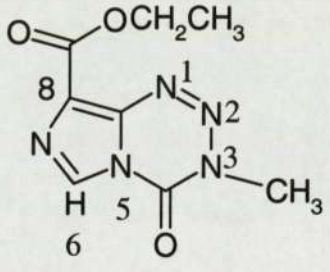
(a) Methyl ester



**$^{1}$H NMR** ($_{d6}$-DMSO/ppm) δ 8.86 (s, 1, H-6), 3.90 (s, 3,C$\underline{H}_3$-O), 3.87 (s, 3, C$\underline{H}_3$-N) (see appendix 1)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 161 ($\underline{C}$OO), 139 (C-4), 137 (C-6), 129 (C-9), 126 (C-8), 52.2 (O$\underline{C}H_2$), 36.4 (N$\underline{C}H_3$) (see appendix 2)

(b) Ethyl ester



**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.45 (s, 1, H-6), 4.52 (q, 2, J= 7.1 Hz, C$\underline{H}_2$-O), 4.04 (s, 3, C$\underline{H}_3$-N), 1.45 (t, 3, J=7.1 Hz, $CH_2$-C$\underline{H}_3$) (see appendix 3)

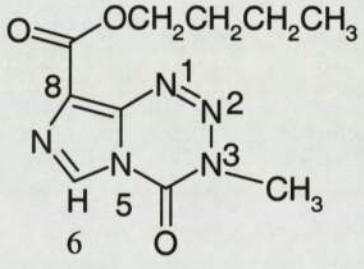
**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 161 ($\underline{C}$OO), 139 (C-4), 137 (C-6), 129 (C-9), 127 (C-8), 60.9 (O$\underline{C}H_2$), 36.4 (N$\underline{C}H_3$), 14.3 ($CH_2\underline{C}H_3$) (see appendix 4)

(c) Propyl ester

**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.46 (s, 1, H-6), 4.41 (t, 2, J= 6.7 Hz, C$\underline{H}_2$-O), 4.03 (s, 3, C$\underline{H}_3$-N), 1.83 (sextet, 2, J= 7.1 Hz, C-C$\underline{H}_2$-C), 1.03 (t, 3, J= 7.4 Hz, C-C$\underline{H}_3$) (see appendix 5)

**$^{13}$C NMR** ($CDCl_3$/ppm) δ 160 ($\underline{C}$OO), 138 (C-4), 136 (C-6), 130 (C-9), 128 (C-8), 67.1 (O$\underline{C}H_2$), 36.5 (N$\underline{C}H_3$), 21.7 ($\underline{C}H_2CH_3$), 8.87 ($CH_2\underline{C}H_3$) (see appendix 6)

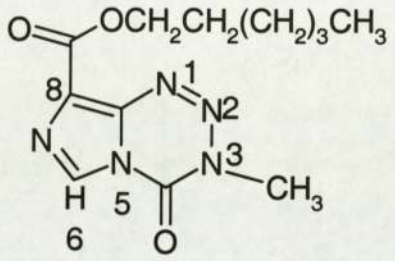
(d) Butyl ester



**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.45 (s, 1 H-6), 4.45 (t, 2, J= 7.1 Hz, C$\underline{H}_2$-O), 4.03 (s, 3, C$\underline{H}_3$-N), 1.79 (quintet, 2, J= 7.4 Hz, C-C$\underline{H}_2$-C), 1.46 (sextet, 2, J= 7.3 Hz, C-C$\underline{H}_2$-$CH_3$), 0.95 (t, 3, J= 7.3 Hz, C-C$\underline{H}_3$) (see appendix 7)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 161 (C=O), 139 (C-4), 137 (C-6), 129 (C-9), 127 (C-8), 64.5 ($OCH_2$), 36.4 ($NCH_3$), 30.3 ($OCH_2CH_2$), 18.7 ($CH_2CH_3$), 13.6 ($CH_2CH_3$) (see appendix 8)

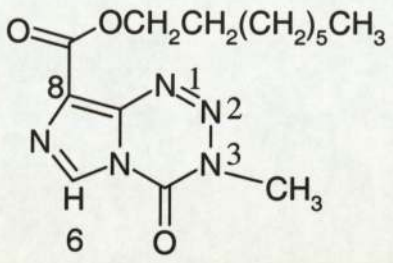
(e) Hexyl ester




**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.49 (s, 1, H-6), 4.45 (t, 2, J= 6.9 Hz, $CH_2$-O), 4.04 (s, 3, $CH_3$-N), 1.79 (quintet, 2, J= 7.1 Hz, C-$CH_2$-C), 1.29-1.40 (m, 6, C-$(CH_2)_3$-$CH_3$), 0.87 (t, 3, J= 6.9 Hz, C-$CH_3$) (see appendix 9)

**$^{13}$CMR** ($_{d6}$-DMSO/ppm) δ 161 (C=O), 139 (C-4), 137 (C-6), 129 (C-9), 127 (C-8), 64.8 ($OCH_2$), 36.4 ($NCH_3$), 30.9 ($OCH_2CH_2$), 28.2 ($O(CH_2)_2CH_2$), 25.1 ($CH_2CH_2CH_3$), 22.1 ($CH_2CH_3$), 13.9 ($CH_2CH_3$) (see appendix 10)

(f) Octyl ester

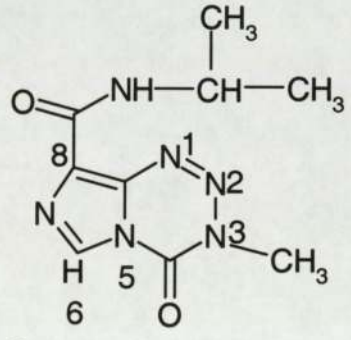


**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.36 (s, 1, H-6), 4.36 (s, 2, C$\underline{H}_2$-O), 3.95 (s, 3, C$\underline{H}_3$-N), 1.74 (s, 2, C-C$\underline{H}_3$-C), 1.19 (s, 10, C-(C$\underline{H}_2$)$_5$-C), 0.78 (s, 3, C-C$\underline{H}_3$) (see appendix 11)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 160 ($\underline{C}$OO), 139 (C-4), 137 (C-6), 129 (C-9), 127 (C-8), 64.8 (O$\underline{C}$H$_2$), 36.4 (N$\underline{C}$H$_3$), 31.2 (OCH$_2\underline{C}$H$_2$), 28.6 (OCH$_2$CH$_2$($\underline{C}$H$_2$)$_2$), 28.2 (O(CH$_2$)$_4\underline{C}$H$_2$), 25.4 ($\underline{C}$H$_2$CH$_2$CH$_3$), 22.1 ($\underline{C}$H$_2$CH$_3$), 13.9 (CH$_2\underline{C}$H$_3$) (see appendix 12)

## B. Temozolomide amide products

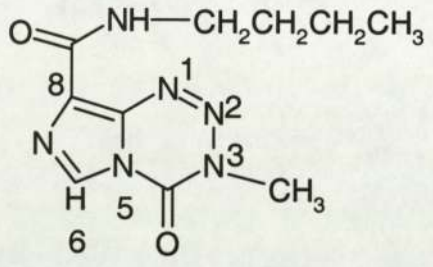
(a) Isopropyl amide



**$^{1}$H NMR** ($_{d6}$-DMSO/ppm) δ 8.83 (s, 1, H-6), 8.21 (d, 1, J= 8.4 Hz, C-N$\underline{H}$-C), 4.13 (sextet, 1, J= 6.9 Hz, N-C$\underline{H}$-(CH$_3$)$_2$, 3.85 (s, 3, C$\underline{H}_3$-N), 1.19 (s, 3, C$\underline{H}_3$-CH), 1.17 (s, 3, C$\underline{H}_3$-CH) (see appendix 13)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 159 (CONH), 139 (C-4), 134 (C-6), 131 (C-9), 128 (C-8), 36.2 ($NCH_3$), 22.2 ($CH(CH_3)_2$) (see appendix 14)

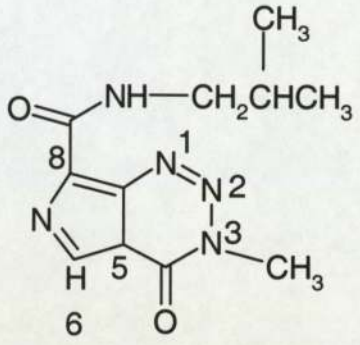
(b) N-butyl amide




**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.38 (s, 1, H-6), 7.35 (m, 1, C-NH-C), 3.99 (s, 3, $CH_3$-N), 3.48 (q, 2, J= 6.7 Hz, N-$CH_2$-C), 1.60 (quintet, 2, J= 7.3 Hz, C-$CH_2$-C), 1.39 (sextet, 2, J= 7.3 Hz, C-$CH_2$-$CH_3$), 0.93 (t, 3, J= 7.3 Hz, C-$CH_3$) (see appendix 15)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 160 (CONH), 139 (C-4), 134 (C-6), 130 (C-9), 128 (C-8), 38.2 ($NHCH_2$), 36.2 ($NCH_3$), 31.4 ($CH_2CH_2CH_3$), 19.6 ($CH_2CH_3$), 13.7 ($CH_2CH_3$) (see appendix 16)

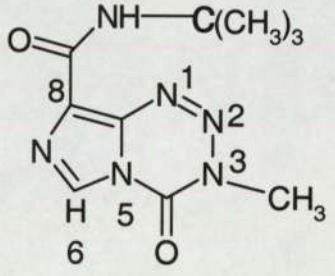
(c) S-butyl amide



**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.35 (s, 1, H-6), 7.46 (m, 1, C-N<u>H</u>-C), 3.99 (s, 3, C<u>H</u>$_3$-N), 3.35 (t, 2, J= 7.1 Hz, NH-C<u>H</u>$_2$-CH), 1.92 (m, 1, C-C<u>H</u>-($CH_3$)$_2$), 0.95 (s, 3, C-C<u>H</u>$_3$), 0.90 (s, 3, C-C<u>H</u>$_3$) (see appendix 17)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 160 (<u>C</u>ONH), 139 (C-4), 134 (C-6), 130 (C-9), 128 (C-8), 45.7 (NH<u>C</u>H$_2$), 35.9 (N<u>C</u>H$_3$), 27.9 (CH$_2$<u>C</u>H), 19.8 (CH(<u>C</u>H$_3$)$_2$) (see appendix 18)

(d) T-butyl amide



**$^{1}$H NMR** ($CDCl_3$/ppm) δ 8.39 (s, 1, H-6), 4.08 (s, 3, C<u>H</u>$_3$-N), 1.42 (s, 9, C-(C<u>H</u>$_3$)$_3$) (see appendix 19)

**$^{13}$C NMR** ($_{d6}$-DMSO/ppm) δ 159 (C=ONH), 139 (C-4), 134 (C-6), 130 (C-9), 128 (C-8), 50.8 (C=O$(CH_3)_3$), 36.2 (N=O$H_3$), 28.5 (C(C=O$H_3)_3$) (see appendix 20)

## 2.3.3 Infrared (IR) analysis

### 2.3.3.1 Introduction

Infrared spectroscopy is mainly used to determine the functional groups present in a compound. It can provide information about possible structures of the compounds under consideration. An infrared spectrum is a record of the light absorbed by a substance as a function of wavelength, which is expressed in term of wavenumber ($cm^{-1}$). The absorptions observed in IR spectra are the result of bond vibration. Absorption of energy from infrared radiation can occur only when there is an exact match between the wavelength of the radiation and the wavelength of the bond vibration. Consequently, each peak in an IR spectrum corresponds to the absorption of the energy by the vibration of a particular bond. For example, if the molecule contains N-H bonds and C=C bonds, the peaks of IR spectrum are likely to present in the region of 3400-2800 $cm^{-1}$and 1600-1850 $cm^{-1}$ respectively. For all compounds, a given type of functional group absorbs in the same general region of the IR spectrum.

### 2.3.3.2 Results

Infrared spectroscopy was performed on an IR Mattson 3000 FT-IR spectrometer, and product samples were pressed into KBr pellets before analysis.

**A. Temozolomide ester products**

(a) Methyl ester

$\nu_{max}$ (KBr): 3489, 2961 (C-H), 1752 (C=O), 1727 (C=O), 1214 (C-O), 1062 (C-O), 828, 556 $cm^{-1}$

(b) Ethyl ester

$\nu_{max}$ (KBr): 3478, 2991 (C-H), 1754 (C=O), 1700 (C=O), 1467 (C-O),1258 (C-O), 1060 (C-O), 844, 561 $cm^{-1}$

(c) Propyl ester

$\nu_{max}$ (KBr): 3122, 2960 (C-H), 1729 (C=O), 1700(C=O), 1457 (C-O), 1200 (C-O), 1174 (C-O), 1052, 942 $cm^{-1}$

(d) Butyl ester

$\nu_{max}$ (KBr): 3156, 2967 (C-H), 1746 (C=O), 1467 (C-O), 1261 (C-O), 1054 (C-O), 823, 561 $cm^{-1}$

(e) Hexyl ester

$\nu_{max}$ (KBr): 2960 (C-H), 1762 (C=O), 1471 (C-O), 1250 (C-O), 1200 (C-O), 1090 (C-O), 850, 530 $cm^{-1}$

(f) Octyl ester

$\nu_{max}$ (KBr):2925 (C-H), 2853 (C-H), 1758 (C=O), 1720 (C=O), 1467 (C-O), 1255 (C-O), 838, 556 $cm^{-1}$

**B. Temozolomide amide products**

(a) Isopropyl amide

$\nu_{max}$ (KBr): 3436 (N-H), 2973 (C-H), 2336, 1758 (C=O), 1652 (C=O), 1471 (C-N), 1263 (C-N), 832, 561 $cm^{-1}$

(b) N-butyl amide

$\nu_{max}$ (KBr): 3350 (N-H), 2957 (C-H), 2358, 1744 (C=O), 1654 (C=O), 1459 (C-N), 1251 (C-N), 842 $cm^{-1}$

(c) S-butyl amide

$\nu_{max}$ (KBr): 3400 (N-H), 2964 (C-H), 1762 (C=O), 1664 (C=O), 1600, 1500 (C-N), 1257 (C-N), 850, 550 $cm^{-1}$

(d) T-butyl amide

$\nu_{max}$ (KBr): 3389 (N-H), 2963 (C-H), 1764 (C=O), 1664 (C=O), 1587 (C-N), 1471 (C-N), 838, 561 $cm^{-1}$

## 2.3.4 Mass spectrum (MS) analysis

### 2.3.4.1 Introduction

Mass spectrometry is used to determine the molecular mass of molecule. The fundamental basis of this technique is that an electron beam of high energy, which is greater than the bond energy of the chemical bonds in the molecule, is applied to the sample resulting in an ejection of electron from the molecule. The obtained molecular ion (M) can be detected by the mass spectrometer. This occurs at the ionic mass value (*m/z*) equal to the molecular mass of the sample molecule. On the other hand, neutral molecules and radicals do not appear as peaks in mass spectrum.

### 2.3.3.2 Results

All samples were dissolved in acetonitrile (HPLC grade), and analyses were performed on a G1034C MS spectrometer.

## A. Temozolomide ester products

(a) Methyl ester

MS:+ ES: m/z = 232 $[M+H]^+$, 214$[M+H-H_2O]^+$

(b) Ethyl ester

MS:+ ES: m/z = 246 $[M+H]^+$, 228$[M+H-H_2O]^+$

(c) Propyl ester

MS:+ ES: m/z = 260 $[M+H]^+$, 242$[M+H-H_2O]^+$

(d) Butyl ester

MS:+ ES: m/z = 274 $[M+H]^+$, 256$[M+H-H_2O]^+$

(e) Hexyl ester

MS:+ ES: m/z = 302 $[M+H]^+$, 284$[M+H-H_2O]^+$

(f) Octyl ester

MS:+ ES: m/z = 330 $[M+H]^+$, 312$[M+H-H_2O]^+$

**B. Temozolomide amide products**

(a) Isopropyl amide

MS:+ ES: m/z = 259 $[M+H]^+$, $[M+H-H_2O]^+$

(b) N-butyl amide

MS:+ ES: m/z = 273 $[M+H]^+$, $[M+H-H_2O]^+$

(c) S-butyl amide

MS:+ ES: m/z = 273 $[M+H]^+$, $[M+H-H_2O]^+$

(d) T-butyl amide

MS:+ ES: m/z = 273 $[M+H]^+$, $[M+H-H_2O]^+$

# Chapter 3

# High performance liquid chromatography (HPLC)

## 3.1 Introduction

HPLC is a widely used analytical technique due to its rapidity, accuracy, high sensitivity, and versatile application. Currently, it has been variously used for applications such as separation, identification, purification, and quantification in biotechnological, biomedical, and biological research. Moreover, several industries, (cosmetics, energy, food, and environmental) mainly consider HPLC as a primary analytical technique. In this study, it was employed for quantitative analysis, and was used to investigate the degradation profiles of the products.

## 3.2 Basic theory and terminology

Chromatography was first used by Tswett in 1903 who applied it to the separation of pigments into coloured bands. It has been defined as a separation method in which the mixtures are resolved by different migration of their constituents during passage through a chromatographic column. The separation process is governed by the distribution of compounds between two phases: the mobile phase (moving phase) and the stationary phase. Fluid entering the column is called *eluent*. Fluid emerging from the end of column is the *eluate*.

### 3.2.1 The basic components and operation of HPLC chromatographic systems

During the past decade, significant advances have been made in the development of HPLC instrumentation. Consequently, the detailed layouts of chromatographic systems are rapidly out of date. However, the basic constituents of HPLC system are outlined in table 3.1, and these components schematically illustrated in figure 3.1.

**Table 3.1** Basic components of HPLC systems

| | |
|---|---|
| 1. Solvent supply system | solvent reservoir, solvent degassing system, pump, gradient elution programmer |
| 2. Sample introduction system | syringe injector, autoinjector |
| 3. Pre-column and analytical column | |
| 4. Detector | |
| 5. Fraction collector | manual or automatic |
| 6. Data handing devices | recorder, integrator, computer |

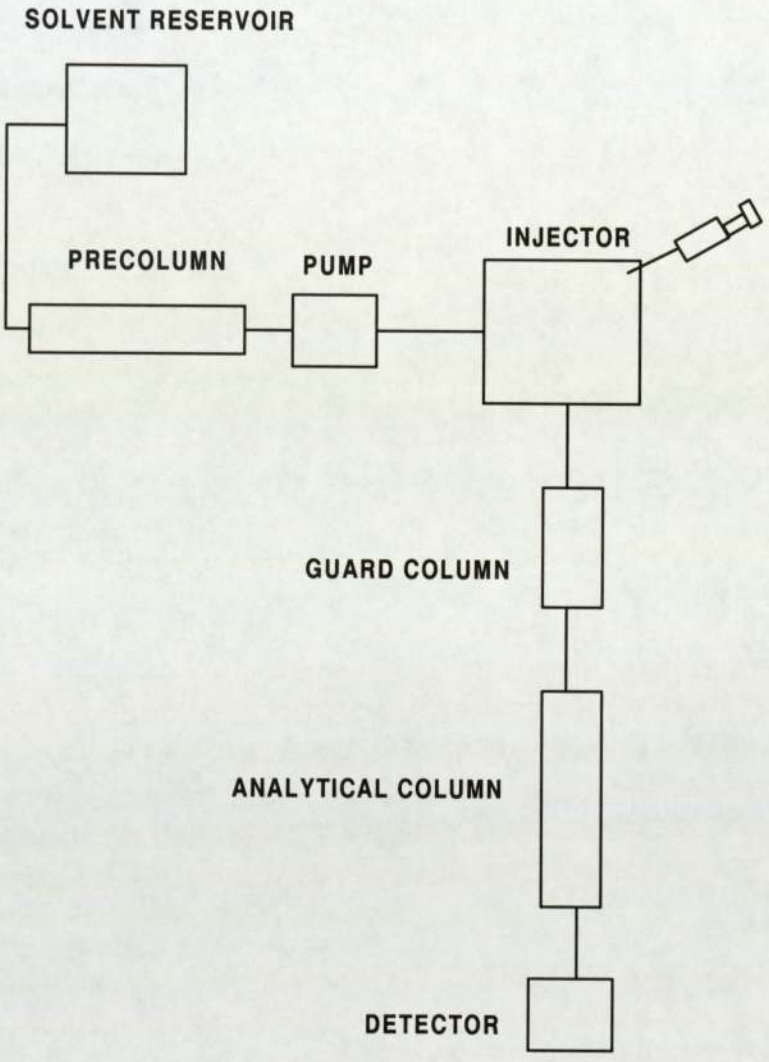



**Figure 3.1** A schematic representation of a high performance liquid chromatograph

The HPLC system shown schematically in figure 3.1 consists of *a solvent reservoir*, containing the mobile phase; *a pump* that is often called a solvent delivery system; *an injector* to introduce the sample into the system; the *analytical column* containing the solid packing (stationary phase); a *detector* to detect the sample compounds.

There are other two components, which are a pre-column and a guard column. These constituents behave as a filter to remove any insoluble materials, impurities or other debris from the mobile phase and sample solution respectively.

To operate the HPLC system, the sample solution is injected into the system and is then pushed through the analytical column by the constant pumping of the mobile phase from the reservoir through the system. The eluent emerging from the column flows through the detector, which generates the response for particular compounds.

### 3.2.2 HPLC chromatographic systems of temozolomide and its derivatives

This chromatographic system consisted of a Waters 600E pump connected to Waters 712 WISP auto-injection and Waters 484 Tunable Absorbance detector set at UV 330 nm. Separations were performed on a reverse-phase HP ODS-hypersil 5μm column (100 × 4.6 mm I.D) equipped with a HP ODS-hypersil 5μm (20 × 4.0 mm) guard column. The computer software was used to record events.

### 3.2.3 The chromatogram

Information about the separation profile of analytes from HPLC chromatographic systems is generally displayed on a chromatogram, which is obtained by converting the detector response to an electrical signal and following this signal on a recorder as a function of time after loading the sample (Rossomando, 1998). Figure 2.3 represents a chromatogram of a sample containing two species of compound.

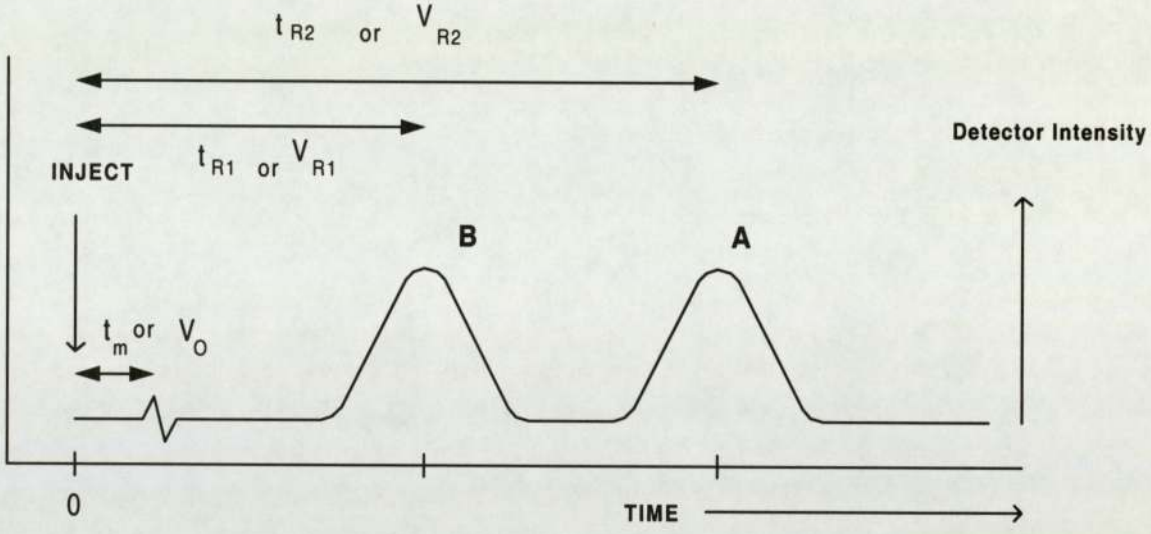



**Figure 3.2** A representative HPLC chromatogram showing the separation of compounds A and B. The time of injection is taken as zero time, and the elution position is shown as a function of time after injection.

A solution mixture of substances A and B is placed onto the column. Compound B traverses the column at a faster rate than compound A. As can be seen, compound B emerges and will be detected by the detector first, and then followed by compound A.

Under these conditions, the chromatogram will show the elution of A and B as a function of time after loading the sample.

