THE DEVELOPMENTAL PHARMACOLOGY OF AMIFOSTINE: PHARMACOKINETICS AND MEASURES OF OXIDATIVE STRESS

by

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ABSTRACT

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Use of the broad-spectrum cytoprotective agent, amifostine, in children may allow more effective cancer treatment by selectively protecting healthy tissue from the detrimental effects of chemotherapy. This effect is mediated via the active metabolite, WR1065, whose mechanism of action may relate to modulation of glutathione levels. Experience with amifostine is limited in pediatrics; therefore, a Phase I study was used to establish pharmacokinetics in children and to evaluate the proposed mechanism of action. Similar systemic exposure to WR1065 was observed in both children and adults after correcting for dose differences, suggesting a similar degree of protection in both populations. In order to evaluate WR1065's mechanism of action, we identified and validated an improved method for glutathione sample preparation, resulting in a reliable assay that will enable us to better assess amifostine's effect on whole blood glutathione levels.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%CV	Percent coefficient of variation
ANC	Absolute neutrophil count
AST	Aspartate transaminase
AUC	Area under the concentration-time curve
$AUC_{0-\infty}$	AUC extrapolated to infinity time
C_{max}	Maximum observed concentration
CNS	Central nervous system
DLT	Dose-limiting toxicity
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	Upstream electrode of electrochemical detector
E2	Downstream electrode of electrochemical detector
EC	Electrochemical
EDTA	Ethylene diamine tetracetic acid (disodium salt, dihydrate)
G-CSF	Granulocyte colony-stimulating factor
GGT	γ-glutamyl transferase
GSH	Reduced glutathione
GSSG	Oxidized glutathione: glutathione disulfide
GSSR	Mixed disulfides
GST	Glutathione S-transferases
HDV	Hydrodynamic voltammogram
HPLC	High pressure liquid chromatography
HPLC-EC	High pressure liquid chromatography-electrochemical detection
HWPC	Humanized whey protein concentrate
i.v.	intravenous
ICE	Ifosfamide Carboplatin Etoposide
k_e	Elimination rate constant
LD_{50}	Dose required to kill 50% of test population
MPA	Metaphosphoric acid
MTD	Maximum tolerated dose

NADP+	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OTZ	L-2-oxothiazolidine-4-carboxylate
P450	Cytochrome P450
PCA	Perchloric acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SH	sulfhydryl
SSA	Sulfosalicylic acid
$t_{½}$	Half-life
TCA	Trichloroacetic acid
t_{max}	Time of maximum observed concentration
UV	Ultraviolet

1.0 INTRODUCTION

1.1 Pediatric Cancer

The incidence of cancer in children is relatively rare as it occurs in approximately 15 out of every 100 000 children and adolescents per year (Health Canada 1999). Between 1991 and 1995, an average of 1 279 Canadian children and adolescents were diagnosed with the disease (Canadian Cancer Statistics 2000). Today, most children with cancer are cured with current therapy. The overall cure rate has increased from 28% in 1963 to 72% in 1993 (Steen 2000). However, up to 30% of all children diagnosed with all forms of cancer will die and this number can be higher for some types of cancers compared to others (Canadian Cancer Statistics 2000). Treatment may fail because of lack of efficacy or due to intolerable short- and/or long-term complications. (Capizzi 1999a).

One of the primary differences between adult and childhood cancers is that childhood cancer can be cured more often (Steen 2000). This is counter-intuitive, as one would expect that further progress could be made in treating a relatively common disease such as adult cancer rather than in treating a much rarer disease such as childhood cancer. This distinction suggests that inherent differences between pediatric and adult cancers exist.

Children are typically diagnosed with cancers that affect stem cells. Certain stem cells may give rise to cancer in children due to spontaneous mutations. Generally, gene mutations that result in childhood cancer are not due to interactions with the environment, but rather, to a genetic aberrance (Steen 2000). In contrast, adults tend to be affected by cancers of the epithelial cells. Cancer in these cells is normally induced by interaction with the environment. This implies that most adult cancers are acquired and that they are the result of lifelong exposure to

cancer-inducing agents (Steen 2000). An additional factor that distinguishes childhood cancer from adult cancer is that children are typically much more resilient than adults so they can tolerate more aggressive therapy. Adults customarily have additional health problems that may hinder treatment that is aggressive enough to achieve a cure (Steen 2000).

1.2 Clinical Trials in Pediatric Oncology

Remarkable progress has been made in recent years in the treatment of childhood cancer. Standard therapy has not only improved cure and survival rates in childhood cancer, but it has also enhanced the quality of life of many patients. However, a proportion of children will die from their disease because they do not respond to this standard therapy. Both ends of this spectrum of patient outcomes are strong motivators for continued evaluation of new therapeutic agents in pediatric oncology (Baruchel and Rowell 1998).