### 3.2.4 Basic equations

(a) Retention time *($t_R$)*

The degree of retardation of a particular compound in a mixture in chromatographic systems can be expressed in terms of retention time *($t_R$)*. Retention time is defined as the time from the sample introduction to the column to the maximum point of the eluted peak (see figure 3.2). Figure 3.2 shows the retention times of compounds B and A are $t_{R1}$ and $t_{R2}$ respectively.

(b) Retention volume *($V_r$)*

The retention volume of the peak is equal to the volume of liquid that passes through the column from the point of loading to the maximum point of the chromatographic band. It is related to the flow rate *($F_v$)* and the retention time *($t_R$)* by the equation:

$$V_r = F_v t_R \tag{3.1}$$

(c) Mobile phase hold up time *($t_m$)* and void volume *($V_o$)*

Mobile phase hold up time is the time required for the mobile phase to pass through the column. Thus, compounds that do not interact with the packing material will be eluted in this time. The volume of mobile phase used to elute the column over the period of hold up time is called void volume *($V_o$)*. However, mobile phase hold up time, $t_m$, and void

volume, $V_o$, are difficult to obtain due to the complexity of the separation mechanisms in chromatographic systems (Krstulovic and Brown, 1982).

*(d)* Capacity factor *(k′)*

The fundamental measure of retention of compounds in liquid chromatography is the capacity factor (or capacity ratio), *k′*.

The capacity factor can be expressed in terms of the retention volume *($V_r$)* of sample compound and the void volume *($V_o$)*. The ratio, which is referred to the capacity factor, *k′*, is given by the following expression:

$$k' = \frac{V_r - V_o}{V_o} \tag{3.2}$$

If the flow rate remains constant during the elution of the sample, the capacity factor can be expressed in terms of retention time through the following expression.

$$k' = \frac{t_R - t_m}{t_m} \tag{3.3}$$

Capacity factor can also be defined as the ratio of the number of molecules of solute in the stationary phase, $N_s$, and the number of molecules in the mobile phase, $N_m$. This can be expressed by the following relationship:

$$k' = \frac{N_s}{N_m} \tag{3.4}$$

By taking into account of the volume of the mobile phase, $V_m$, and stationary phase, $V_s$, in the column, the capacity factor can be related to the partition coefficient $K$ of the solute between the mobile phase and stationary phase through the following equation.

$$k' = K\frac{V_s}{V_m} \tag{3.5}$$

## 3.3 High-performance liquid chromatography (HPLC) method development

The theoretical principle of HPLC can be simply described as one in which a mobile phase is delivered through a chromatographic column. When a sample solution is placed onto the latter component, distribution of a compound between the mobile phase and stationary phase will occur. The elution performance depends on the physicochemical characteristics of analytes and the properties of the chromatographic system. Compounds which have a strong interaction with the stationary phase, will exist in the system longer than others that favourably distribute in the mobile phase. Nevertheless, the chromatographic system should be chosen so that the partitioning of the compounds between these two phases is sufficiently different to permit the component separation (Olson, 1990).

The development of a HPLC protocol can be achieved by modifying the mobile phase and the stationary phase. In this project, only one column was used as a stationary phase, and the mobile phase was altered in solvent composition depending on the nature of each product.

### 3.3.1 Stationary phase selection

The stationary phase, known as the chromatographic column, consists of the solid support packing in the column, which is normally a stainless steel tube. There are several materials that can be used as stationary phases to generate the different modes of separation. For example, porous silica, is the most commonly used in *normal phase* liquid chromatography. The polar hydroxyl functional groups (silanol) present on the surface of the solid will have more affinity for interaction with hydrophilic compounds than hydrophobic compounds. Therefore, more lipophilic compounds elute more quickly than hydrophilic compounds. Conversely, the column in *reverse phase* chromatography (RPC) is composed of silica particles covalently bonded on an n-alkyl chain, which commonly is C-8 (octyl), or C-18 (octadodecyl) chain. Thus, hydrophilic compounds pass through the column more quickly than hydrophobic compounds. RPC is the most useful chromatographic technique for pharmaceutical separations. This is attributed to its employment of an aqueous mobile phase thereby a large amount of water can be used. As a result, a wide-ranging polarity of analytes can be separated by this system, and it is generally compatible with most aqueous samples, which can be directly injected onto the

column without pre-treatment. Additionally, a very polar compound that may not be eluted from a normal phase system can be accommodated by RPC.

Generally, the choice of mode of chromatographic systems can be simplified in figure 3.3. The type of chromatographic systems, which may be considered for each compound is based primarily on the molecular weight, the polarity, and the ionic character of the compound. Traditionally, size exclusion chromatography is recommended for the separation of compounds where molecular weight is greater than 1000 daltons, and this is still the method of choice for determination of the size distribution of polymers (Riley, 1995). Conventional reverse phase chromatography can also be used for the separation of high molecular weight compounds (*e.g.* proteins).

The main factors to be considered in the choice of mode of chromatography for the separation of small molecules are the ionic character and polarity of the compound. Ionic compounds are normally the best separated by ion exchange or reverse phase chromatography. Non-polar compounds are mainly recommended to be separated by normal phase chromatographic systems. However, in practical terms, pragmatic consideration is the principal criterion to select the type of chromatographic system employed.

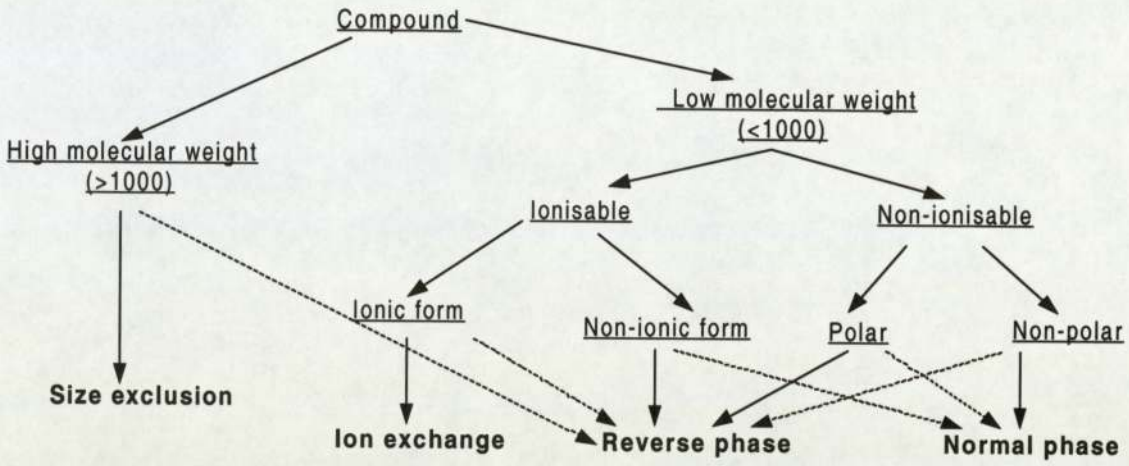



**Figure 3.3** A simplified diagram indicating the choice of mode of chromatographic systems.

In this study, a reverse phase column, in which silica was treated with octadodecyl ligand was selected.

## 3.3.2 Mobile phase selection

The composition of mobile phases in HPLC systems can be adjusted in order to manipulate the distribution of the solute between the mobile phase and stationary phase leading to achieving the aim of the application. Modification of the mobile phase depends on the physicochemical properties of products.

There are several criteria for a solvent being considered for use as a suitable mobile phase component of HPLC systems. Firstly, the solvent must be able to completely dissolve the sample components without chemically reacting with them. An appropriate solvent should also be readily available, pure, and non-toxic. Finally, the solvent should have low viscosity to minimise any pressure drop during the operation.

There are a few common solvents, which are water, various alcohols, acetonitrile and tetrahydrofuran, used in HPLC systems. The simplest mobile phase will generally be composed of two solvents, a weak solvent A and a strong solvent B. For instant, a typical *reverse phase* eluent consists of a mixture of water, which is the weaker solvent, and a stronger organic solvent modifier. Similarly, a *normal phase* eluent will normally comprise a mixture of a weak solvent such as n-heptane and a stronger modifier such as chloroform or ethyl acetate.

According to an increase in the lipophilic property of the temozolomide products in this project, acetonitrile was introduced as an organic modifier. Varying compositions of acetonitrile and water were employed to optimise peak resolution and retention time. The pH value of mobile phase is generally crucial for weak acid or weak base compounds. Adjusting pH value can merely generate solely neutral or non-ionised form of the compounds resulting in production of a symmetrical sharp peak. However, for all of temozolomide products, which are neutral compounds, pH values are unlikely to have any effect.

### 3.3.3 Detector selection

Numerous kinds of detectors, for instance, refractive index (RI), ultra-violet (UV), and fluorescence can be used to detect the analytes eluted from the column. Selection depends on the properties of each compound. In this study, a UV detector was employed due to the high absorbance sensitivity of UV light by temozolomide and its derivatives.

## 3.4 Quantitative analysis using HPLC systems

### 3.4.1 Introduction

Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a known solution. It involves injecting a series of known concentrations of interest compound solution (standard solution) onto the HPLC for detection. The obtained chromatograms will give a series of peaks that correlate to the concentrations of standard solution injected. The area under the chromatogram peaks is calculated to develop a calibration curve.

The calibration curve is obtained by plotting calculated peak area values against a series of concentrations of the standard solution. The concentrations of unknown can thus be calculated from the equation of the calibration curve.

### 3.4.2 Calibration curves and internal standards

For the optimisation of HPLC conditions for quantitative analysis, an internal standard (IS) should be added to the system. The content of a compound in a sample can be accurately determined by a relative assay in which content of analye is compared against a pure reference internal standard (Lough and Wainer, 1995). In the internal standard method, the intensity of detector response at known concentrations of pure IS compound is investigated by determining the peak area of the obtained chromatogram. Prior to loading the samples onto the column, the IS solution with a known concentration is added to each sample. Thus, the peak of the IS can be compared to the peak of analyte on the chromatogram of each sample. For this method to efficiently work, it is crucial to choose a suitable internal standard. Ideally, an internal standard peak must be completely resolved from the analyte peak and any other peak that might reasonably be expected to arise from other component in the sample (*i.e.* metabolite peak). It should also have a detector response factor similar to the analyte. Finally, internal standards should elute from the column relatively close to the interest compounds.

To generate the calibration curve, the ratios of peak areas of standard interest compound and internal standard are plotted *versus* a series of concentrations of standard interest compound.

In this study, mitozolomide, which is the progenitor of temozolomide and anthracene were used as the internal standards for the HPLC determination of temozolomide (free drug form and acid form) and its derivatives respectively.

## 3.5 Experimental

### 3.5.1 Preparation of stock and internal standard (IS) solutions

#### 3.5.1.1 Standard stock solutions

(a) Sample 1, 2, 4a, 4b, 4c, 4d, 3a, 3b, 3c, 3d (see table 3.2) stock solution:

Each sample 0.002g of sample was dissolved in 2ml 10% w/v of propylene glycol in water and then water was added to 50 ml.

(b) Sample 4e, 4f (see table 3.2) stock solution:

Each sample 0.002g of sample was dissolved in 5ml 10% w/v of propylene glycol in water and then 5ml of acetonitrile was added as a co-solvent, finally it was diluted with water to 50ml.

### 5.1.2 Internal standard solutions

(a) Mitozolomide internal standard solution

Mitozolomide 0.0018g was dissolved in 150ml 0.5% v/v acetic acid.

(b) Anthracene internal standard solution

Anthracene 0.002g was dissolved in 75ml acetonitrile.

## 3.5.2 Preparation of calibration solutions

Standard stock solutions (40μg/ml) of each sample were diluted to various concentrations, which were generally 5-6 standards (20, 8, 4, 2, 0.04 μg/ml). Standard working solutions (1ml) were added to an injection vial and then internal standard solution (1ml) was added to it. The solution mixture (200 μl) was injected onto the column.

Calibration curves for HPLC conditions of temozolomide, temozolomide acid, and temozolomide products have been shown in figures 3.4-3.15.

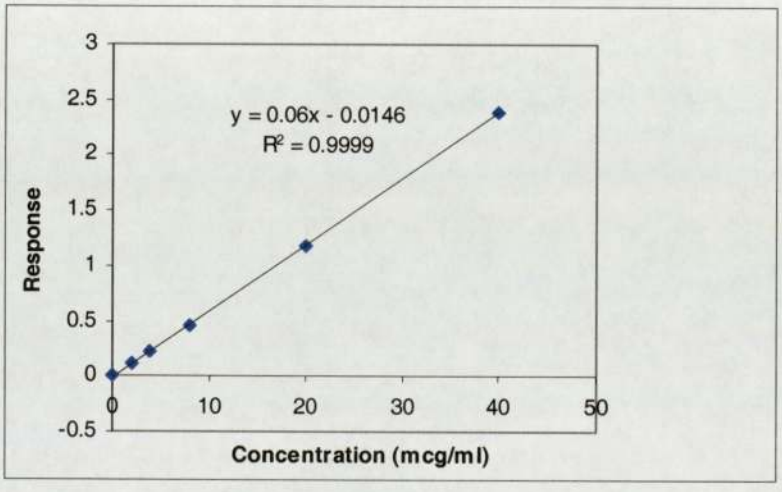



**Figure 3.4** A typical calibration curve for HPLC detection of methyl ester product

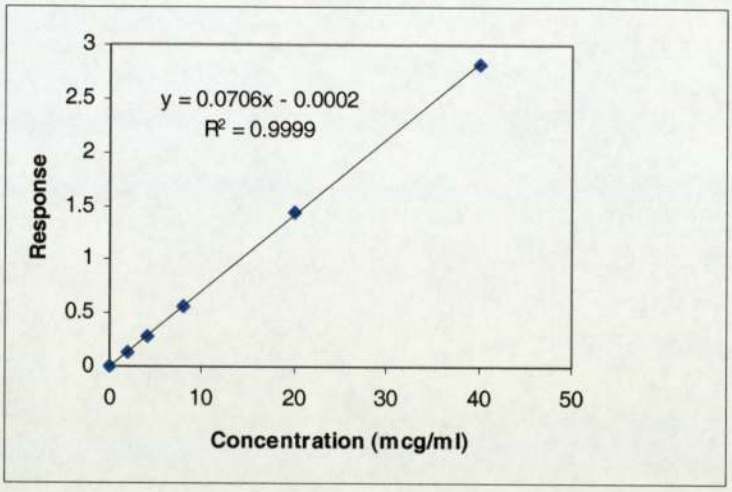



**Figure 3.5** A typical calibration curve for HPLC detection of ethyl ester product

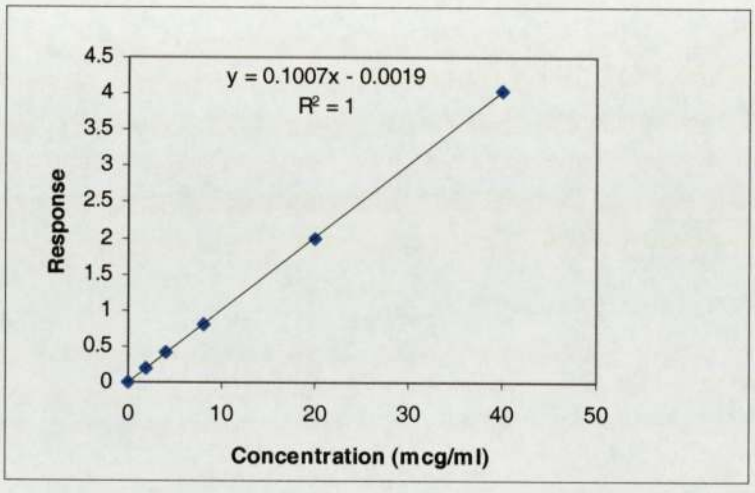



**Figure 3.6** A typical calibration curve for HPLC detection of propyl ester product

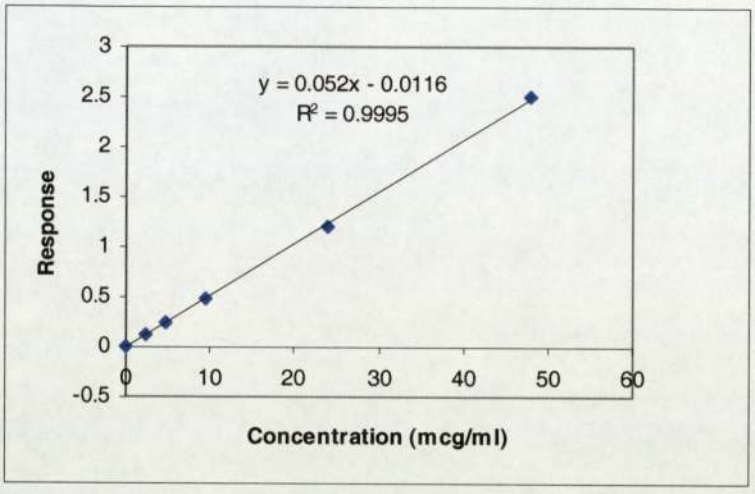



**Figure 3.7** A typical calibration curve for HPLC detection of butyl ester product

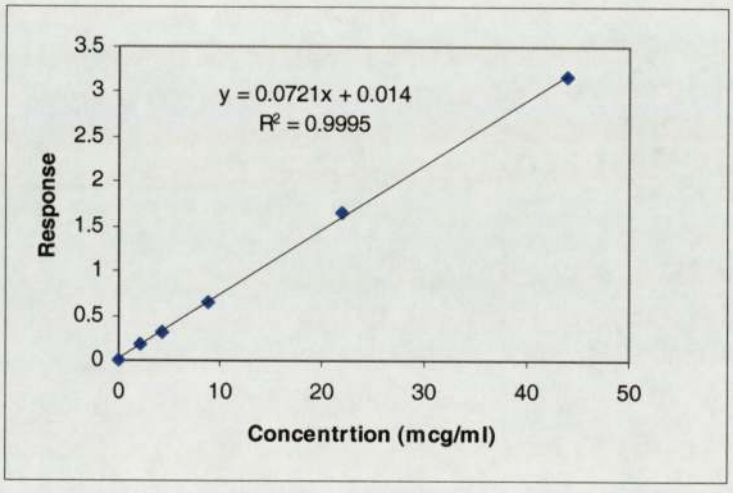



**Figure 3.8** A typical calibration curve for HPLC detection of hexyl ester product

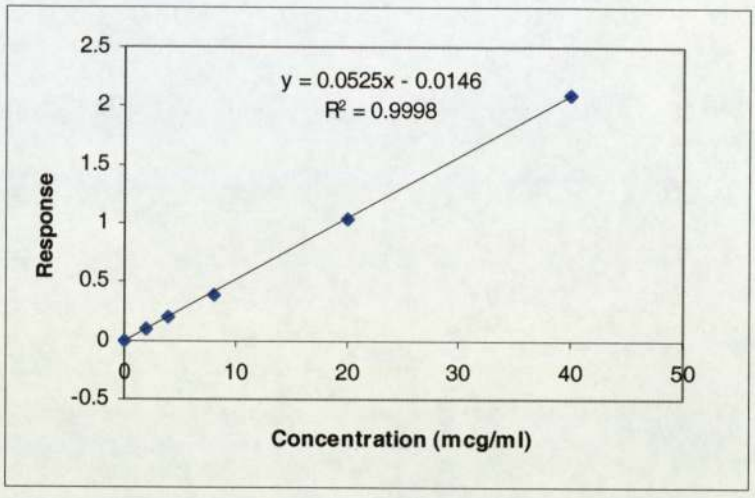



**Figure 3.9** A typical calibration curve for HPLC detection of octyl ester product

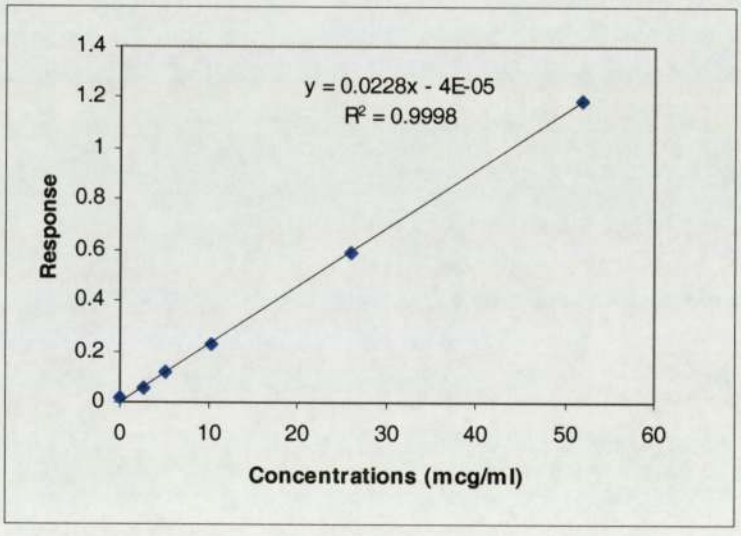



**Figure 3.10** A typical calibration curve for HPLC detection of isopropyl amide product

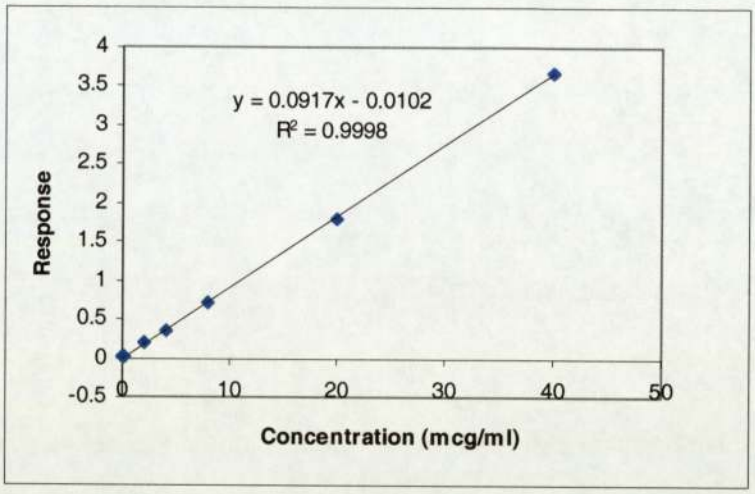



**Figure 3.11** A typical calibration curve for HPLC detection of n-butyl amide product

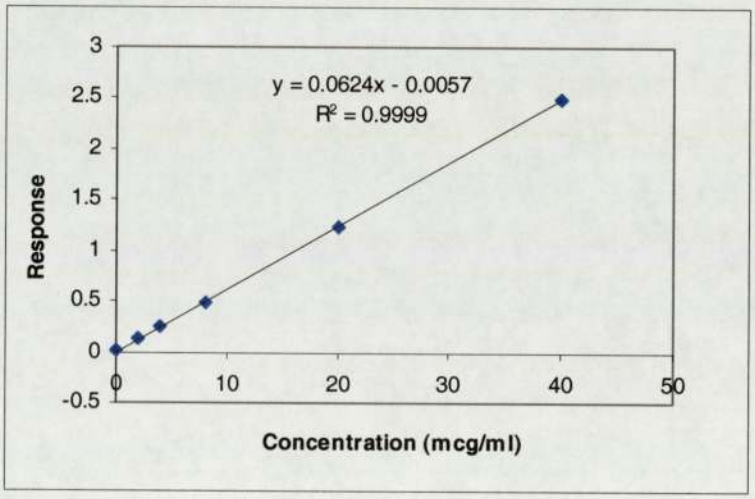



**Figure 3.12** A typical calibration curve for HPLC detection of s-butyl amide product

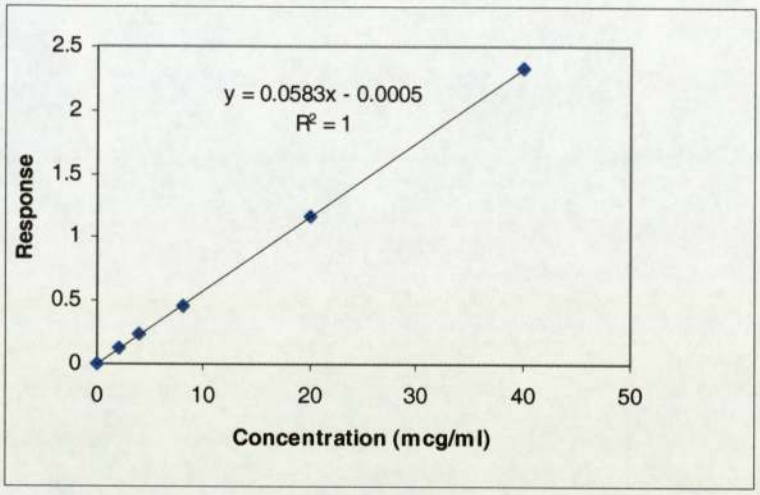



**Figure 3.13** A typical calibration curve for HPLC detection of t-butyl amide product

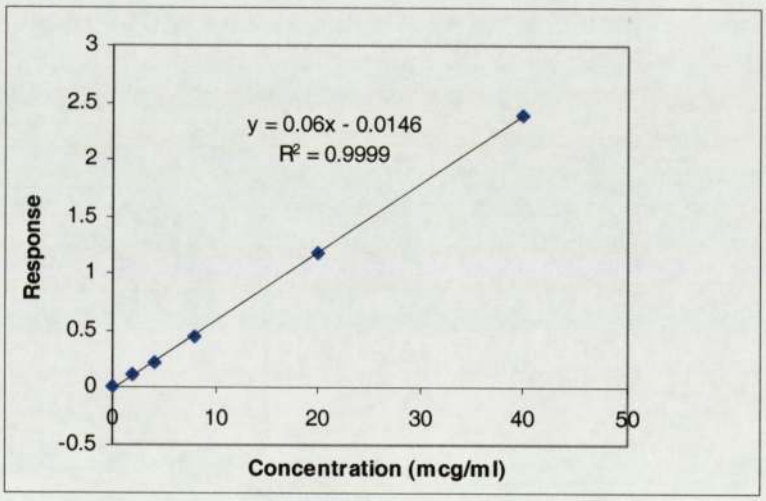



**Figure 3.14** A typical calibration curve for HPLC detection of temozolomide

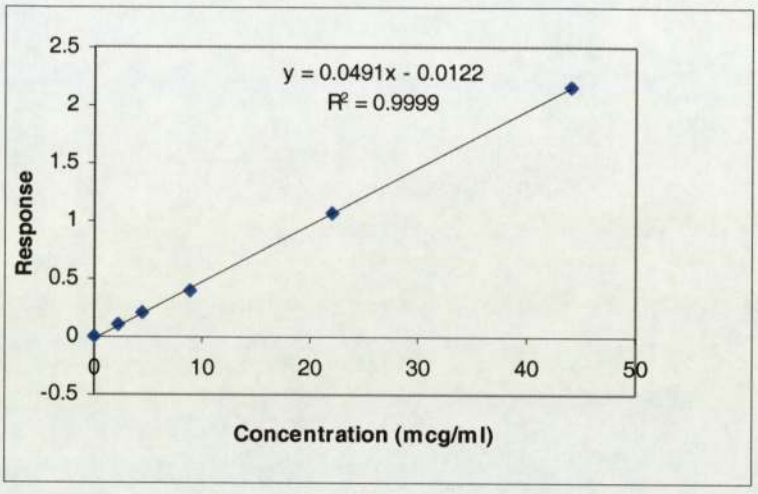



**Figure 3.15** A typical calibration curve for HPLC detection of temozolomide acid

A typical chromatogram of each sample compound with internal standard is shown in appendices 21-32.

## 3.6 Results and discussion

### 3.6.1 The selection of HPLC conditions

Table 3.2

| *Sample (S)* | *Internal standard (IS)* | *Mobile Phase* | *Retention time (mins) S / IS* |
|---|---|---|---|
| Temozolomide *1* | **Mitozolomide** | **0.5% AcOH :MeOH 90:10 v/v** | **5.5 / 16.5** |
| Temozolomide Acid *2* | | | **5 / 16.5** |
| Derivatives | **Anthracene** | **MeCN : water 70:30 v/v** | **2.1 / 7.5** |
| Methyl ester *4a* | | | |
| Ethyl ester *4b* | **Anthracene** | **MeCN : water 80:20 v/v** | **2.2 / 5** |
| Propyl ester *4c* | | | **2.3 / 5** |
| Butyl ester *4d* | | | **2.4 / 5** |
| Iso-propyl amine *3a* | | | **2.1 / 5** |
| N-butylamine *3b* | | | **2.2 / 5** |
| Sec-butylamine *3c* | | | **2.2 / 5** |
| T-butylamine *3d* | | | **2.2 / 5** |
| Hexyl ester *4e* | | | **2.8 / 5** |
| Octyl ester *4f* | | | **3.5 / 5** |

0.5% AcOH = 0.5% v/v of acetic acid in water MeCN = acetonitrile MeOH=methanol

To develop HPLC conditions for each sample compound, the trial and error approach, based on the principle theory of chromatography was employed to optimise HPLC separation and quantification. As previously stated (see section 3.3.1), for all of temozolomide derivatives, one reverse phase column was used, and varying the composition of mobile phase was performed to optimise peak resolution and retention time.

With regard to the HPLC conditions for temozolomide free drug form and, the mobile phase, which was 10% v/v methanol in 0.5% v/v acetic acid, was used to generate excellent chromatograms (Shen *et al.*, 1995).

Temozolomide free drug form (***1***) (see table 3.2) is insoluble in either aqueous or most of organic solvents (Rathbone, 1999). However, it can be readily dissolved in 0.5% v/v acetic acid, pH~5, (Shen *et al.*, 1995).

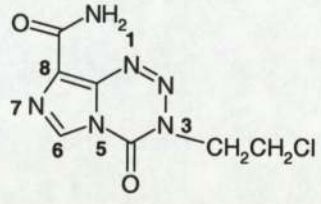
Amide and ester derivatives (***4a-f, 3a-d***) have higher lipophilic properties than (***1***). Therefore, in order to develop HPLC conditions for such derivatives, MeCN was introduced to the aqueous mobile phase. This would be advantageous because MeCN has more lipophilicity than MeOH and all of temozolomide derivatives can be decomposed in MeOH (Stevens, *et al.*, 1984).

As can be seen in table 3.2, introducing a large amount of MeCN (70-80%) to the aqueous mobile phase successfully generated excellent chromatograms of all

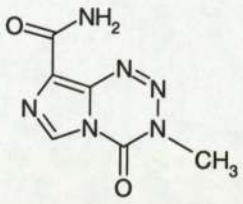
temozolomide derivatives. The retention time for each derivative was around 2 minutes. However, some exceptions are temozolomide hexyl (***4e***) and octyl ester (***4f***) products. Their retention times were obtained around 3 minutes. This may be because their lipophilic characteristics are significantly higher than those of other products. The highly lipophilic substances will have more affinity for interaction with the hydrophobic stationary phase resulting in eluting a slower elution.

The degradation profiles of ester products were also studied using HPLC conditions for temozolomide derivatives (80% v/v MeCN in water as mobile phase). In permeation tests (see section 5.2.3), ester products were completely hydrolysed and generated temozolomide acid, which was eluted from the column faster than ester products. The retention time for this metabolite was around 1 minute.

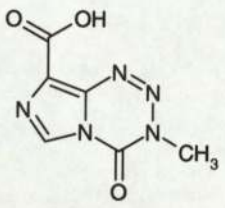
## 3.6.2 Selection of internal standards



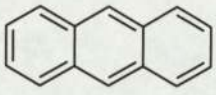
**Mitozolomide**



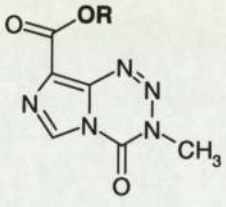
***(1)***



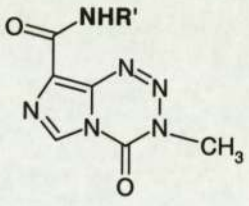
***(2)***



**Anthracene**



**ester derivative of (*1*)**



**amide derivative of (*1*)**

**Figure 3.16** The chemical structures of internal standards and compounds of interest

Mitozolomide, which is the predecessor of (*1*), was used as the internal standard in the HPLC measurement of (*1*) and (*2*). As can be seen in figure 3.16, the chemical structure of mitozolomide is relatively close to (*1*) and (*2*). Obviously, it has a UV absorbance in the same range as (*1*). However, the ethyl chloride group at position 3 (see figure 3.16) of mitozolomide confers a higher lipophilicity than (*1*) and (*2*). This lipophilicity difference results in a good resolution.

The diverse range of derivatives of (*1*) were tested using the HPLC conditions for ester and amide derivatives and were expected as internal standard to generate a good resolution. However, the chromatogram peaks for such agents (retention time~2 minutes) overlapped the chromatogram peaks of interest compounds. Finally, anthracene was successfully introduced as the internal standard conferring a good resolution. It is likely that it has a vast difference of character from temozolomide analogues. The fused three-membered ring with conjugated double bonds represents a highly lipophilic property. Anthracene was thus eluted from the column more slowly than temozolomide derivatives.

# Chapter 4

# *In vitro* esterase enzymes investigation

## 4.1 Introduction

Esterases are a diverse group of Phase I metabolising enzymes. These enzymes mediate reactions involved in the hydrolysis of acid esters to the free acid and alcohol. It has been established that the skin possesses considerable esterase activity (Hotchkiss, 1999), and this property has been exploited in the development of prodrugs that require activation by skin esterases (Higuchi and Yu, 1987). Prodrugs such as ester derivatives are generally highly lipophilic compounds compared to their predecessors. The hydrophobic prodrugs can readily partition into the skin, where they are metabolised by esterase enzymes, and transformed to the active free acid form. To ensure the existence of esterase enzymes within the skin, esterase enzyme inhibitor studies have been conducted to examine the effect on the hydrolysis of a salicylic acid diester (Hotchkiss, 1992). In the absence of inhibitors, the diester is completely hydrolysed to the monoester and finally to salicylic acid in mouse and human skin. The addition of inhibitors was seen to inactivate esterase enzyme activity within the skin.

Additionally, several permeation studies have demonstrated that many ester products such as corticosteroid (Higuchi and Yu, 1987), salicylate (Behrendt and Kampffmwyer, 1988), and oestradiol (Valia *et al.*, 1985) can be extensively hydrolysed, transformed into the free acid form, whenever there is an esterase enzyme within the skin.

Consequently, temozolomide ester products could be metabolised, and converted to temozolomide acid by esterase enzymes within the skin. To investigate this speculation,

an enzyme experiment was performed *in vitro*. A temozolomide amide derivative was also investigated to study the effect of esterase enzymes on this product.

## 4.2 Experimental

### 4.2.1 Materials and instrumentation

#### 4.2.1.1 Materials

Pig liver esterase (Sigma): suspension in 3.2 M ammonium sulphate solution pH 8; 200 unit/mg protein (14mg protein/ml)

Ammonium phosphate (Sigma-Aldrich Co. Ltd. UK)

Sodium phosphate (Sigma-Aldrich Co. Ltd. UK)

Sodium phosphate monobasic anhydrous (Sigma-Aldrich Co. Ltd. UK)

Temozolomide methyl ester (synthesised as outlined in section 2.2.3)

Temozolomide n-butyl amide (synthesised as outlined in section 2.2.3)

DMSO (Fisher Scientific Ltd. UK)

#### 4.2.2.2 Instrumentation

Bruker NMR AC 250 spectrometer

## 4.2.2 Preparation of solutions for enzyme study and diluted solutions

3.2M $(NH_4)_2SO_4$ solution: $(NH_4)_2SO_4$ (8.46g) was dissolved in 20 ml distilled water.

0.5M phosphate buffer solution pH 7.4: $Na_2HPO_4$ (205mg) and $NaH_2PO_4$ (169 mg) were dissolved in 5ml deuteriated water ($D_2O$)

$(NH_4)_2SO_4$ diluted solution: 3.2M $(NH_4)_2SO_4$ solution (300µl) was added to 0.5ml buffer solution

Enzyme diluted solution: (300µl) esterase enzyme was added to 0.5ml buffer.

## 4.2.3 Incubation method

Temozolomide methyl ester and the n-butyl amide derivative were chosen to investigate the degradation profiles of ester and amide derivatives respectively.

(a) Enzyme experiment: Temozolomide methyl ester or n-butyl amide (2mg) was dissolved in a small amount of DMSO in a NMR tube, and then 0.5ml buffer solution was added. Finally, 50µl of the enzyme solution was added into the mixture solution. The tube was incubated at 37°C in a water bath.

**(b)** Control experiment: Temozolomide methyl ester or n-butyl amide (2mg) was dissolved in a small amount of DMSO in an NMR tube, and then 0.5ml buffer

solution was added. Finally, 50µl of the $(NH_4)_2SO_4$ diluted solution was added into the mixture solution. The tube was incubated at 37°C in a water bath.

Periodically (every 5 minutes) the tubes were removed from the water bath and [$^1$H]-NMR spectra were obtained.

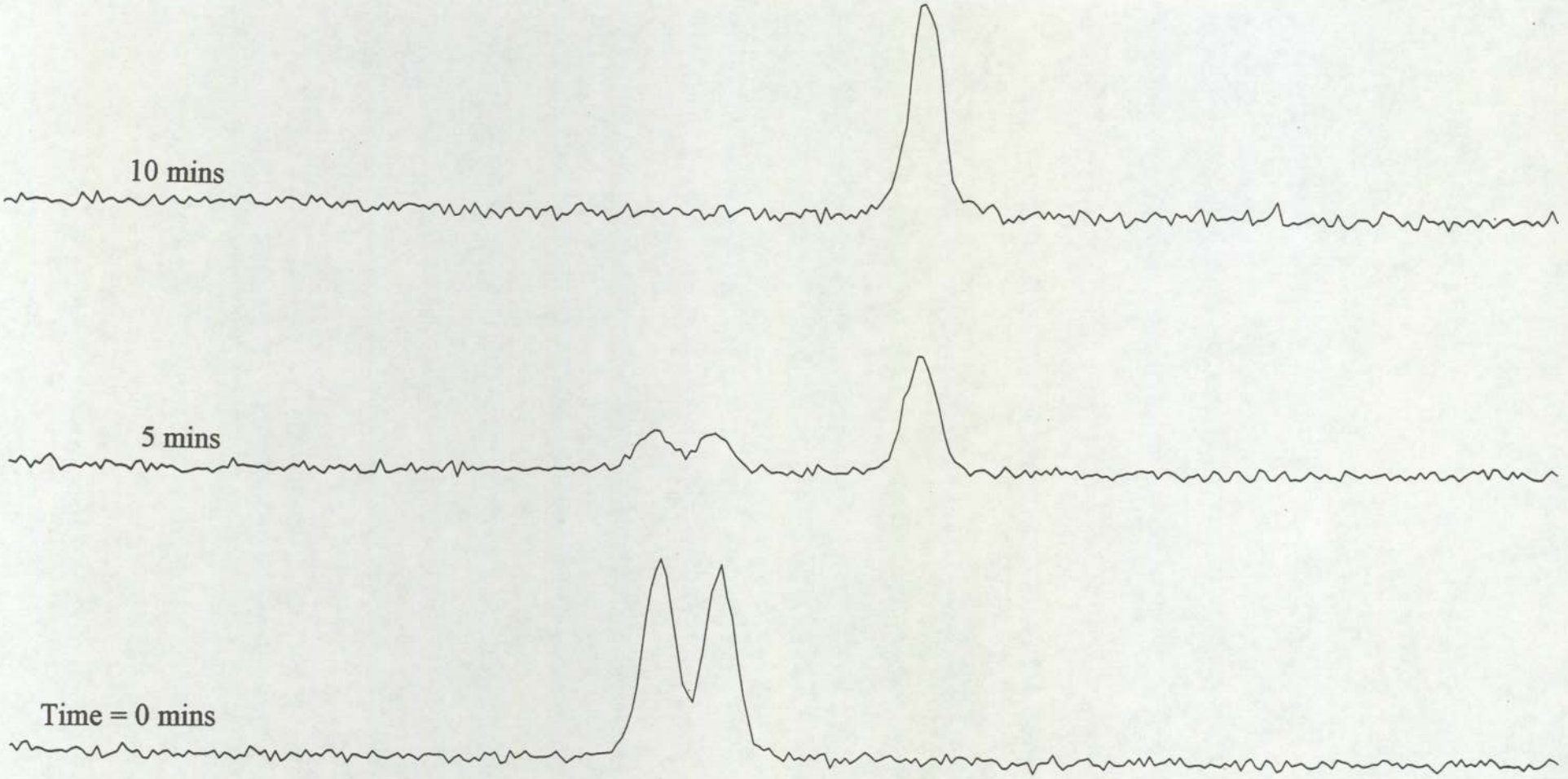
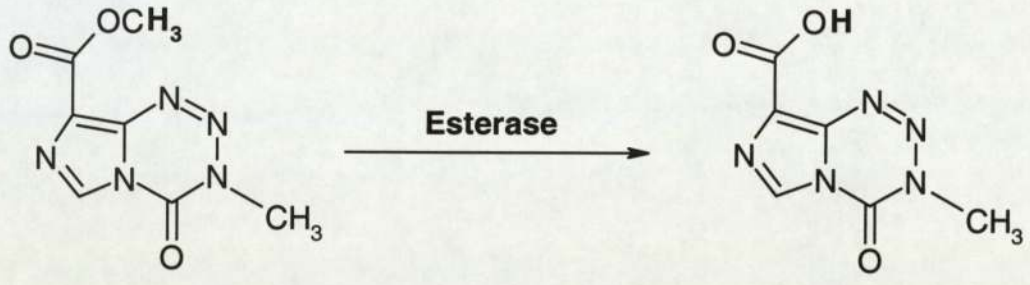



FIGURE 4.1 THE DEGRADATION PROFILE OF METHYL ESTER BY ESTERASE ENZYME

## 4.3 Results and discussion




**Temozolomide methyl ester** **Temozolomide acid**

**Scheme 4.1** The degradation mechanism of temozolomide ester product by esterase enzyme

Figure 4.1 shows the [$^1$H]-NMR of the degradation profile of methyl ester product. At time 0 mins, the resonance frequency of the two singlets (at 3.63 and 3.65 ppm) which are responsible for the two methyl groups of the methyl ester molecule, suggests the presence of methyl ester product. After 5 mins, the singlet (at 3.58 ppm) which corresponds to the methyl group of the temozolomide acid molecule indicates the presence of temozolomide acid, while, the two singlets of methyl ester product were gradually reducing. Finally, at time 10 mins, the methyl ester product had been eliminated. Hence, only the singlet of temozolomide acid remained. This result clearly demonstrates that methyl ester had completely degraded releasing temozolomide acid when exposed to pig liver esterase enzyme (see scheme 4.1).

However, the n-butyl amide product gradually decomposed without the observation of temozolomide acid after 8 hours incubation (results not shown). This is probably due to the high stability of amide product to esterase enzymes.

# Chapter 5

# Skin delivery study

## 5.1 *In vitro* permeability test

### 5.1.1 Introduction

*In vitro* techniques are widely used in the assessment of percutaneous and transdermal absorption particularly for potentially toxic chemicals which cannot be tested in humans. The major advantage of *in vitro* investigations in this area is that the experimental conditions can be controlled precisely, and the only variables are the skin and test materials. In addition, absorption rates and skin metabolism can be measured more accurately in an *in vitro* system since sampling is performed directly beneath the barrier layer. Skin metabolism can also be studied in viable skin without interference from metabolic systemic processes. Finally, absorption measurements are more easily obtained from diffusion cells than from analysis of biological specimens from clinical studies. Thus, they can be the fundamental data used to precisely predict the permeability characteristics of the samples or to select the compounds to be tested in an *in vivo* system.

Numerous experiments demonstrate a good correlation between *in vitro* and *in vivo* percutaneous absorption. For example, a recent review by Bronaugh and Maibach (1987) details experiments reporting a similarity between *in vivo* and *in vitro* absorption measurement with tributyl phosphate in rat, rabbit, and pig skin.

Ideally, human skin should be used in such investigations in order to provide as good representation as possible of an *in vivo* study. However, human skin is not easily

obtainable. Moreover, the great variability in several parameters such as age, site, hairiness, thickness, and condition may present the difficulty in interpreting the results (Brain *et al.*, 1998; Wester and Maibach, 1999). Therefore, in this study, silicone membrane and full-thickness rat skin were used for investigating the permeation profile of all samples. However, for comparative purposes temozolomide free drug and some of its derivatives were repeated in human skin.

Silicone membrane is a purely lipophilic membrane, which behaves as the skin principal barrier (stratum corneum). The advantage of using a synthetic membrane is that it provides a less variable system leading to providing an excellent preliminary interpretation of results.

Animal skins are widely used because they are easier to obtain than human, and less variable in most parameters. Notwithstanding, animal skins are more permeable than human skin, and their barrier properties are different among animal species (Bronaugh, 1998; Bronaugh, *et al.*, 1999).

### 5.1.1.1 *In vitro* skin diffusion cells

The most common technique for evaluation of *in vitro* percutaneous penetration uses diffusion cells. There are different designs of diffusion cells, which have been used for percutaneous absorption studies. However, there are only two basic types (Bronaugh, 1996): the one-chambered and the two-chambered cell. The several kinds of two-

chambered cells have been widely used for many years to create conditions in which the permeant diffuses from the vehicle in donor chamber on one side of membrane to the other side (receptor chamber) that contains receptor fluid. Normally, an infinite dose (*i.e.* one is that large enough to maintain constant concentration during the course of experiment) is added to one side of membrane and its rate of diffusion across the concentration gradient into a receiver on the opposite side is determined. The two-chambered cell is applicable to the measurement of absorption of compounds that are applied to skin at infinite dose (*i.e.* saturated solutions or transdermal delivery devices) leading to producing a steady rate of delivery. The one-chambered cell has a chamber beneath the skin, which is allowed to be exposed to the environment above. Some substances are intentionally applied to the skin in creams or lotions. The amount of penetrating substance on the skin is relatively small, thereby, a steady-state rate of absorption is not attained. In these samples, absorption of the chemical can be only studied in a one-chamber cell. The surface of the skin in this cell type is exposed to the environment, thus thin layers of material can be applied in vehicles relevant to *in vivo* exposure. The skin is not excessively hydrated by continued exposure to an aqueous solution as in the two-chambered cell. The chamber under the skin serves as the container for the receptor fluid, which is taken off to determine the absorption rates of permeant.

### 5.1.1.2 Receptor fluid

The receptor chamber dimensions are constrained by the requirement of guaranteeing that the receptor phase can act as a sink throughout an experiment. As a general rule, the concentration of the permeant in the receptor fluid should not be allowed to exceed ~10% of the concentration of donor solution (Brain *et al.*, 1998) because excessive receptor phase concentration leads to a decrease in the rate of absorption.

Receptor fluids that can be used range from water alone to isotonic phosphate buffer solution. Occasionally, for some test compounds, which have high lipophilicity (water solubility < 10 μg/ml), solubilising agents may be introduced to the receptor fluid so that skin absorption can be more easily determined by simply sampling the receptor solution. However, it is important to consider the possibility that the solubilizers may damage the barrier properties of the skin (Bronaugh *et al.*, 1999).

### 5.1.1.3 Application of permeant

Topical formulations of a substance applied to the skin surface can affect its permeation. Several factors should be considered in selecting a suitable application procedure including the nature of vehicle, the permeant concentration, topical dosage forms (*i.e.* ointments, creams, and lotions) and the exposure time. In this study, to facilitate delivery of drug molecules, solutions of sample compounds were prepared using 10% v/v propylene glycol in water as a solvent. Propylene glycol (PG) is usually added to topical

formulations (from 5-15%) to increase the solubility of lipid-like materials (usually active ingredients) (Flynn, 1995). PG itself can penetrate *via* the intercellular lipid and lead to expansion in the lipid multilayers. However, at the lower levels of the stratum corneum, the lipid domains are only disrupted focally (Menon *et al.*, 1998). Additionally, enhancing effects of PG are only apparent at high concentrations (Barry, 1983).

## 5.2 Materials and methods

### 5.2.1 Materials

Temozolomide free drug, temozolomide ester homologous series (***4a-f***), and temozolomide amide derivatives (***3a-d***) were studied.

### 5.2.2 Solubility determination

The sample solutions that were used as donor phases should be saturated in order to maintain high concentration gradients leading to optimisation of the rate of absorption.

Due to limitations in the availability of temozolomide and its synthesised derivatives, solubility was determined on a small sample of each in order to minimise loss of ingredients.

An excessive amount of each sample (5-10mg) was stirred in 1 ml of 10% v/v propylene glycol in water, at ambient conditions, for about 4-5 hours, and then the suspension was allowed to stand for 2-3 hours. The supernatant was removed and the concentration of the sample was assayed by using HPLC.

### 5.2.3 Permeation procedure

#### 5.2.3.1 Preparation of membrane barriers

##### 5.2.3.1.1 Silicone membrane

Synthetic silicone membrane, which was 0.05 inches thick, was cut to fit to the size of the diffusion cell cap, and was thoroughly washed before use with distilled water.

##### 5.2.3.1.2 Rat skin

Full-thickness skin was obtained from male Wistar rats, aged 10 to 12 weeks and weighing 250 to 300g. The animals were sacrificed by cervical dislocation and the abdominal region of each rat was carefully shaved using electric clippers. The whole-thickness intact skin was excised using a sharp pair of scissors and then the underlying connective tissue and fat tissue were removed from the undersurface. The skin was cut into a small piece fitting to the size of diffusion cell cap, and it was used immediately.

#### 5.2.3.1.3 Human skin

The whole epidermis of human skin was purchased from The Stephen Kirby Skin Bank. The donor was a 72-year-old Caucasian man. The skin was obtained from the legs, and it was stored at –80°C before using within 1 week. Before using, the skin was defrosted thoroughly at room temperature and then it was cut into a small piece fitting to the size diffusion cell cap, and was used immediately.

### 5.2.3.2 Preparation of receptor phase solution

Double distilled water was boiled to get rid of the air bubbles and then it was allowed to cool down to room temperature. Finally, it was sonicated to completely de-gas.

### 5.2.3.3 Preparation of diffusion cell

The membrane (as detailed above) was mounted on the diffusion cell, on which the surface area available for diffusion of each cell was about 2.8-3.4 $cm^2$. The cell cap was placed on the membrane, and it was sealed with Parafilm® connecting the cell cap, membrane, and receptor cell body (see figure 5.1). The capacity of each receptor cell was around 25-26 ml, which was filled with receptor solution, which was completely in contact with the membrane sheet. During the course of experiment, the solution was continuously stirred by a teflon-coated magnetic bar placed in the cell. The temperature was maintained at 37°C by a thermostatic water pump that circulated the water through

the jacket surrounding the cell body. The system was allowed to equilibrate under these conditions for half an hour. Finally, the prepared sample solution (2ml) was added into the donor cap cell, and it was sealed with Parafilm® to minimise the evaporation of donor solution. 1ml of receptor solution was removed every 30 minutes for 5 hours and then every 60 minutes for a further 3 hours. The volumes withdrawn were always replaced with equal volumes of fresh receptor solution to maintain constant volume. The concentration of each sample solution was determined by using HPLC.

For every sample, six determinations were performed on human and rat skin, and three determinations were carried out through artificial silicone membrane. The mean values were used for correlation and comparison purposes.

As stated above, after the removal of receptor solution, the receiver compartment was refilled with an identical volume of receptor fluid. This causes of the dilution of receptor solution leading to an under determination of the sample concentration. As a consequence, it was necessary to mathematically correct each successive sample concentrations, which was determined by applying the following equation:

$$C_t = C_{mt} + \left[ V_s . \frac{\sum_{t=1}^{t=n-1} C_m}{V_r} \right] \qquad (5.1)$$

where $C_t$ is the actual current concentration of drug in the receptor phase at time t, $C_{mt}$ is the apparent (*i.e.* measured) current concentration of drug in the receptor phase, $V_s$ is the sample volume withdrawn for analysis, $V_r$ is the volume of receptor solution, and $\Sigma C_m$ is the summed total of the previous measured concentrations.

Finally, the amount of drug penetrating the skin *per* unit area was calculated by dividing the obtained concentrations by the surface area available for diffusion cell and this value was different for each individual cell. In this study, results were expressed in term of % cumulative amount permeated (μg for permeation test through silicone membrane, and μ mol for rat and human skin) *per* square centimetre of surface area of diffusion cell ($cm^2$) (Bodor and Sloan, 1982).

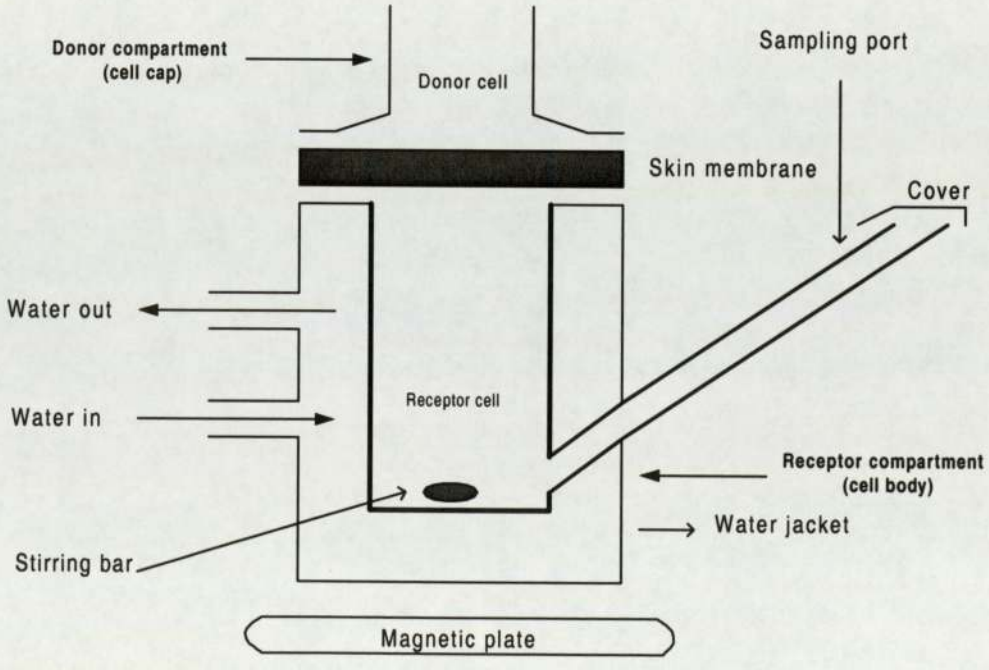



**Figure 5.1** Schematic illustration of a diffusion cell

## 5.3 Results and discussion

### 5.3.1 Solubility of temozolomide and its derivatives in 10% v/v propylene glycol

**Table 5.1** Solubility of temozolomide and its derivatives in 10% v/v propylene glycol under ambient conditions

| Compound | Concentration mg/ml |
|---|---|
| Temozolomide free drug | 3.0 |
| **Ester products** | |
| Methyl ester | 3.0 |
| Ethyl ester | 2.5 |
| Propyl ester | 2.0 |
| Butyl ester | 1.4 |
| Hexyl ester | 0.4 |
| Octyl ester | 0.3 |
| **Amide products** | |
| Isopropyl amide | 2.8 |
| N-butyl amide | 1.8 |
| Sec-butyl amide | 1.8 |
| T-butyl amide | 1.4 |

Table 5.1 represents the solubility of temozolomide and its derivatives in 10% v/v propylene glycol in water. As can be seen, with the exception of methyl ester, the solubility of the homologous series of ester products ($C_2$-$C_8$) decreases significantly in aqueous solution as the number of $CH_2$ groups in molecule increased. The aqueous solubility of octyl ester is the lowest, around 10 times lower than free temozolomide. As expected, this is largely attributed to an increase in lipophilicity of temozolomide as the length of the alkyl chain increased. The solubility of butyl amide products (N-, Sec-, and T-butyl amides) in the aqueous solution is considerably lower than free temozolomide. As is also the case with ester derivatives, attaching alkyl groups to the parent molecule is primarily the cause of the increase in lipophilicity. However, the isopropyl amide derivative possesses only slightly lower aqueous solubility than free temozolomide.

### 5.3.2 Permeation of temozolomide and its ester products through silicone membrane

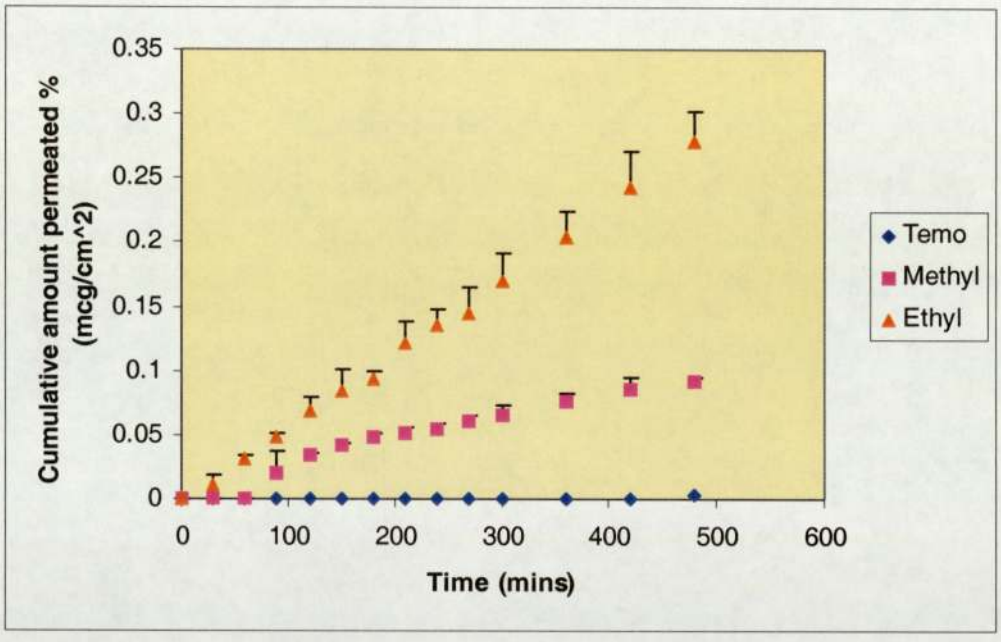



**Figure 5.2** Permeation of temozolomide, methyl and ethyl ester products through synthetic silicone membrane (n=3, mean +s.d.)

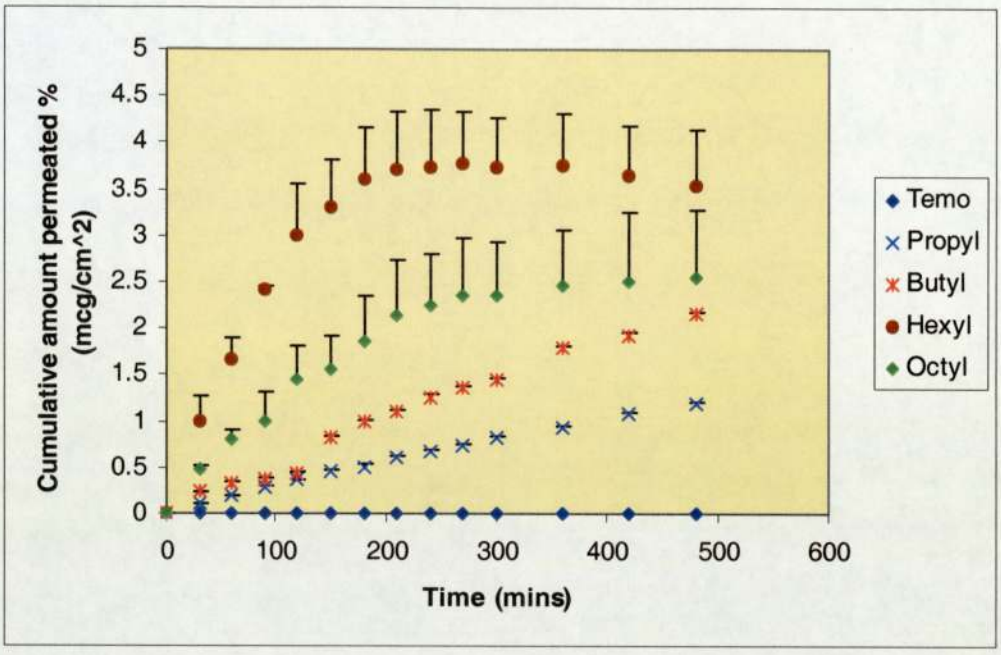



**Figure 5.3** Permeation of temozolomide, propyl, butyl, hexyl, and octyl ester products through synthetic silicone membrane (n=3, mean +s.d.)

Figures 5.2 and 5.3 show the permeability characteristics of temozolomide and a homologous series of its ester products *via* purely lipophilic silicone membrane. As can be seen, free temozolomide, methyl and ethyl esters could not pass easily through the synthetic membrane (see figure 5.2). Conversely, propyl, butyl, hexyl, and octyl esters passed through the membrane far more easily (see figure 5.3). These results can be explained by considering the lipophilicity of each compound. Increasing the chain length of the hydrocarbon group is the likely cause of increased permeation through the membrane. However, one exception was octyl ester. Its permeability was significantly

less than hexyl ester. This can be described by considering the size and the interaction with the membrane of the compounds. For small size species (molecular weight <1000 daltons), the effect of size on the diffusion (D) in liquids may be viewed in terms of the following Stokes-Einstein equation in which M is molecular weight and C is constant.

$$D = C.M^{-1/3} \tag{5.2}$$

From equation 5.2, it is clear that higher molecular weight leads to lower diffusion of molecules. Therefore, the octyl ester product may not easily diffuse across the membrane because it has higher molecular weight. However, for the rest of the ester products, molecular weight seems to have less of an effect. In addition, octyl ester may have high affinity for the lipophilic silicone membrane resulting in lower permeation.

This result indicates that there is an optimum lipophilicity for permeation at the $C_6$ hydrocarbon group. Temozolomide ester derivatives, which have lipophilicity higher or lower than this optimum value, will permeate through the membrane with more difficulty. Similar conclusions can be deduced from some results in the literature. The optimum flux ($J_s$) of alkanols in aqueous solution across an isolated epidermis was found at hexanol ($C_6$) (Chien, 1992). Diez-Sales *et al.* (1993) also reported that the optimal permeability coefficient value for aniline corresponds to 4-*n*-butyl aniline through full-thickness rat skin. Recently, the permeation study through epidermal rat skin of phenol compounds indicates that there is an optimum permeability coefficient value at 5-phenyl pentanol (Lopez *et al.*, 1998).

### 5.3.3 Permeation of temozolomide and its amide products through silicone membrane

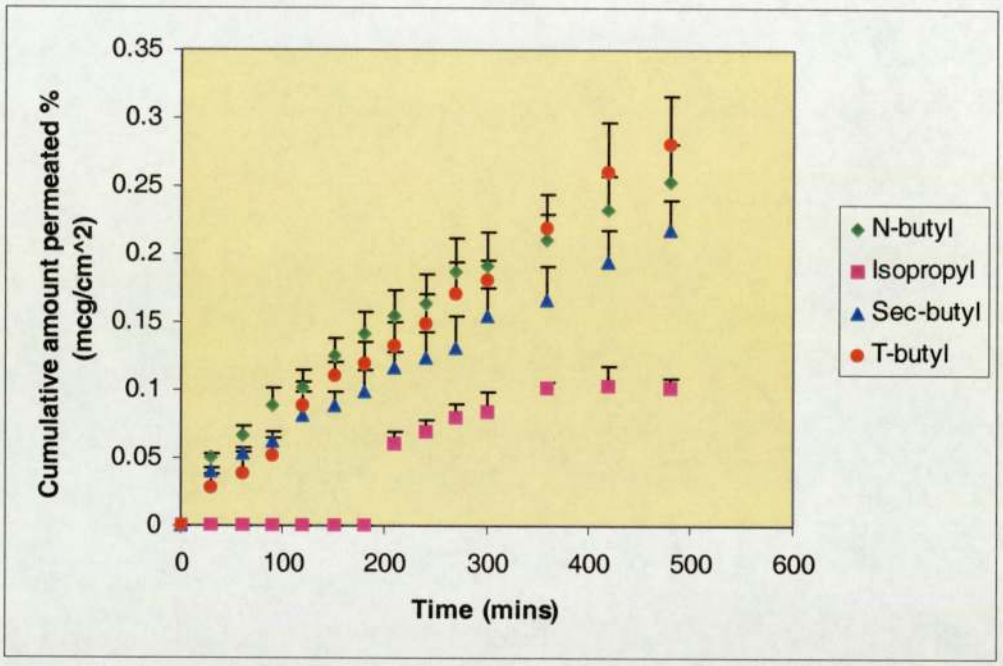



**Figure 5.4** Permeation of temozolomide and its amide products through synthetic silicone membrane (n=3, mean +s.d.)

Figure 5.4 depicts the permeability characteristics of temozolomide and its amide products *via* the synthetic silicone membrane. As revealed by this figure, the permeation of temozolomide was the lowest, and the permeation of isopropyl amide product was inferior to butyl amide products. As expected, the longer carbon chain length is principally responsible for the higher permeation. Based on the solubility data (see section 5.3.1), it can be seen that butyl amide products are more lipophilic than the

isopropyl amide product. As a result, these derivatives might penetrate the lipophilic silicone membrane more readily. The difference in constitutional isomers (straight and branched chains) of butyl amide products was also investigated and was expected to indicate differences in permeation. Butane and isobutane are different compounds with different properties. For example, the boiling point of butane is –0.5 °C, whereas that of isobutane is –11.7 °C (Loudon, 1995). However, they have the same molecular formula, $C_4H_{10}$. Therefore, attaching different constitutional isomers to the parent molecule is likely to indicate different properties. However, the results suggest that the differences in constitutional isomers do not significantly change the permeability characteristics. This probably indicates that the differences of constitutional isomers do not alter the lipophilic properties of these compounds.

### 5.3.4 Permeation of temozolomide and its ester products through full-thickness rat skin

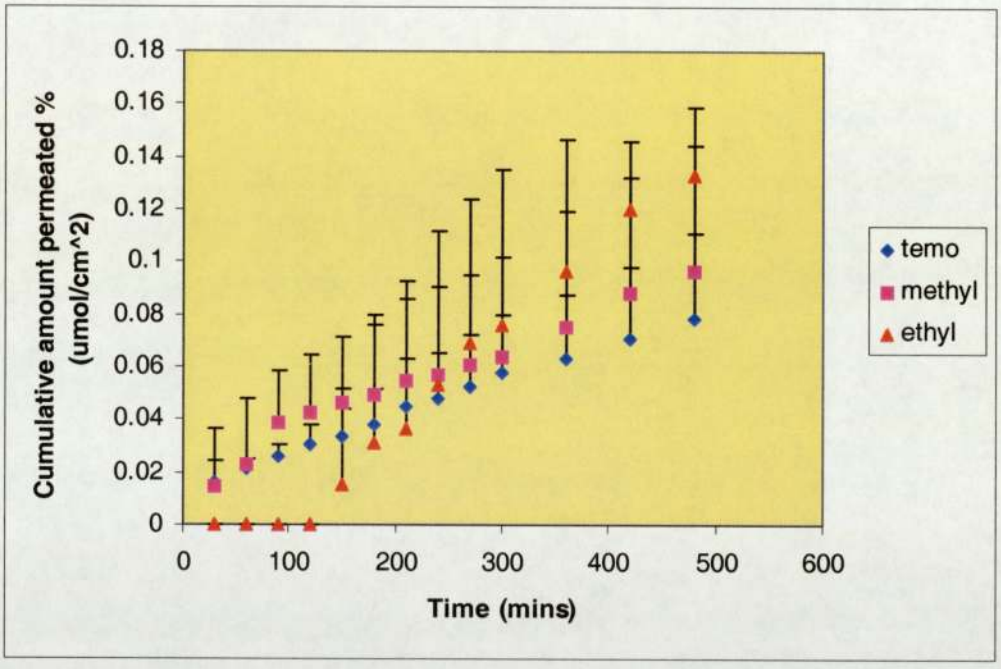



**Figure 5.5** Permeation of temozolomide, methyl and ethyl ester products through full-thickness rat skin (n=6, mean +s.d.)

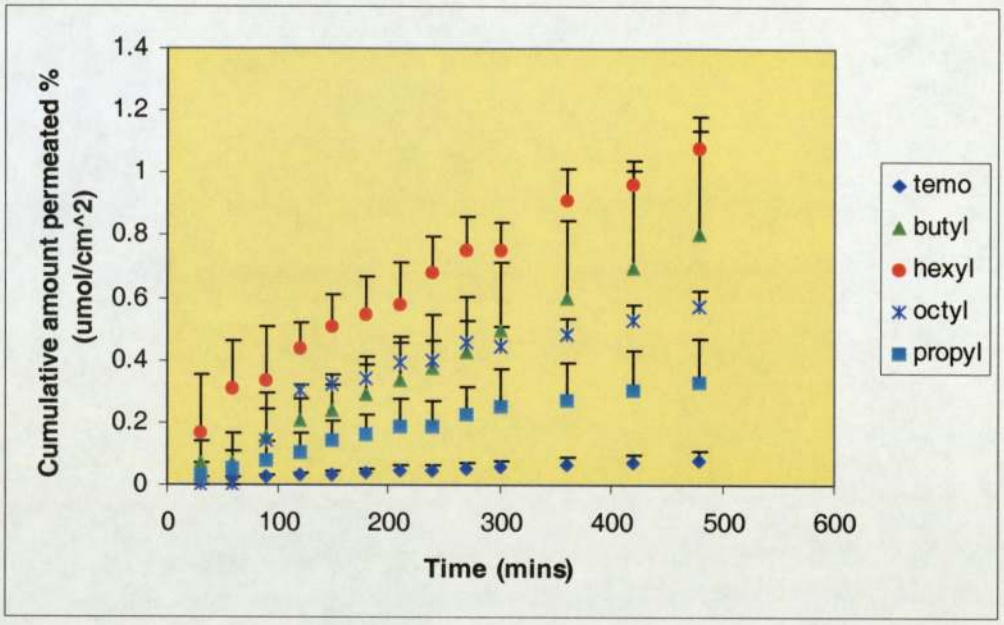



**Figure 5.6** Permeation of temozolomide, propyl, butyl, hexyl and octyl ester products through full-thickness rat skin (n=6, mean +s.d.)

Figures 5.5 and 5.6 illustrate the permeability characteristics of temozolomide and its ester derivatives through whole rat skin. As can be seen, the permeation profiles of all compounds were similar to those of the experiment *via* silicone membrane. Clearly, the presence of -$CH_2$- groups from 3 to 8 on the temozolomide molecule caused increased permeation of this agent through the skin (see figure 5.6). As the alkyl chain length increased, the cumulative amount permeated (%) increased to a maximum value at n=6 (hexyl ester, see figure 5.7). These results indicate that the balance exists between the permeability and lipophilicity with a maximum amount permeated of hexyl ester products. As expected, all ester products were extensively transformed into temozolomide free acid form. This finding demonstrates that ester derivatives are

metabolised by esterase enzymes existing within the skin. This is similar to many corticosteroid drugs (*i.e.* betamethasone, prednisolone, and triamsinolone), animal and human skin rapidly hydrolyse corticosteroid ester to the free corticosteroid acid (Hotchkiss, 1998).

Permeability studies of amide derivatives were also tested through rat skin. However, these compounds degraded during the experiment. There were two overlapped peaks displayed on the chromatogram. The receptor solution was then examined using HPLC condition for temozolomide acid. There was no temozolomide acid compound detected over 8 hr. This seems that amide products undergo bioconversion (*i.e.* by hydrolytic or enzymatic transformation) within viable rat skin and transform to a metabolite that is not temozolomide acid.

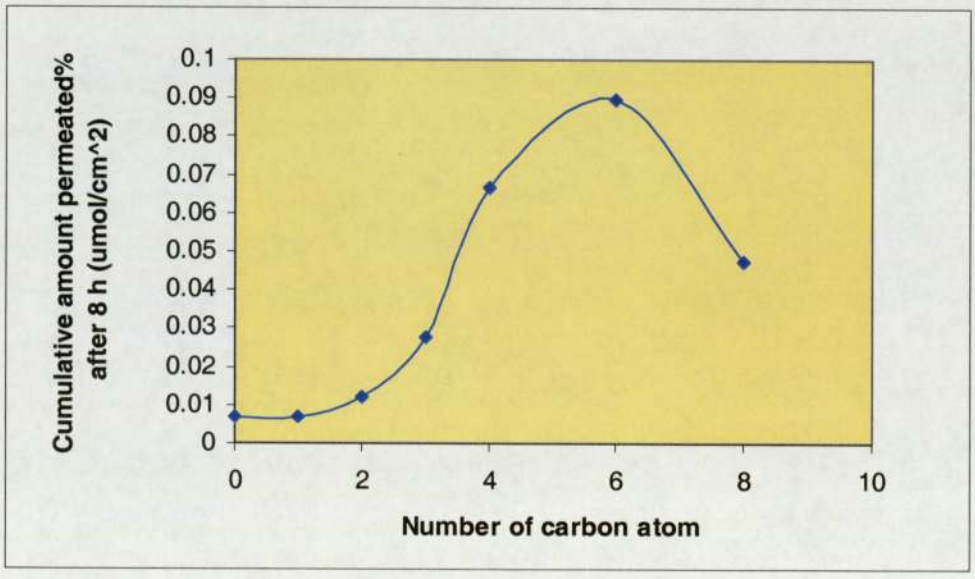



**Figure 5.7** Dependence of cumulative amount permeated % after 8 h across the full-thickness rat skin on the alkyl chain length of temozolomide ester products.

### 5.3.5 Permeation of temozolomide, hexyl ester, and n-butyl amide through human skin

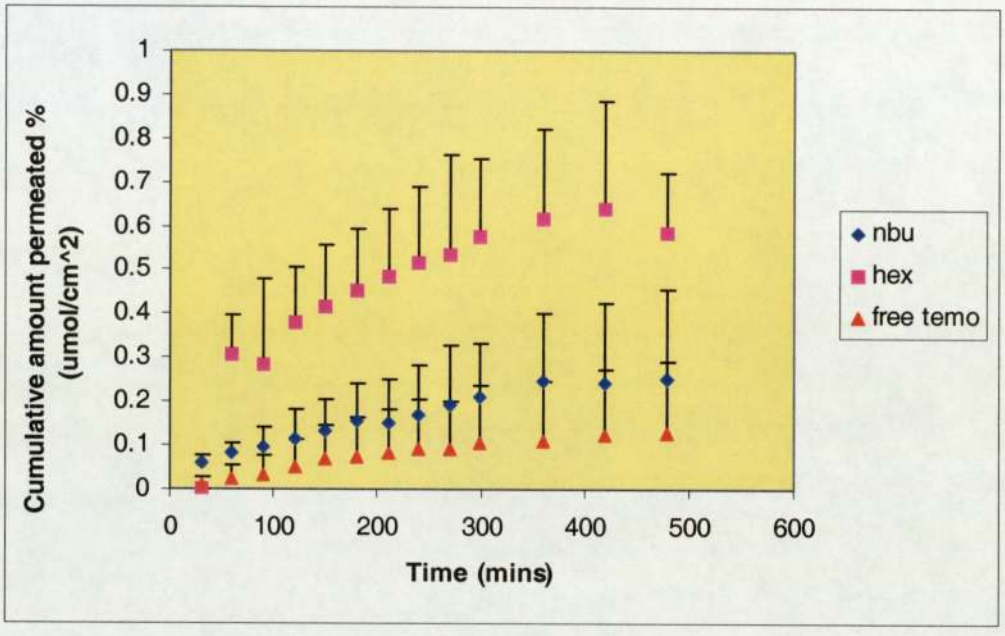



**Figure 5.8** Permeation of temozolomide, hexyl ester, and n-butyl amide through human skin (n=6, mean + s.d.)

Figure 5.8 shows the permeability profiles of temozolomide free drug *versus* its hexyl ester and n-butyl amide lipophilic derivatives. Temozolomide hexyl ester was chosen due to its greatest permeability through rat skin (see section 5.3.4). The straight chain butyl amide product (n-butyl amide) was also selected in order to compare its permeability with the straight chain hexyl ester product. It is clear that the permeation of both ester and amide products notably increased compared with temozolomide in the free drug form. Obviously, attaching hydrocarbon chain on temozolomide molecule to increase its lipophilicity is the reason for the rise of the permeation. Similarly to rat skin as described

in section 5.3.4, the hexyl ester derivative was completely metabolised, converted to temozolomide acid by esterase enzyme within the skin. However, the n-butyl amide product was resistant to metabolism by this enzyme.

# Chapter 6

# Conclusions

Temozolomide ester and amide derivatives were successfully synthesised using Pybrop® and DMAP as coupling agents.

To develop HPLC conditions for each derivative, the trial and error approach, based on the principle theory of chromatography was employed to optimise HPLC separation and quantification. Varying compositions of acetonitrile and water was performed to optimise peak resolution and retention time.

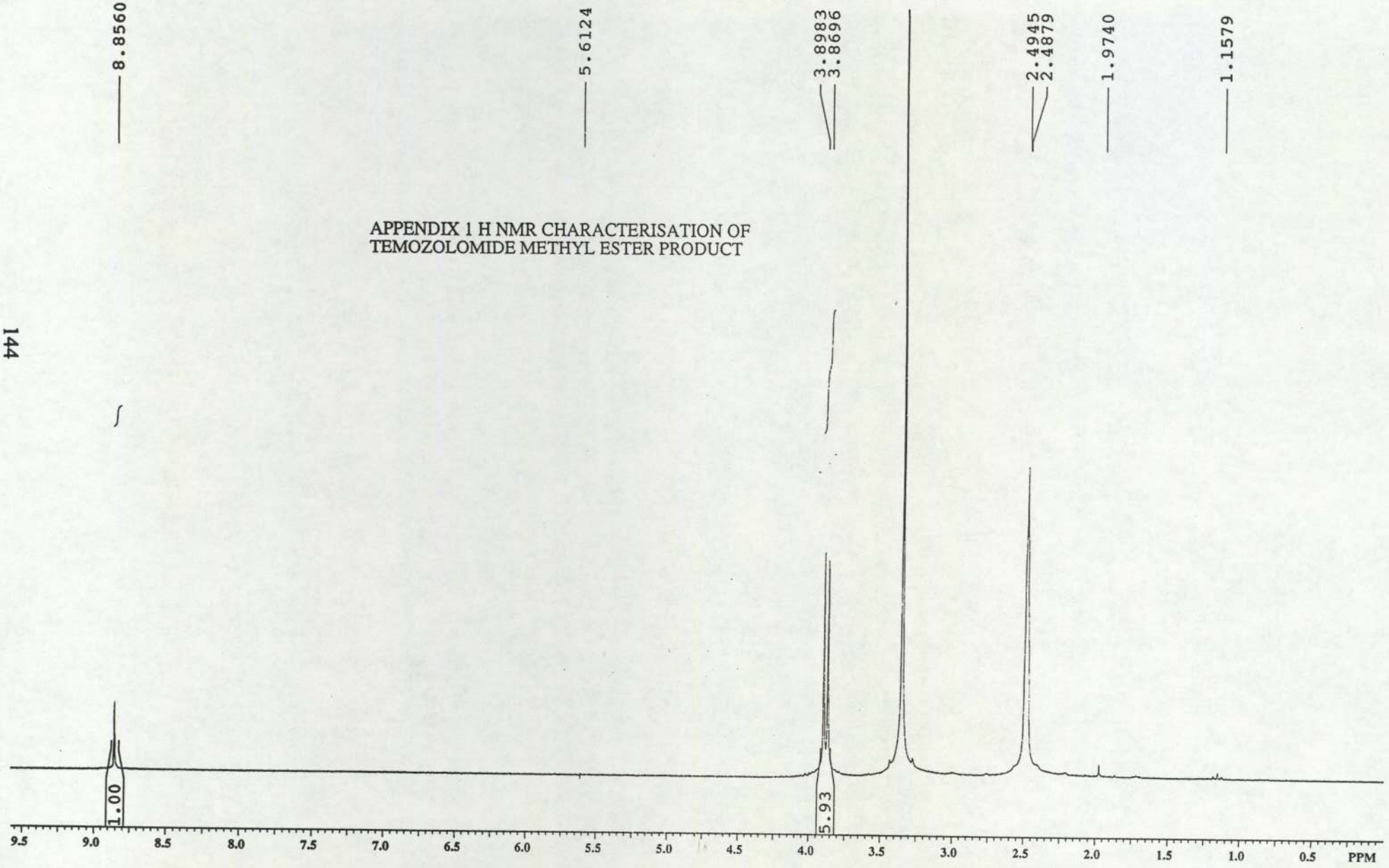
*In vitro* enzyme experiments were performed on methyl ester and butyl amide products. Methyl ester products had completely degraded, releasing temozolomide acid when exposed to pig liver esterase enzyme. However, the butyl amide product was resistant to metabolism by this enzyme.

In this study, it is clear that the permeation of both ester and amide products increased compared with temozolomide in the free drug form. Obviously, attaching a hydrocarbon chain on the temozolomide molecule to increase its lipophilicity is the reason for the increase in the permeation. Similarly to many corticosteroid drugs, the ester derivatives were completely metabolised and converted to temozolomide acid by esterase enzyme within viable rat and human skin. However, n-butyl amide product was resistant to metabolism by this enzyme.

The hexyl ester derivative demonstrated the greatest permeability through rat and human skin. This compound is thus the promising prodrug to be tested in an *in vivo* system.

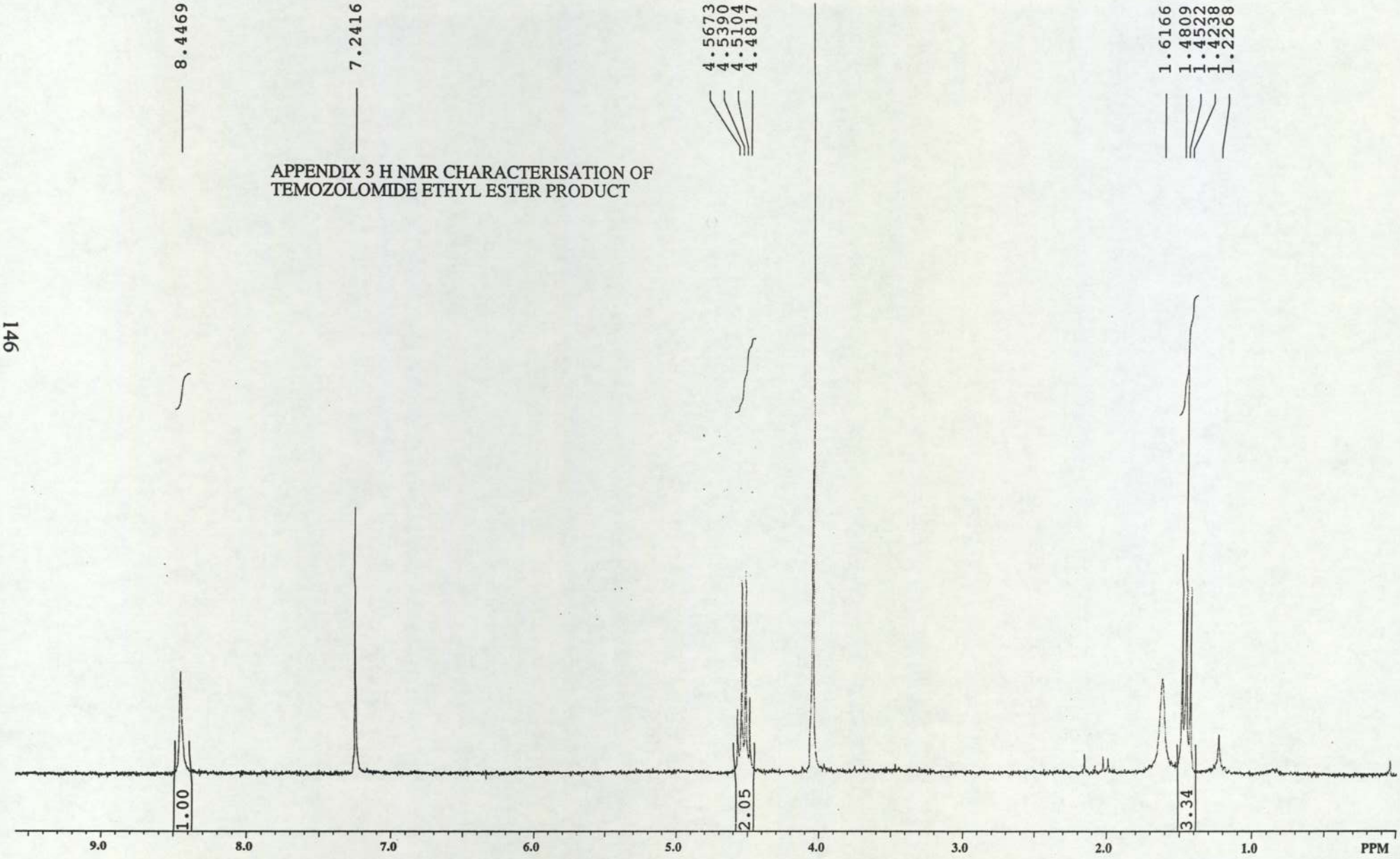
# Appendices

APPENDIX 1 H NMR CHARACTERISATION OF
TEMOZOLOMIDE METHYL ESTER PRODUCT

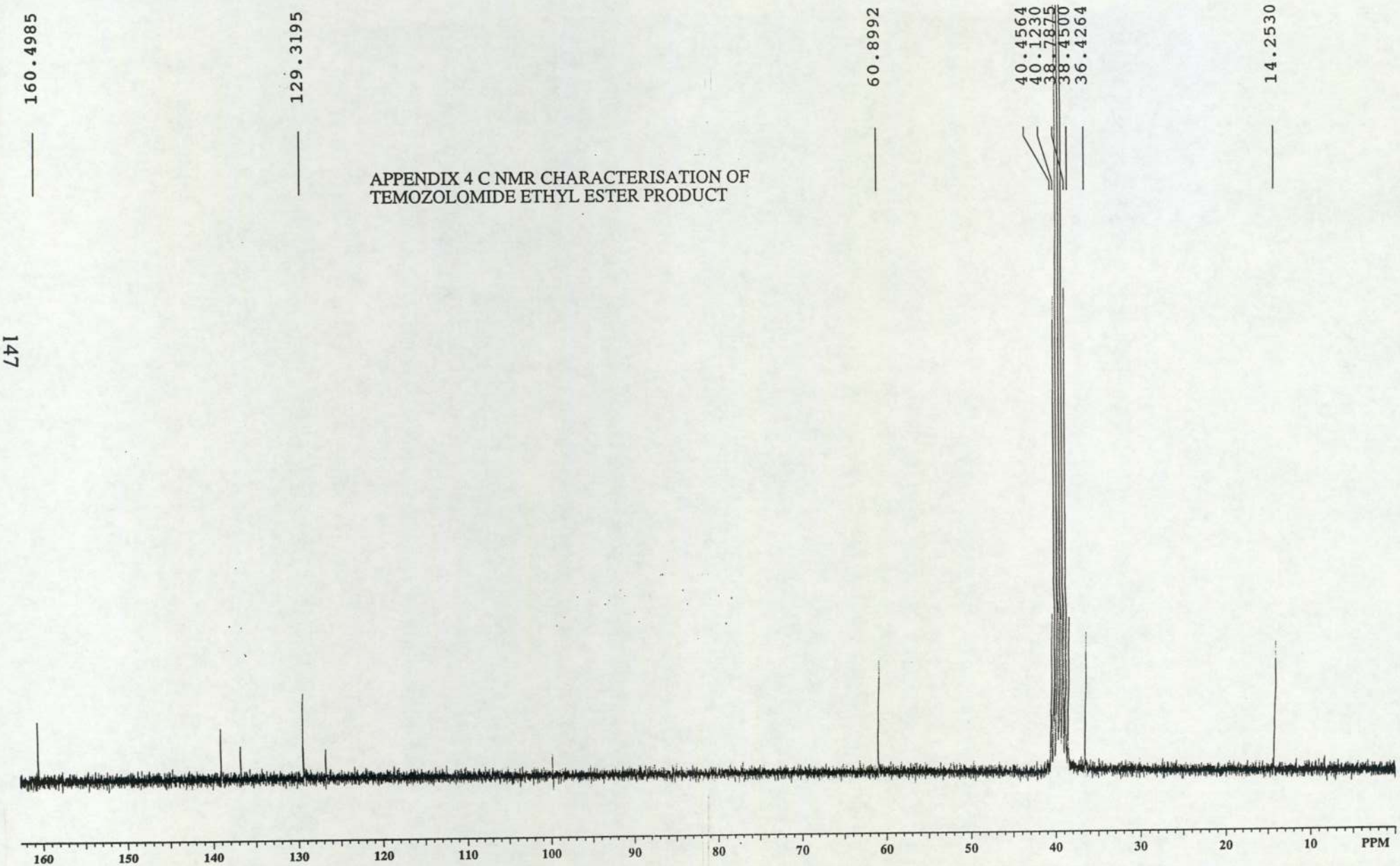


APPENDIX 2 C NMR CHARACTERISATION OF TEMOZOLOMIDE METHYL ESTER PRODUCT

APPENDIX 3 H NMR CHARACTERISATION OF TEMOZOLOMIDE ETHYL ESTER PRODUCT

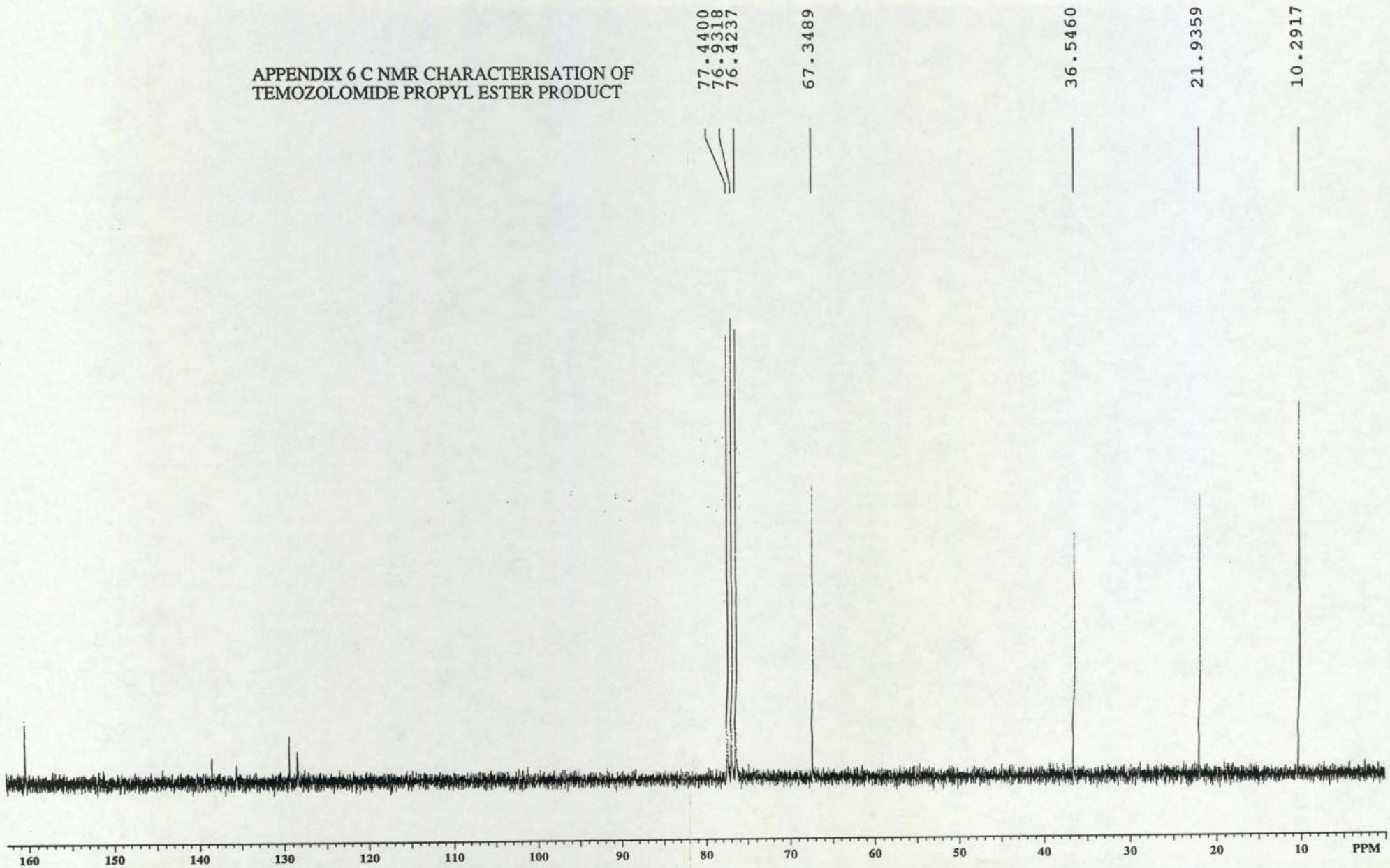


APPENDIX 4 C NMR CHARACTERISATION OF TEMOZOLOMIDE ETHYL ESTER PRODUCT



APPENDIX 5 H NMR CHARACTERISATION OF TEMOZOLOMIDE PROPYL ESTER PRODUCT

APPENDIX 6 C NMR CHARACTERISATION OF TEMOZOLOMIDE PROPYL ESTER PRODUCT

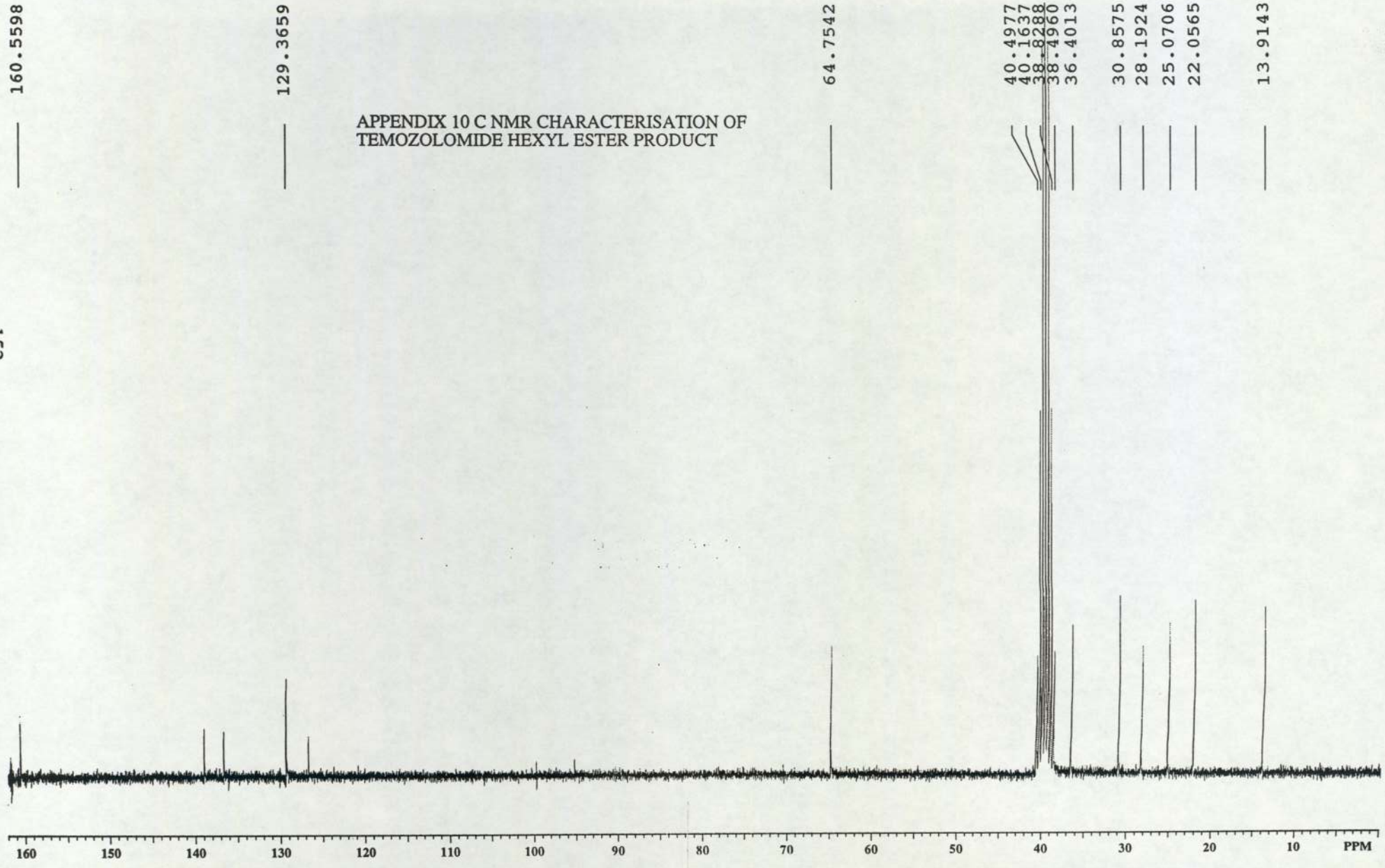


APPENDIX 7 H NMR CHARACTERISATION OF TEMOZOLOMIDE BUTYL ESTER PRODUCT

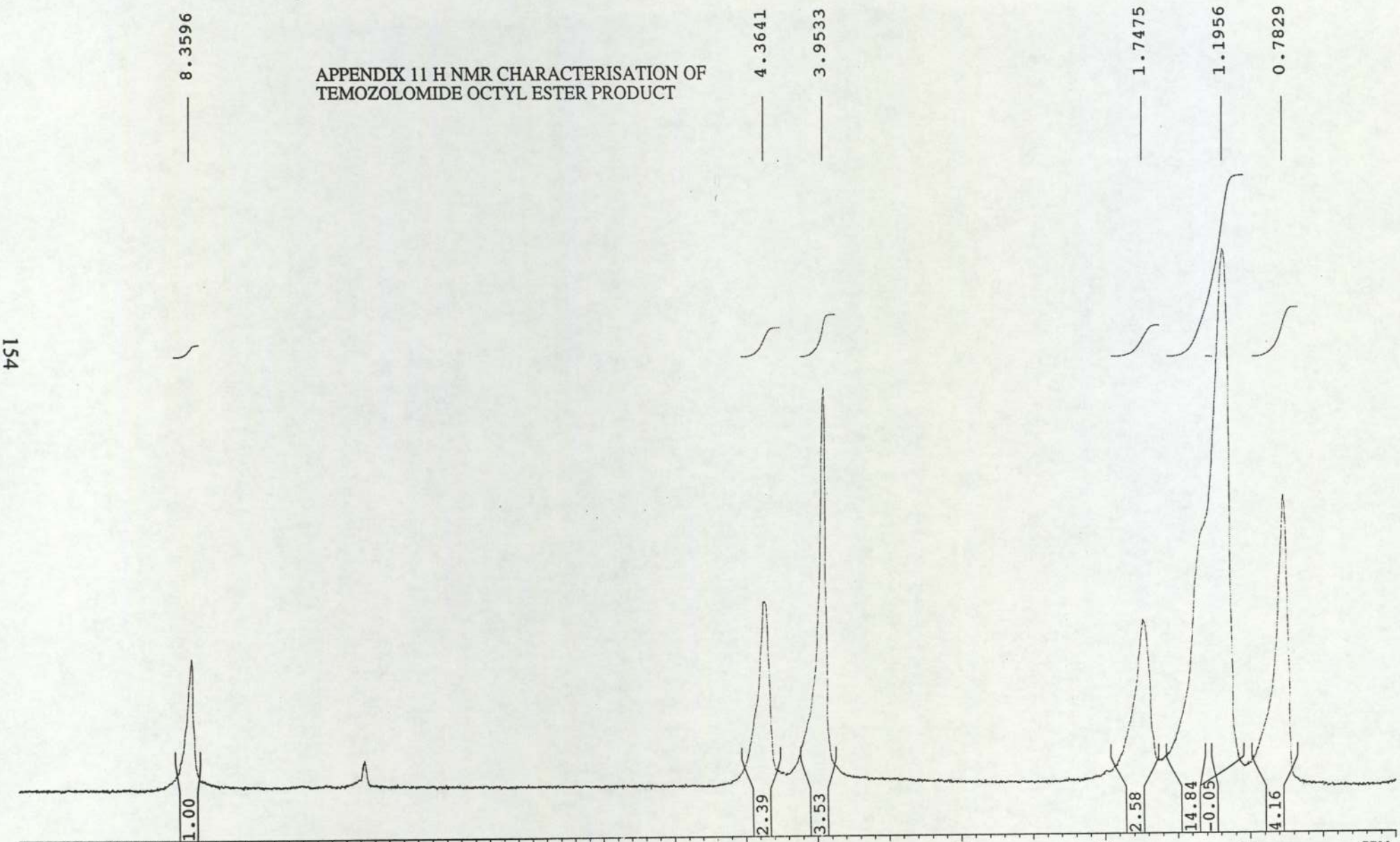
APPENDIX 8 C NMR CHARACTERISATION OF TEMOZOLOMIDE BUTYL ESTER PRODUCT

APPENDIX 9 H NMR CHARACTERISATION OF TEMOZOLOMIDE HEXYL ESTER PRODUCT

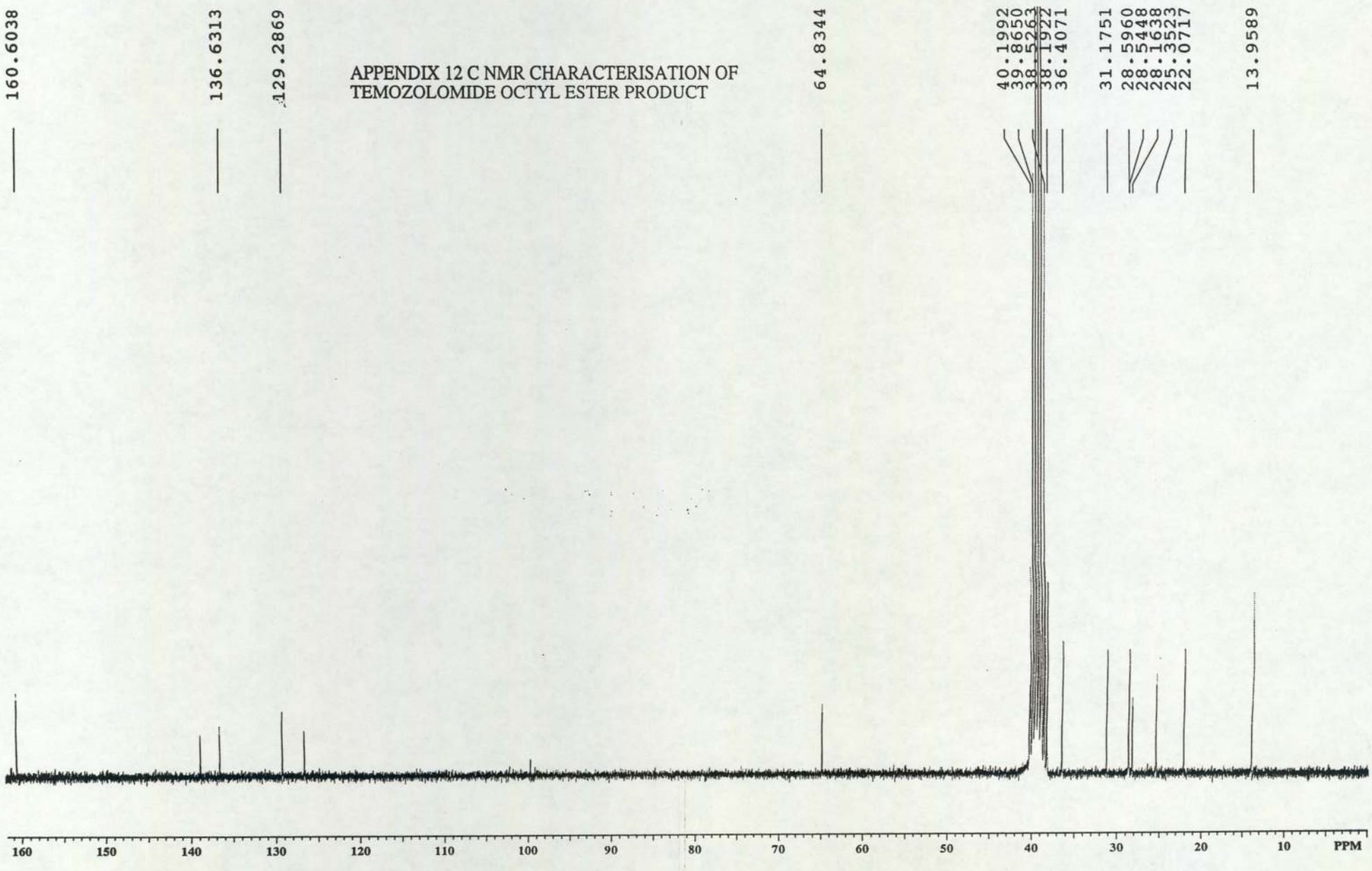
APPENDIX 10 C NMR CHARACTERISATION OF TEMOZOLOMIDE HEXYL ESTER PRODUCT



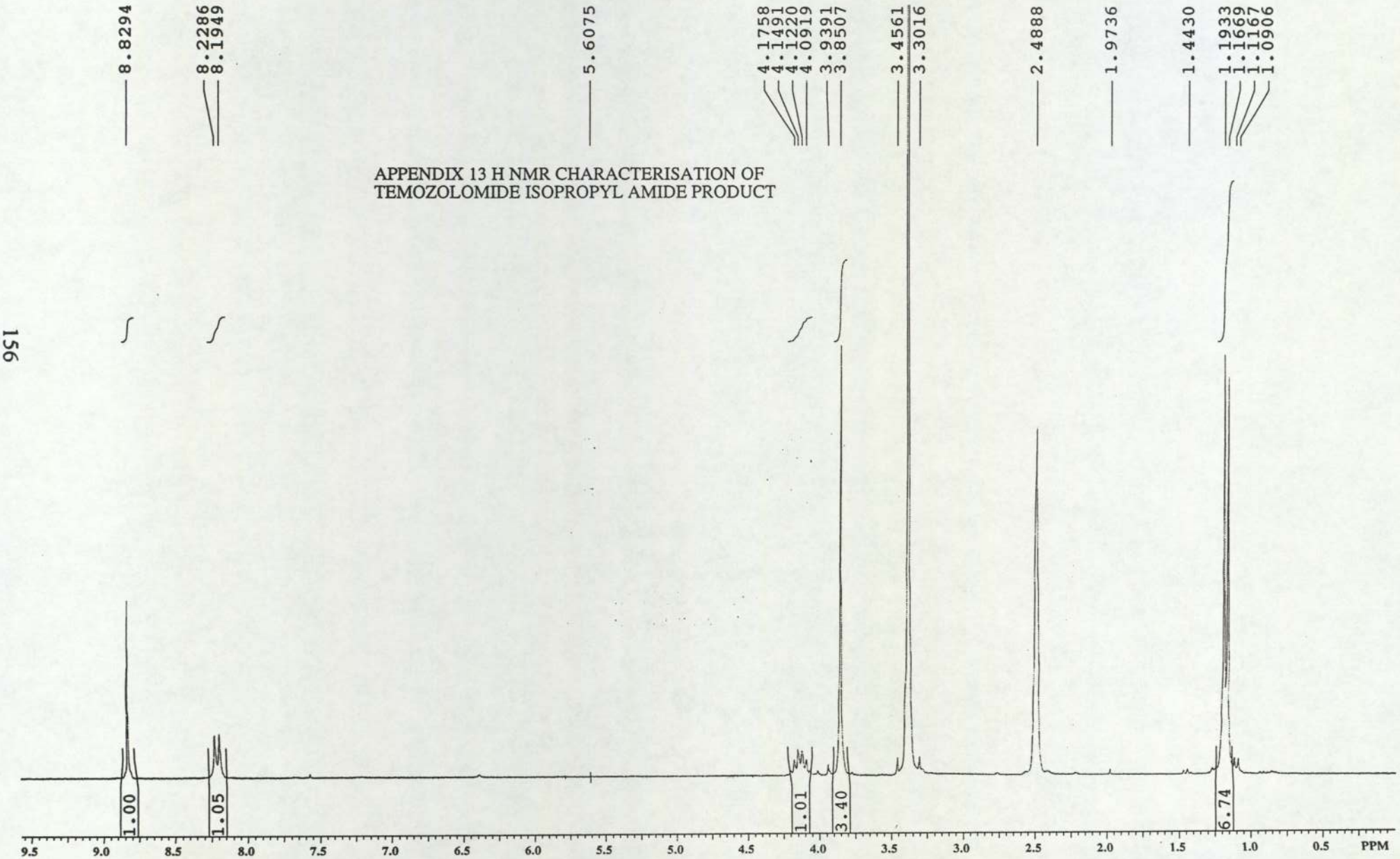
APPENDIX 11 H NMR CHARACTERISATION OF TEMOZOLOMIDE OCTYL ESTER PRODUCT



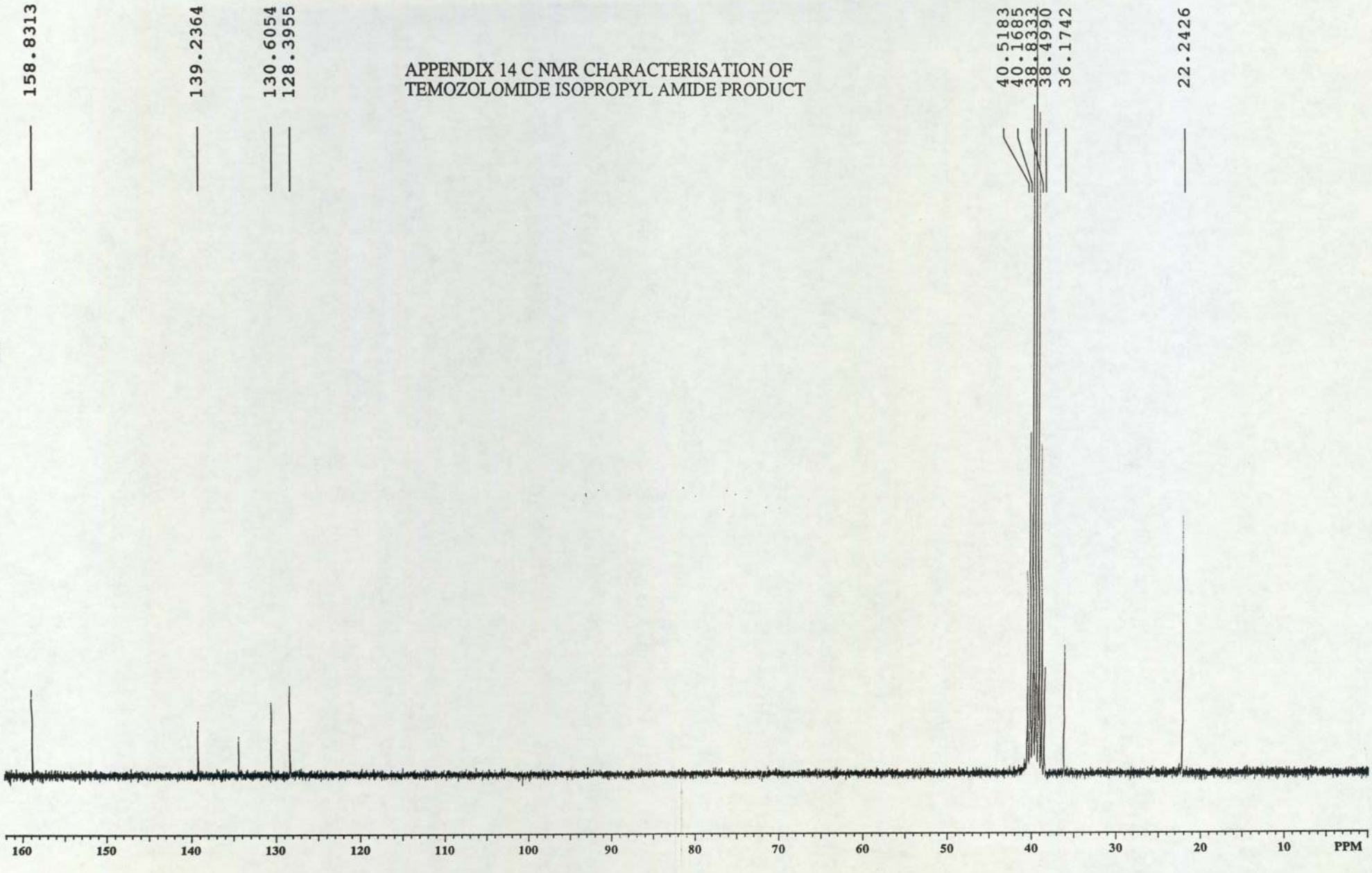
APPENDIX 12 C NMR CHARACTERISATION OF TEMOZOLOMIDE OCTYL ESTER PRODUCT



APPENDIX 13 H NMR CHARACTERISATION OF
TEMOZOLOMIDE ISOPROPYL AMIDE PRODUCT

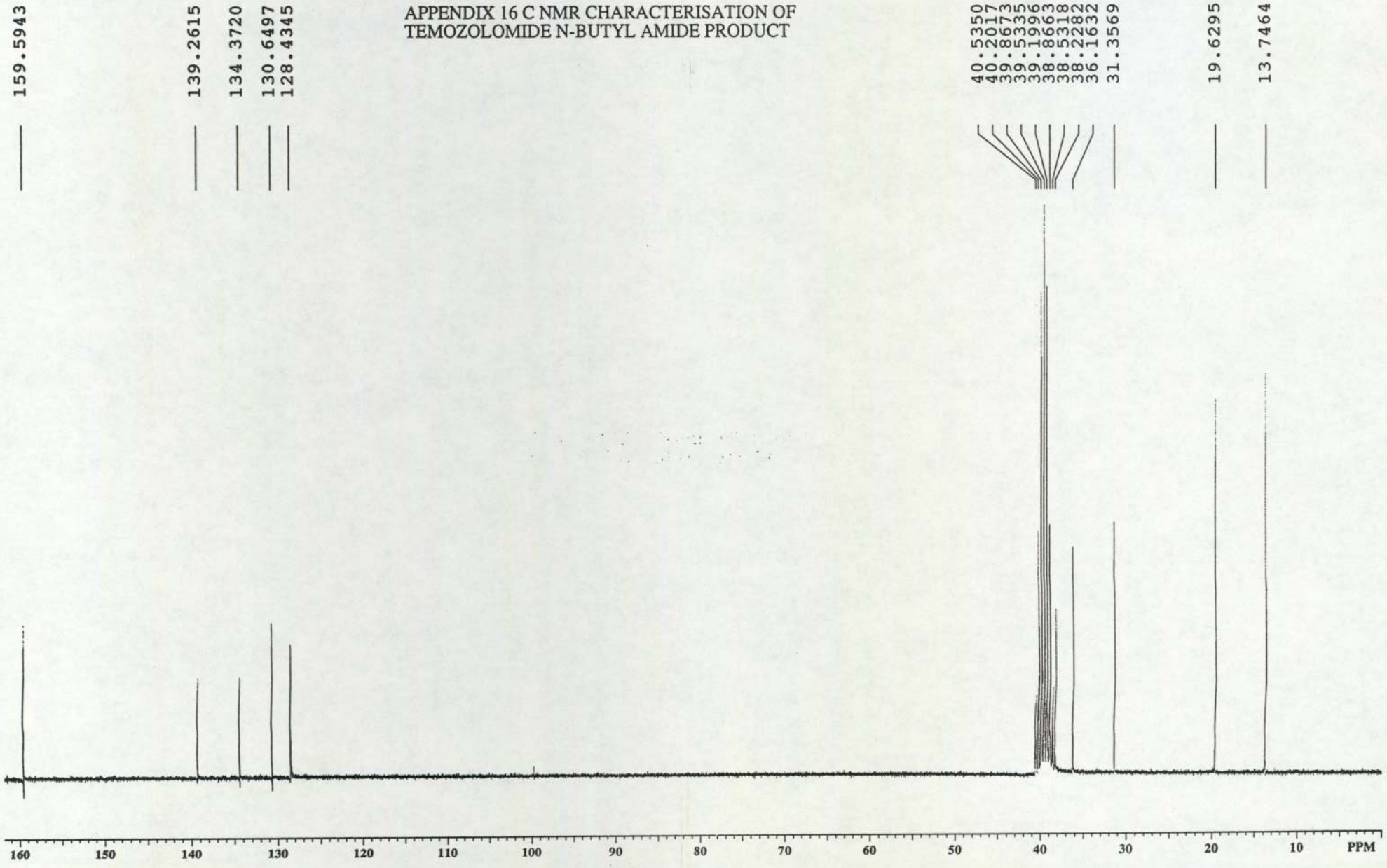


APPENDIX 14 C NMR CHARACTERISATION OF TEMOZOLOMIDE ISOPROPYL AMIDE PRODUCT



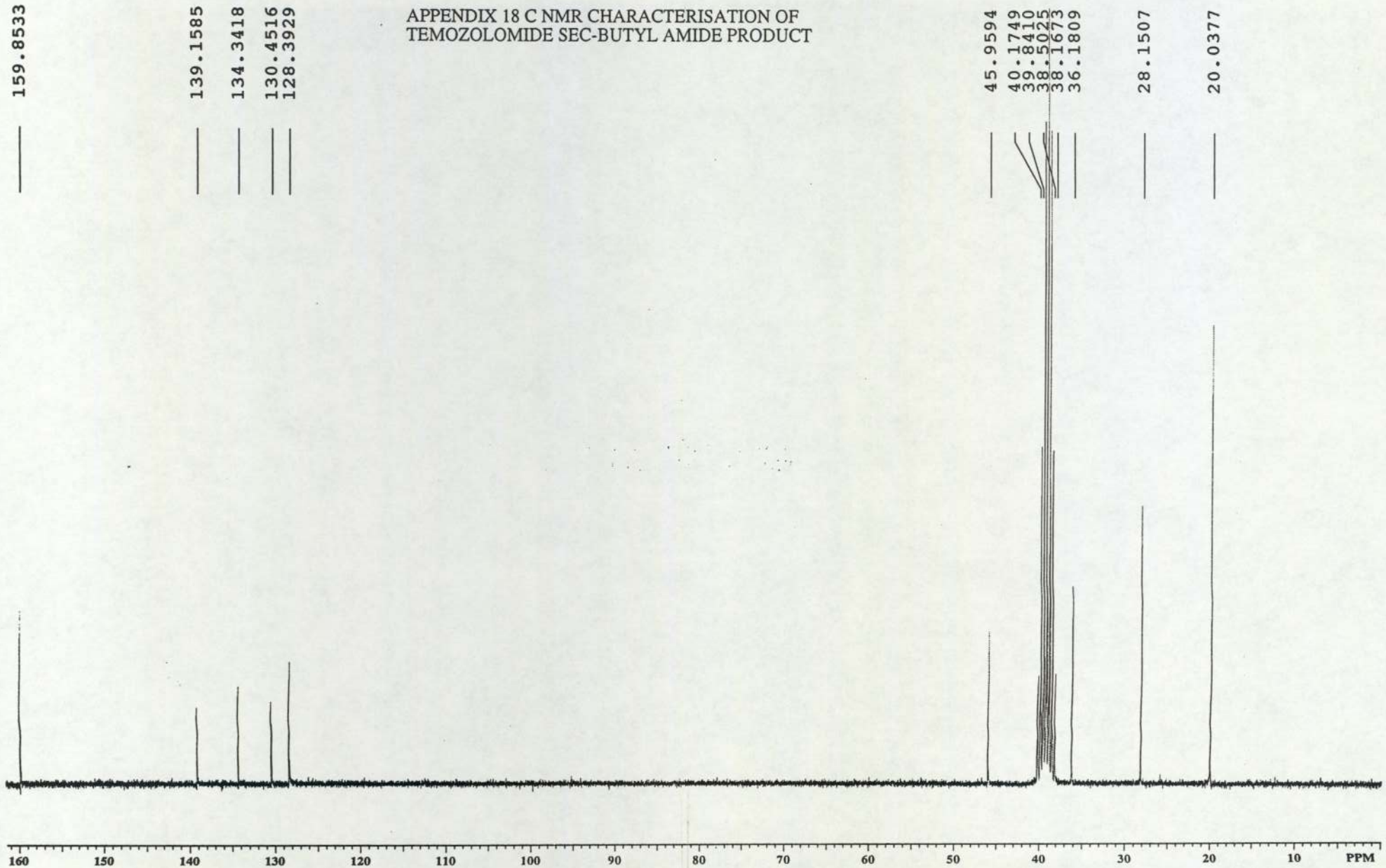
APPENDIX 15 H NMR CHARACTERISATION OF TEMOZOLOMIDE BUTYL AMIDE PRODUCT

APPENDIX 16 C NMR CHARACTERISATION OF TEMOZOLOMIDE N-BUTYL AMIDE PRODUCT

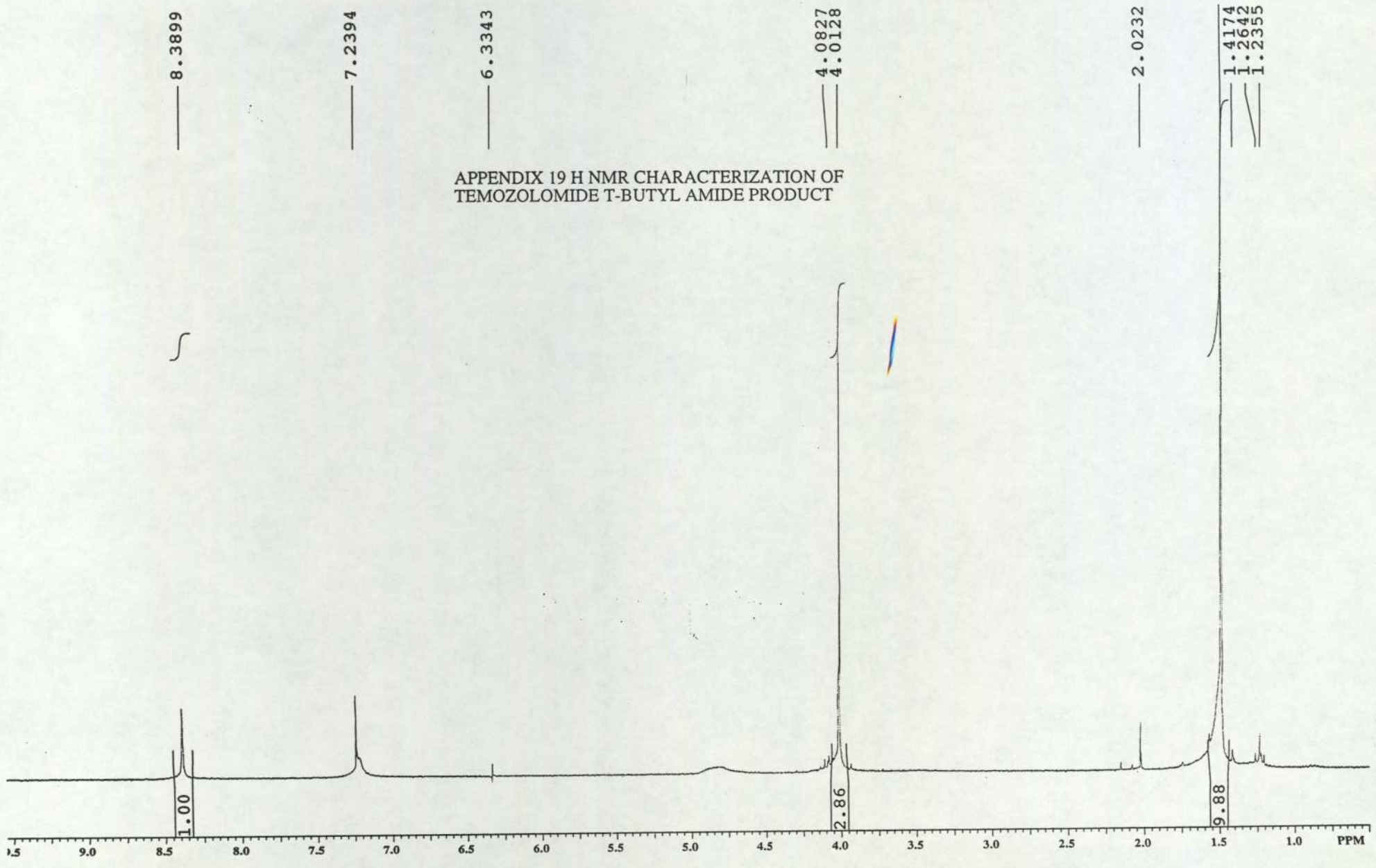


APPENDIX 17 H NMR CHARACTERISATION OF TEMOZOLOMIDE SEC-BUTYL AMIDE PRODUCT

APPENDIX 18 C NMR CHARACTERISATION OF TEMOZOLOMIDE SEC-BUTYL AMIDE PRODUCT

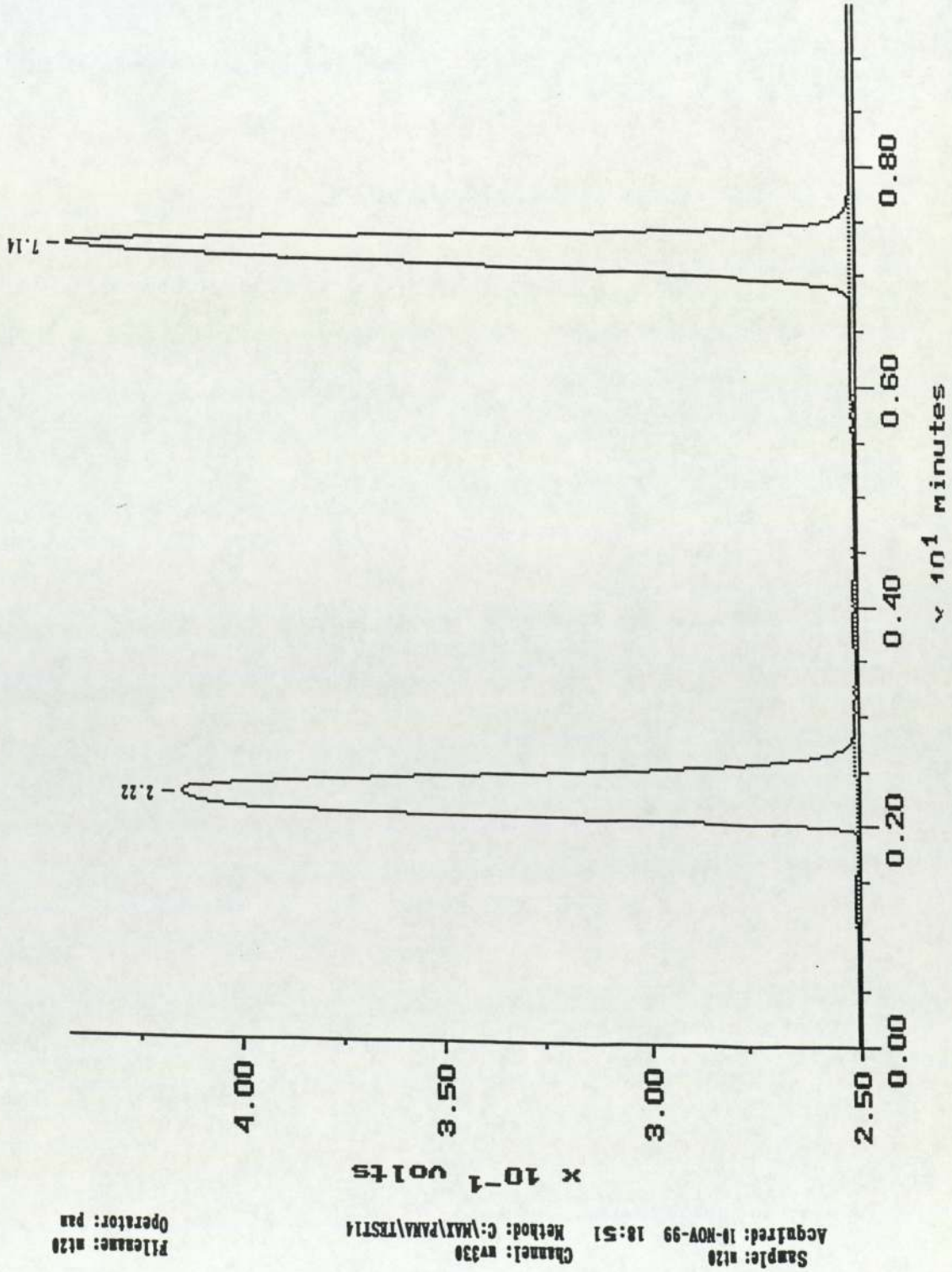


APPENDIX 19 H NMR CHARACTERIZATION OF
TEMOZOLOMIDE T-BUTYL AMIDE PRODUCT

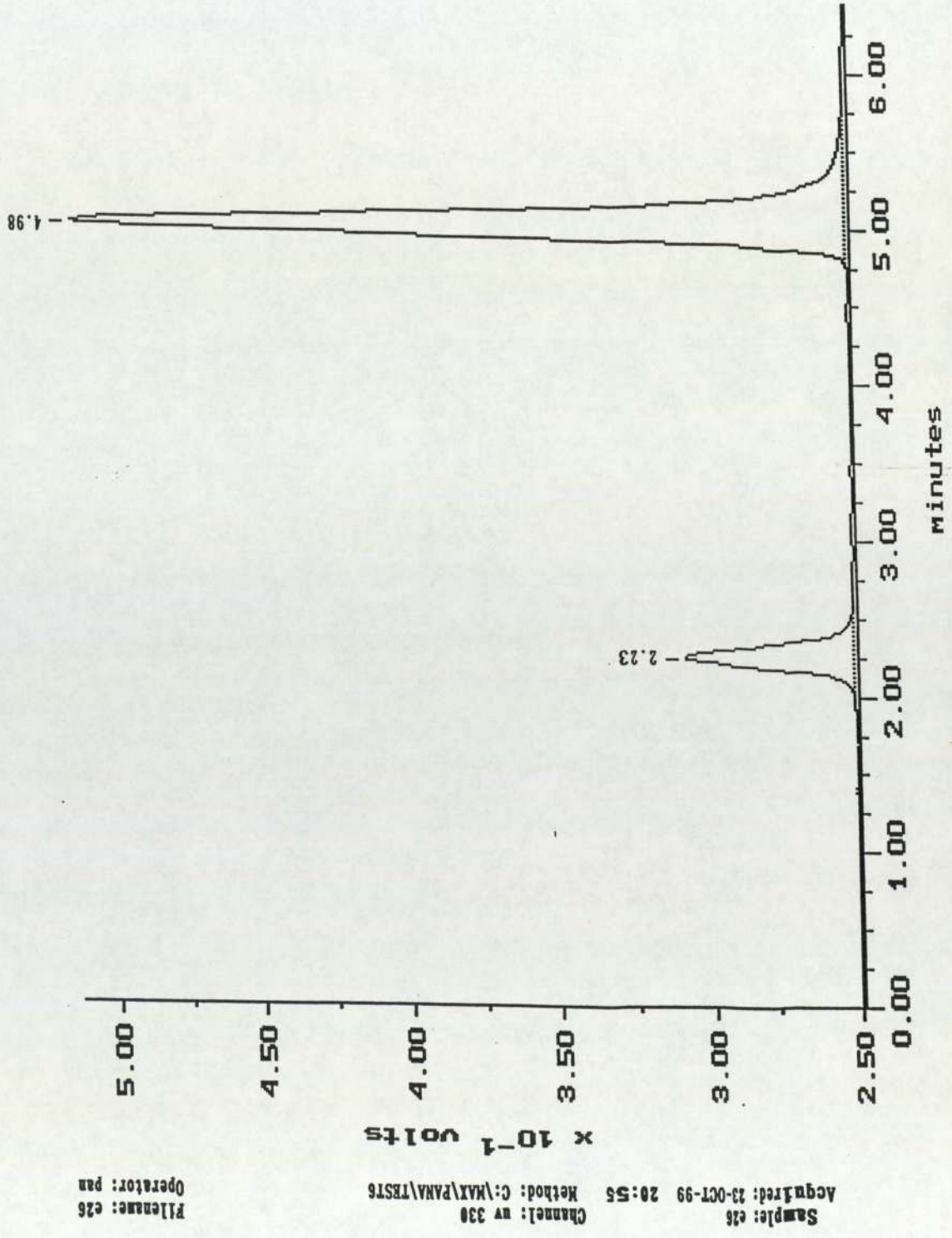


APPENDIX 20 C NMR CHARACTERISATION OF TEMOZOLOMIDE T-BUTYL AMIDE PRUDUCT

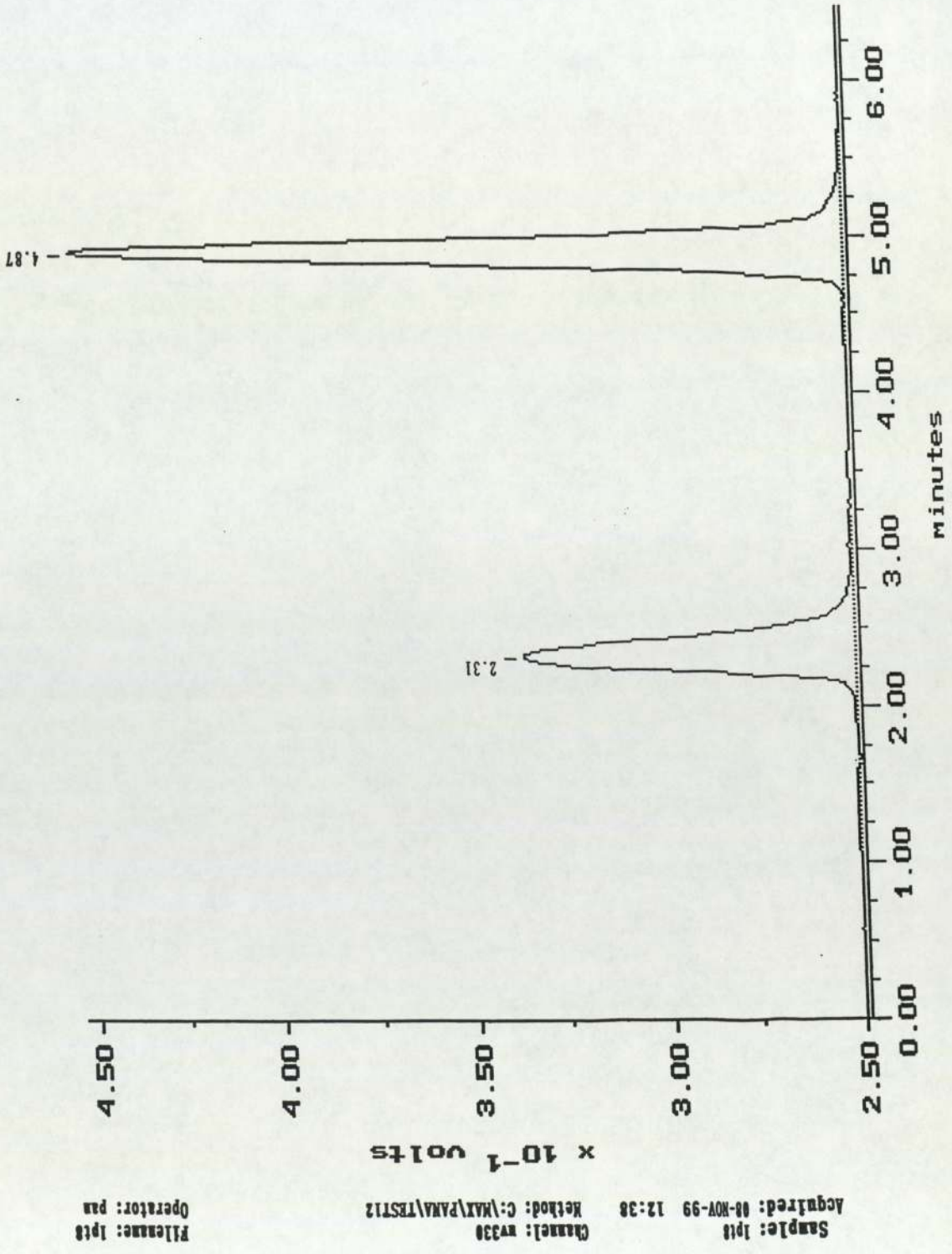
Appendix 21 A typical chromatogram of methyl ester product (2.22 mins) and internal standard (7.14 mins)



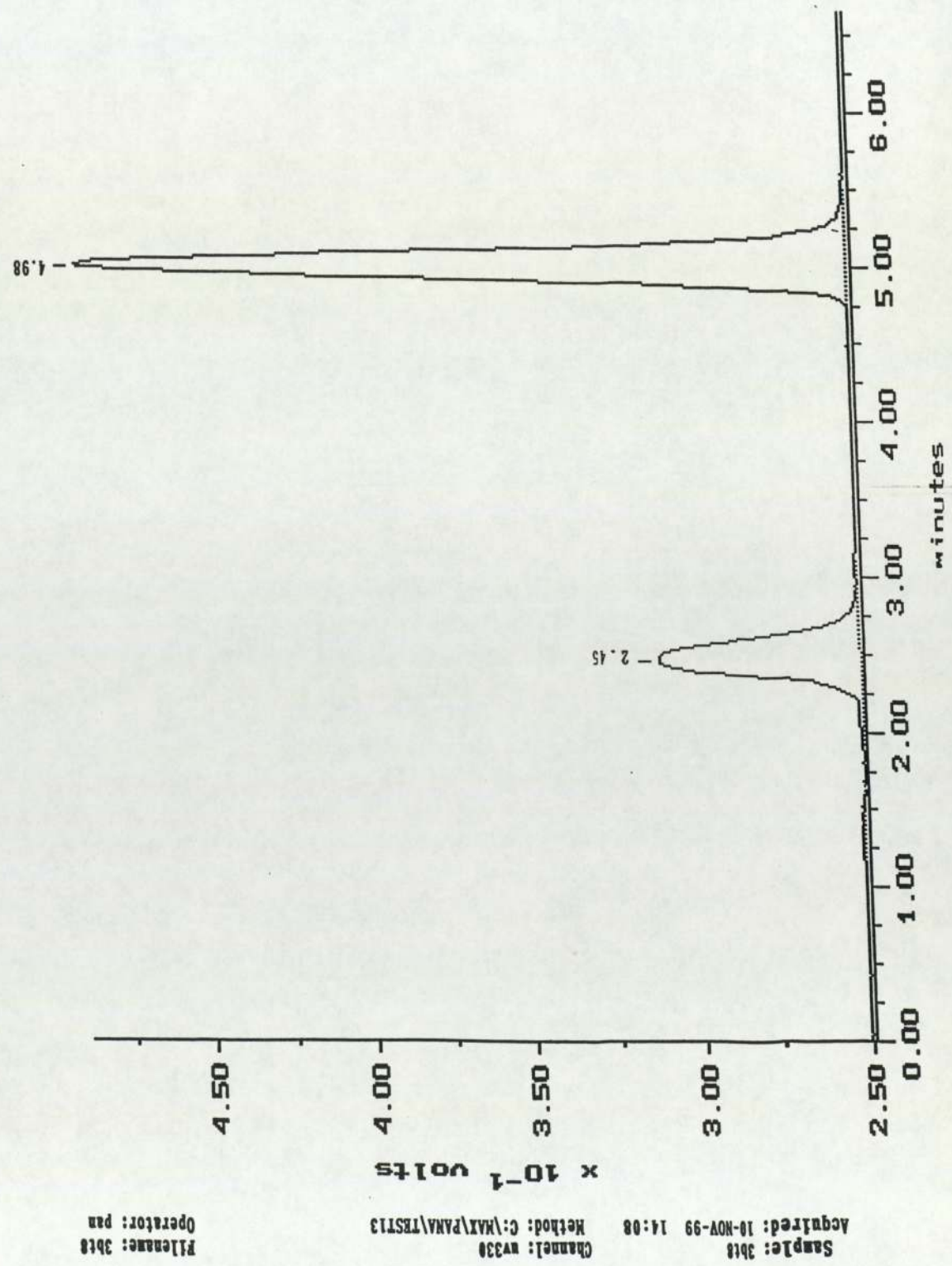
Appendix 22 A typical chromatogram of ethyl ester product (2.23 mins) and internal standard (4.98 mins)



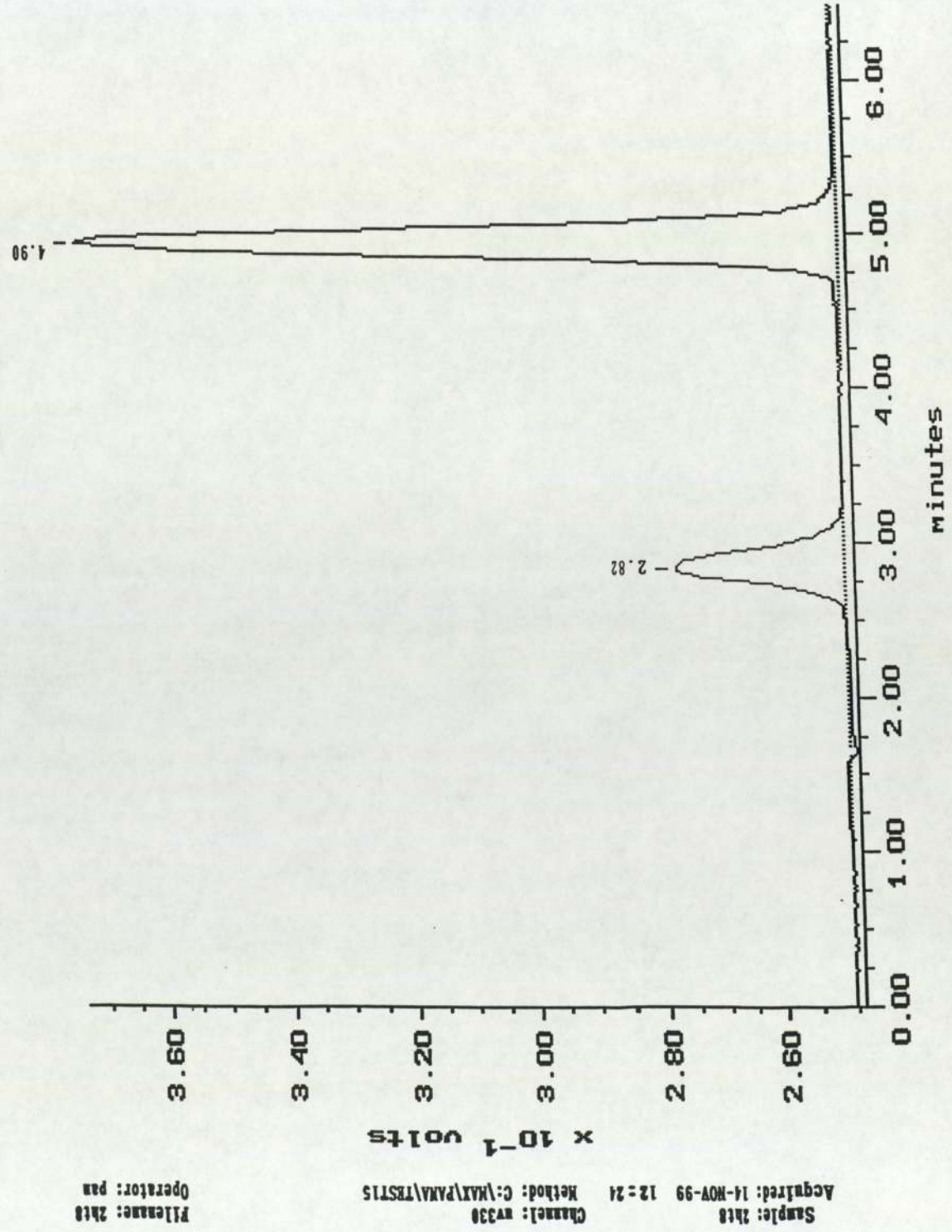
Appendix 23 A typical chromatogram of propyl ester product (2.31 mins) and internal standard (4.87 mins)



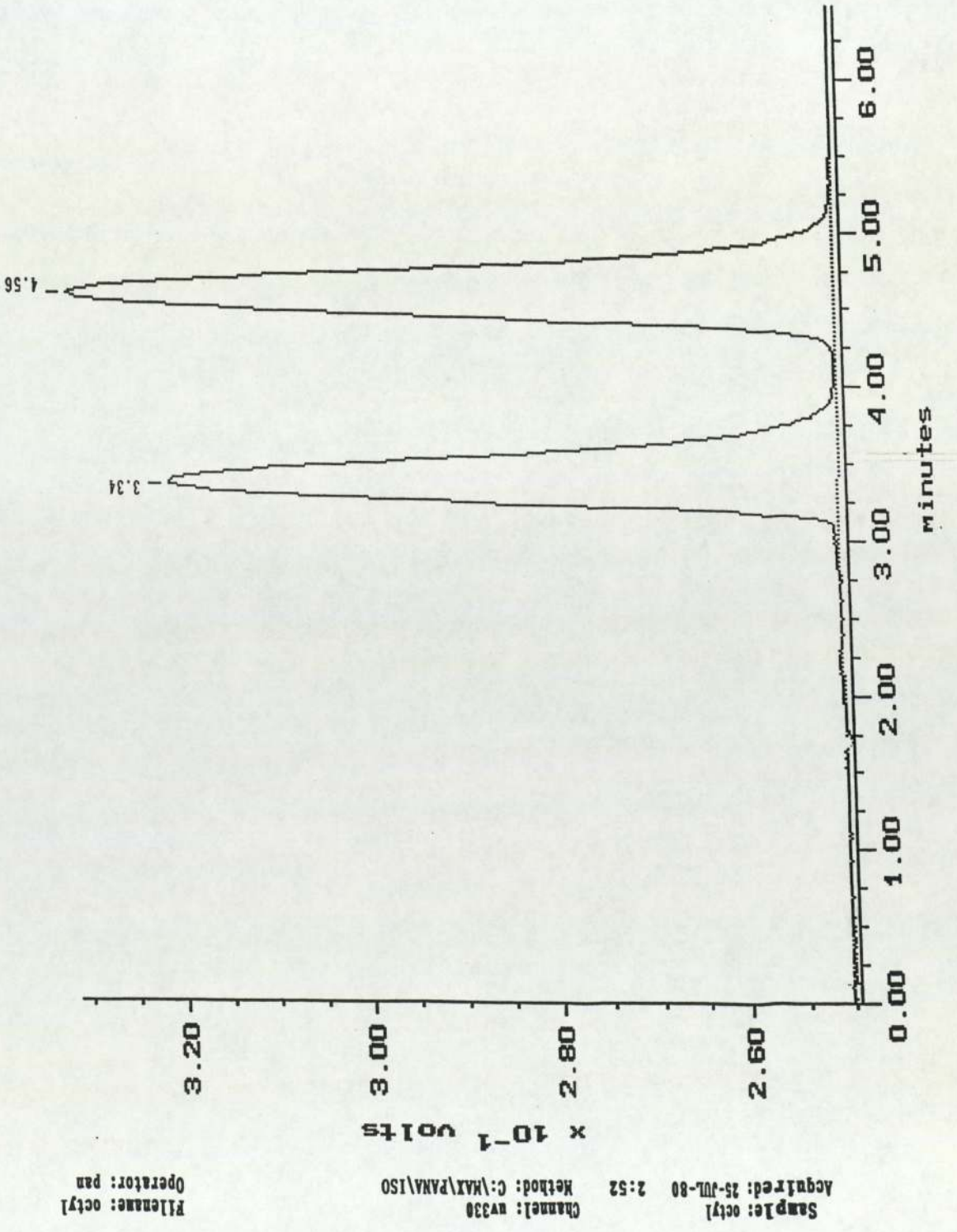
Appendix 24 A typical chromatogram of butyl ester product (2.45 mins) and internal standard (4.98 mins)



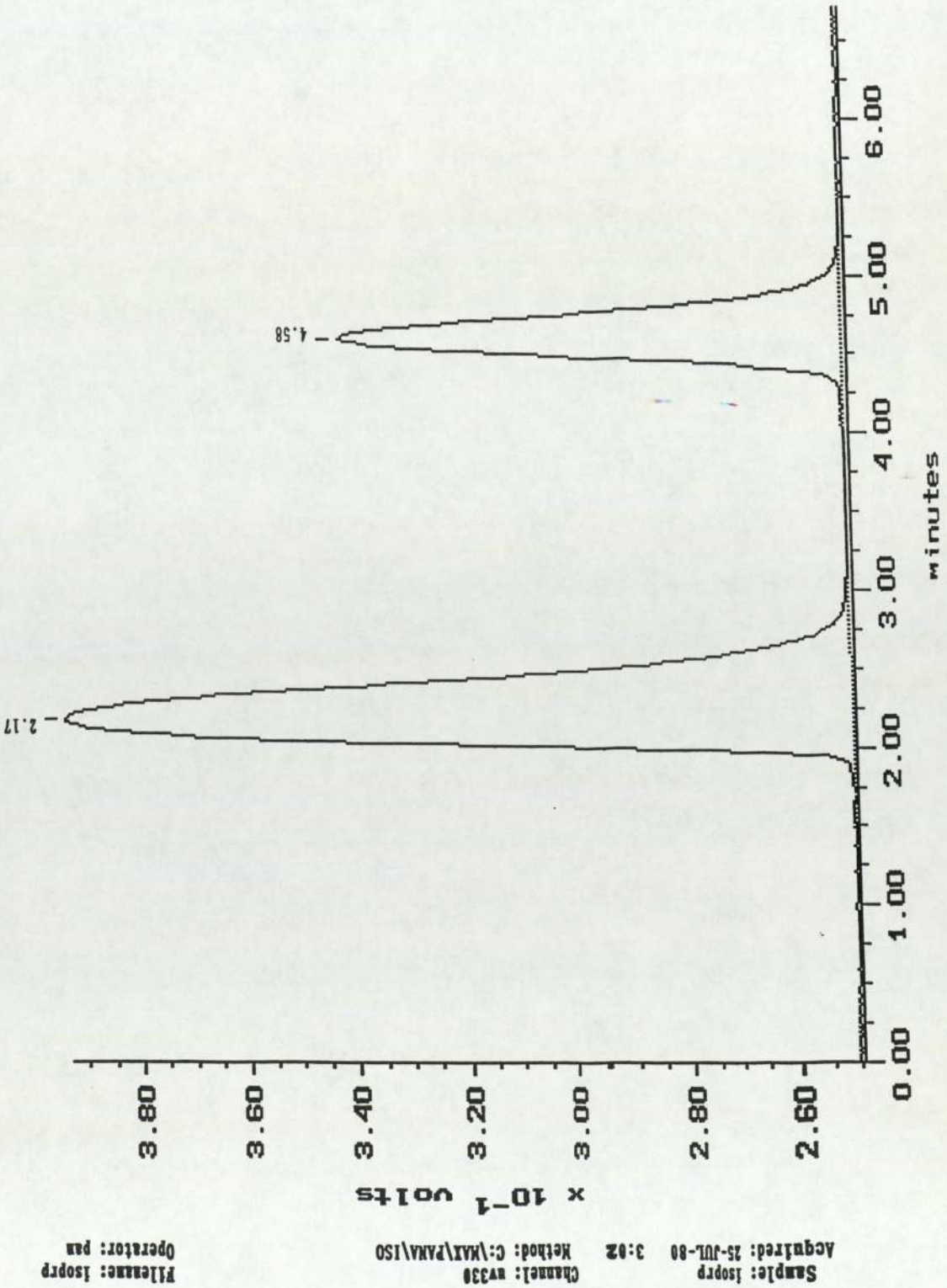
Appendix 25 typical chromatogram of hexyl ester product (2.82 mins) and internal standard (4.90 mins)



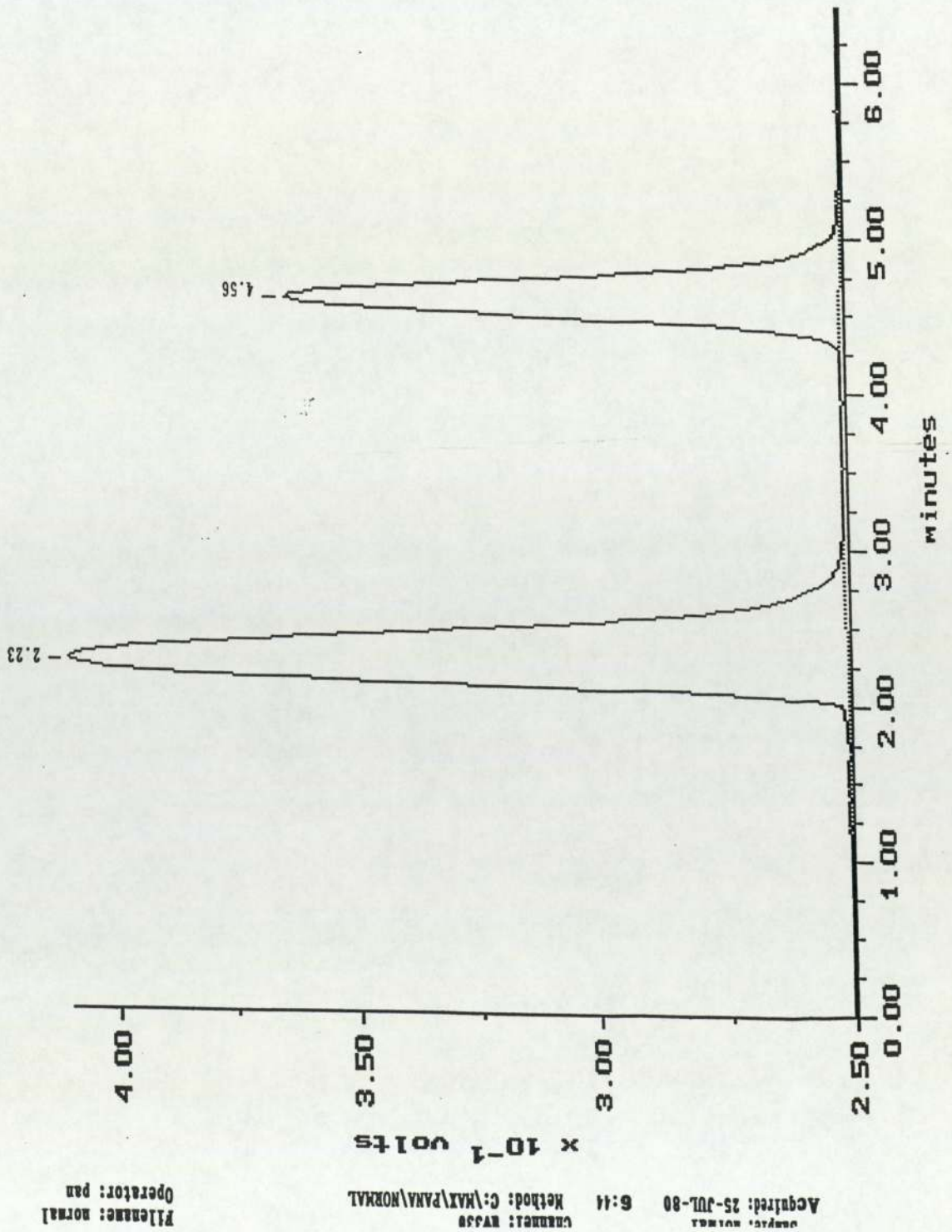
Appendix 26 A typical chromatogram of octyl ester product (3.34 mins) and internal standard (4.56 mins)



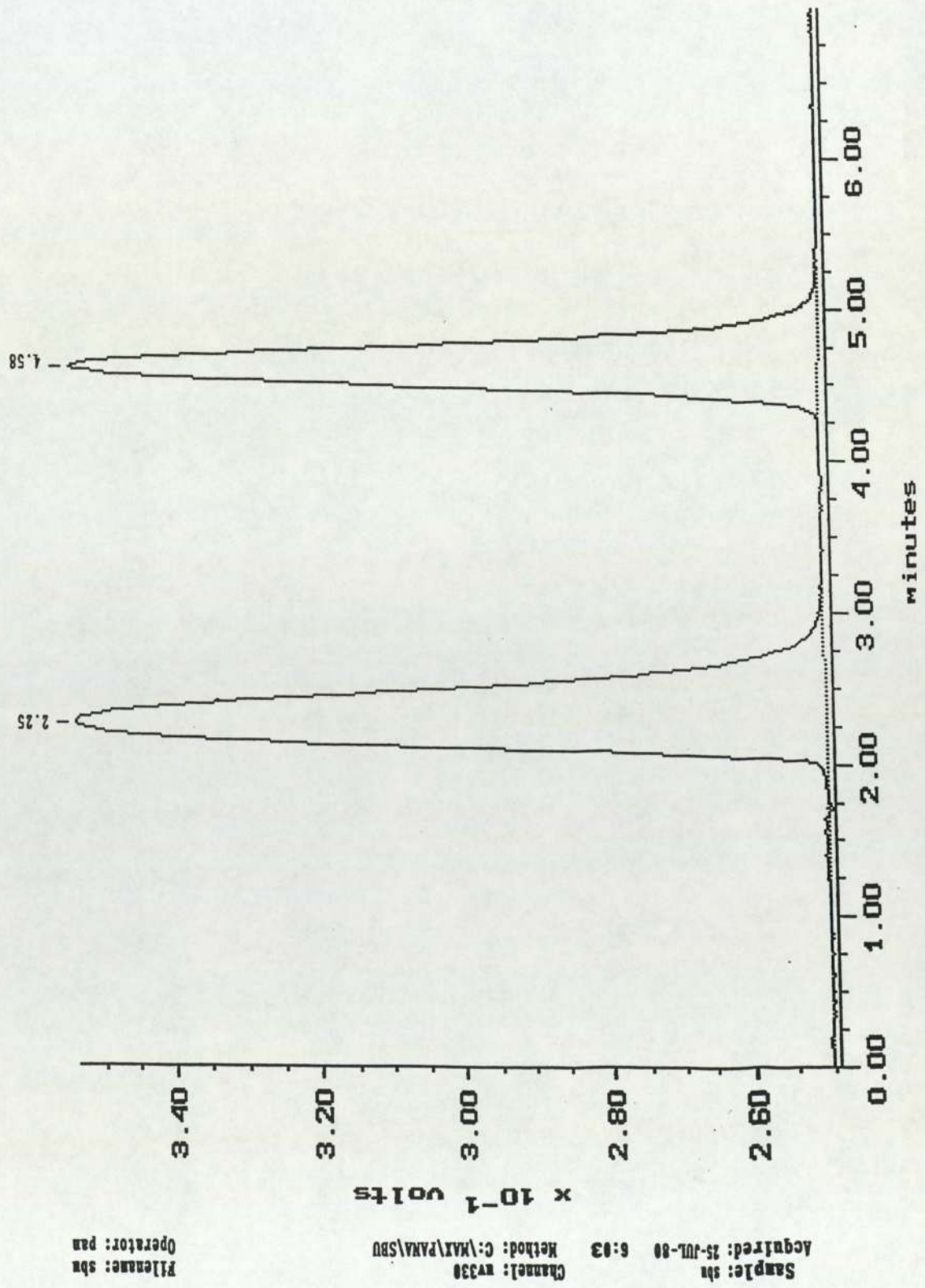
Appendix 27 A typical chromatogram of isopropyl amide product (2.17 mins) and internal standard (4.58 mins)



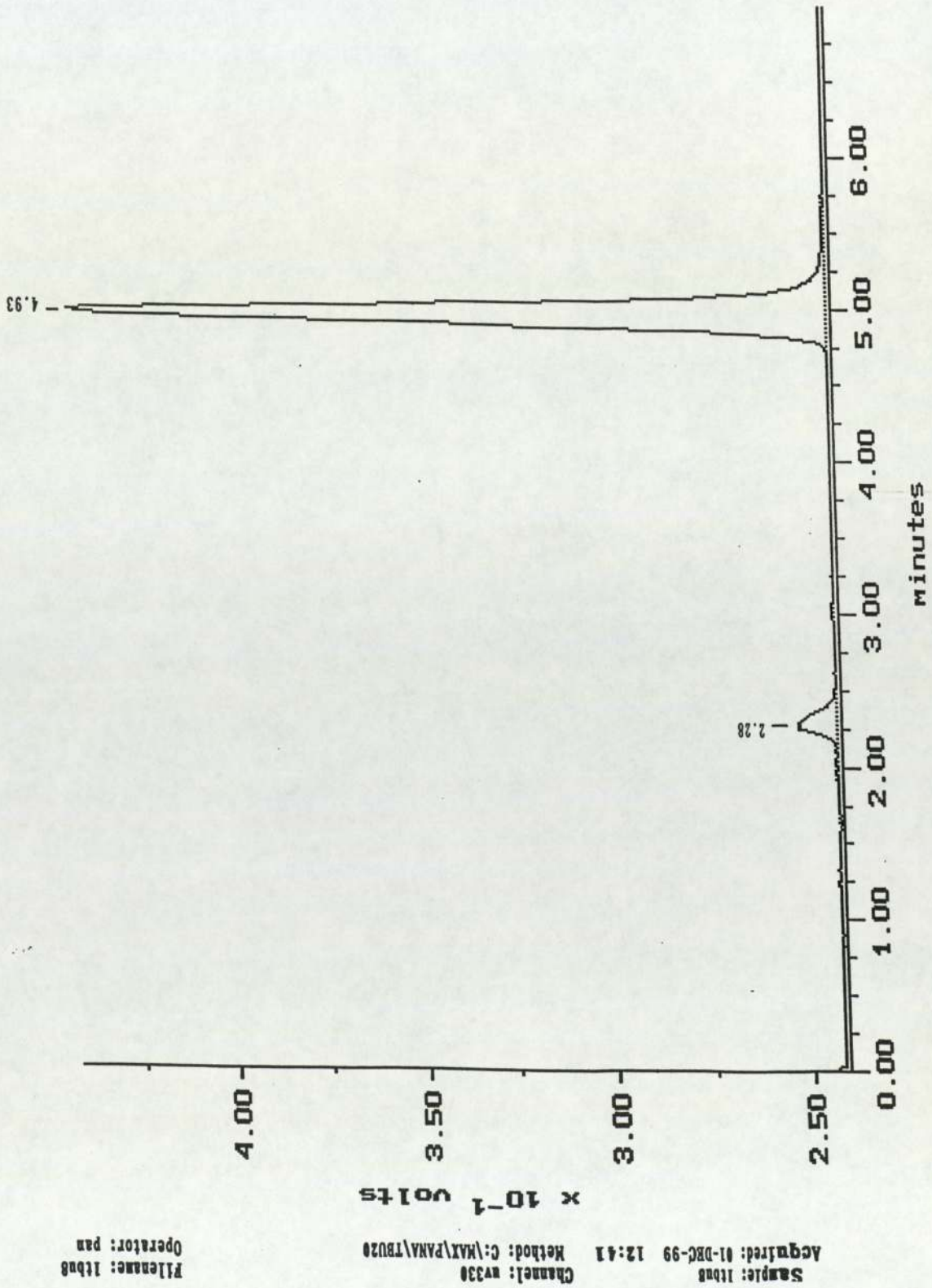
Appendix 28 A typical chromatogram of *n*-butyl amide product (2.23 mins) and internal standard (4.56 mins)



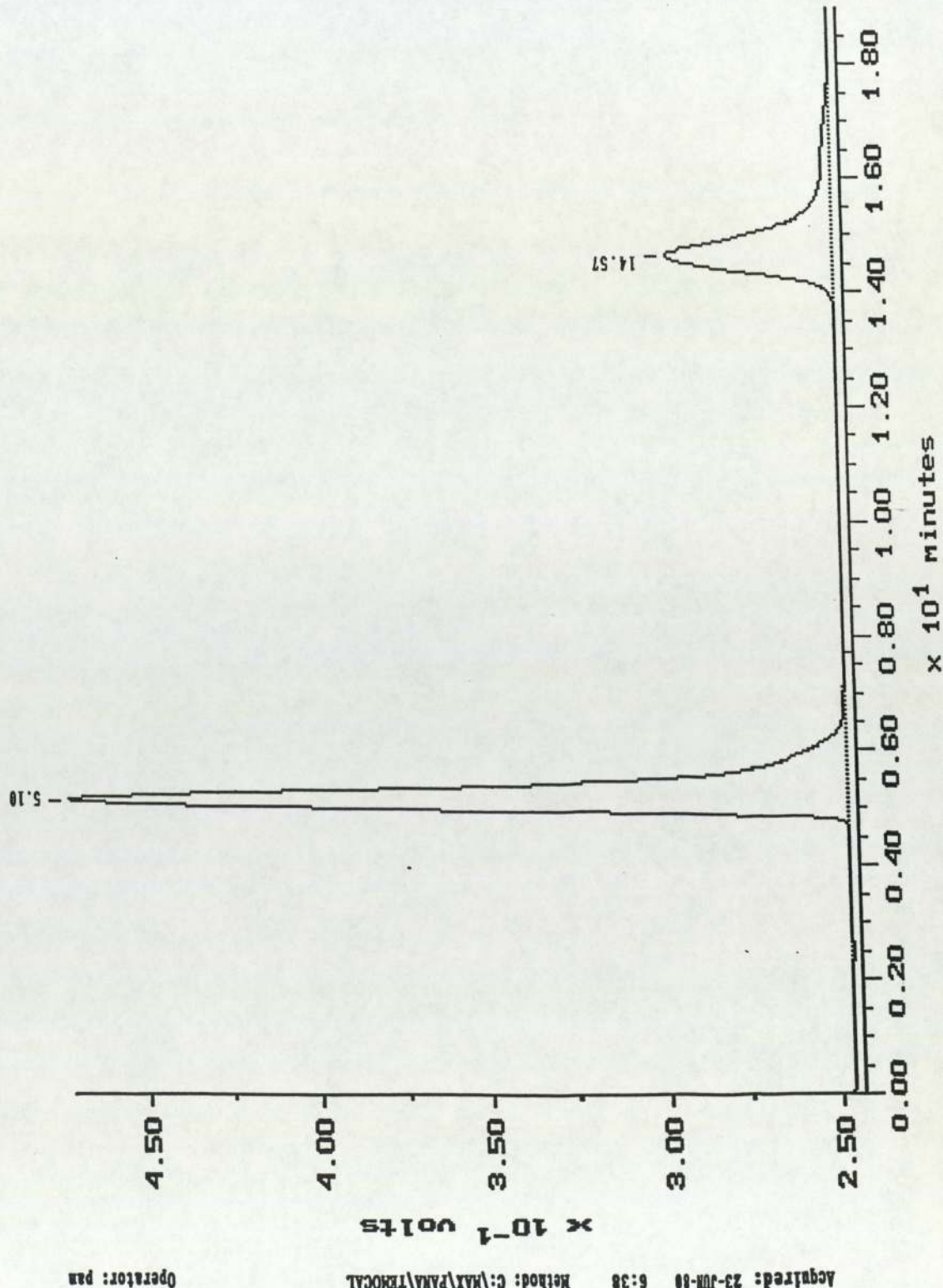
Appendix 29 A typical chromatogram of sec-butyl amide product (2.25 mins) and internal standard (4.58 mins)



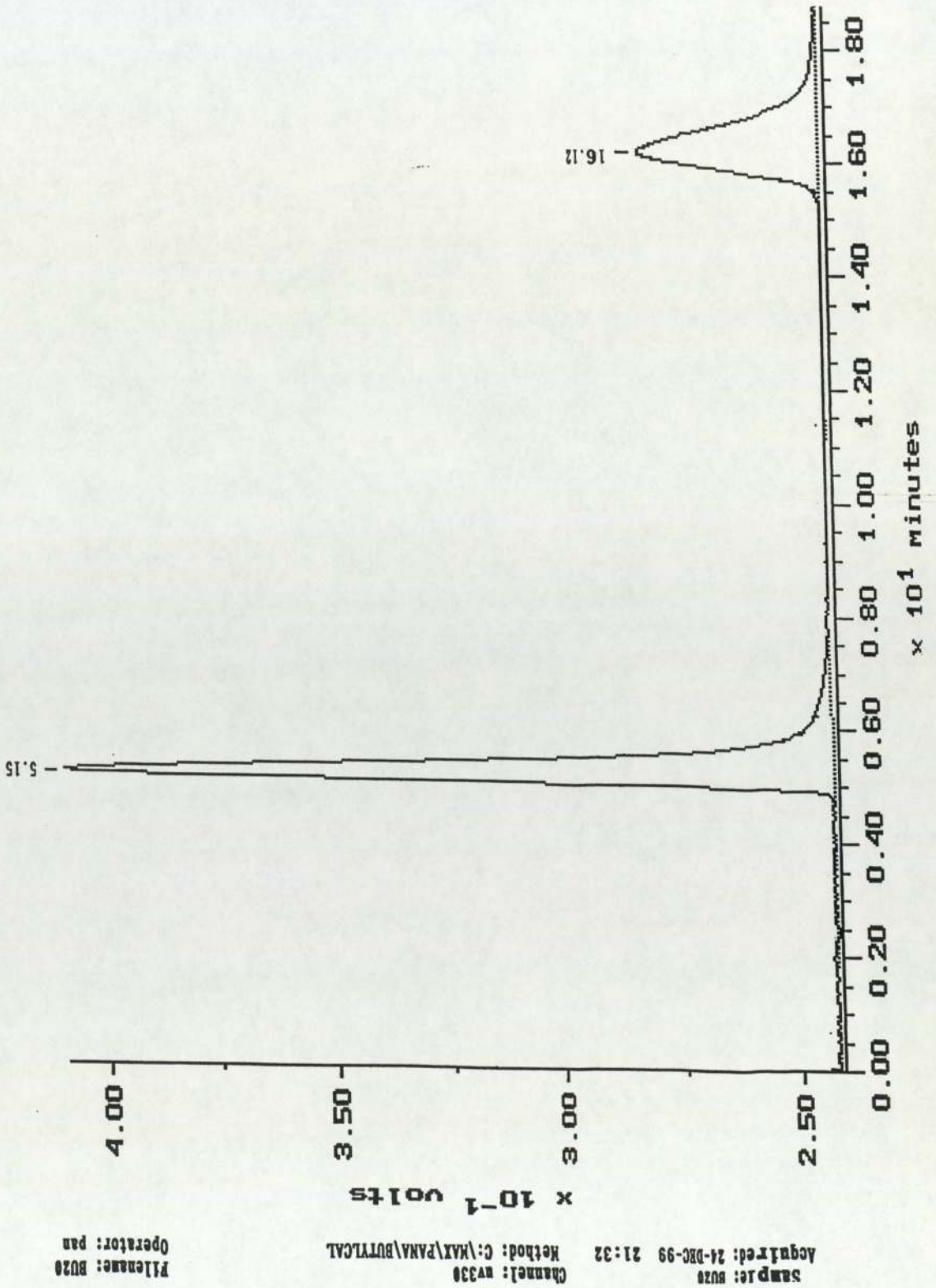
Appendix 30 A typical chromatogram of *t*-butyl amide product (2.28 mins) and internal standard (4.93 mins)



Appendix 31 A typical chromatogram of temozolomide free drug (5.10 mins) and internal standard (14.57)



Appendix 32 A typical chromatogram of temozolomide acid (5.15 mins) and internal standard (16.12 mins)



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