A drug enters the first of three phases of clinical trials for use in humans only after it has undergone extensive preclinical testing in animals (Hughes 2000). The definitions of the phases for clinical trials for cancer treatments differ from those for other therapies. While the main objectives in both types of Phase I trials are to collect pharmacokinetic and safety information, the main difference lies in the subjects that are used. Cancer Phase I trials involve the use of patients diagnosed with the disease (Hughes 2000). These patients are usually terminally ill and their disease is refractory to available treatment. In contrast, other Phase I studies involve the use of healthy, adult volunteers (Naranjo and Janecek 1998). This main difference does not allow investigators to make observations about the efficacy of the drug, which is a possibility in oncology Phase I studies although it is not a specific goal. A table summarizing the goals and objectives of the phases of cancer therapy trials is included in Appendix 1. Small Phase I trials in adults are usually the first step in translating basic research into clinical applications. This

usually leads to larger studies in adults, which can be concurrent with Phase I studies in children. Even though similar studies have been carried out in adults, specific Phase I studies in children are needed because there are differences between these two populations. Firstly, many more adults than children have cancer. Furthermore, adults tend to become eligible for Phase I trials earlier in the course of their disease since there usually is a lack of effective therapy for these patients. In contrast, when a child is initially diagnosed with the disease, he or she is treated with curative intent. Only after additional treatment attempts fail (e.g., Phase II trials, bone marrow transplantation *etc.*) do children become candidates for Phase I trials. Finally, experience has shown that the results of adult trials are relatively poor predictors of potential toxicities in children (Baruchel and Rowell 1998).

By definition, as alluded to above, a Phase I trial does not imply therapeutic intent. This raises moral and ethical issues. However, it can be argued that while the primary aim of a Phase I trial is to evaluate the maximum tolerated dose (MTD) of a new agent and to identify toxicity, this does not preclude a secondary therapeutic aim. It is reasonable to expect that an anti-tumor effect would be achieved in some patients. This can provide some potential benefit for the child when he or she is faced with failure of all existing therapies. Therefore in a Phase I trial, it can be maintained that there are two purposes: a research component and an additional therapeutic benefit (Baruchel and Rowell 1998).

Most Phase I studies also seek to define the pharmacokinetics of the investigational agents. Variability in systemic exposure among patients should be defined in the scope of a Phase I study. This may help in delineating sub-groups of patients within the trial who are especially susceptible to drug toxicity or lack of efficacy. This information can lead to dose modification. Furthermore, pharmacokinetic studies in Phase I trials may allow modification of sampling strategies that will facilitate the study of pharmacokinetic-pharmacodynamic

relationships in larger populations of children should the investigational agent proceed to Phase II trials (Baruchel and Rowell 1998).

1.3 Anticancer Therapy

Surgery, cytotoxic chemotherapy and radiation represent three modalities in the control and cure of neoplastic diseases. While the skill in using these techniques has improved over the past 30 years, precisely targeting malignant tissue without damaging normal tissue remains a major challenge (Capizzi 1999b). The effectiveness of anticancer therapy depends on how toxic the drugs are to the tumor relative to healthy cells in the body. Bone marrow cells, as well as the rapidly dividing cells of the gastrointestinal tract and hair follicles, are often the most susceptible in addition to tumors. Nevertheless, many other tissues in the body are also affected by chemotherapeutic agents to a certain extent (Wolf *et al* 1987).

Chemotherapeutic agents are usually administered at their MTD as this often displays the greatest efficacy against the tumor. Furthermore, the delivery of adequate doses of chemotherapy is often compromised by the narrow therapeutic index of these cytotoxic agents (Capizzi 1999b). Damage to normal cells results in dose-limiting toxicity (DLT) which compromises the patient's quality of life and may prevent delivery of adequate doses to control the tumor (Links and Lewis 1999). Despite the fact that improved supportive care measures have lessened the toll of treatment on patients, further research is still required to ensure that patients receive optimal therapeutic benefit (Capizzi 1999b).

1.3.1 Cytoprotective Agents

Cytoprotective agents have been developed as a means of reducing the toxicity associated with chemotherapeutic agents. The aim of cytoprotective agents is to improve the therapeutic index of the cytotoxic drugs by reducing the DLT to normal tissue (Links and Lewis 1999). By

definition, a cytoprotective agent must not compromise the antitumor efficacy of the chemotherapeutic drug (Links and Lewis 1999). Ideally, it should be easily administered, have a reasonable safety profile of its own and cannot interfere with the delivery of chemotherapy (Capizzi 1999a; Links and Lewis 1999). By improving the patient's tolerance to anticancer therapy, the cytoprotective agent may offer multiple benefits including potential dose intensification and prolongation of therapy, which may improve response and/or cure rates. As a result, the costs associated with supportive care in the event of complications are reduced significantly and quality of life is improved (Capizzi 1999a).

The concept of cytoprotection was first introduced by Patt *et al.* (1949) who reported that rats pretreated with the sulfhydryl amino acid cysteine were protected from lethal doses of radiation. This sparked the interest in using thiol agents as potential cytoprotectants (Capizzi 1999a). Thiol groups contain a nucleophilic sulfur, which provides an alternative target for the reactive intermediates formed following treatment with radiation (oxygen free radicals) or alkylating agents (electrophilic intermediates). These highly reactive species are responsible for both the efficacy and toxicity of anticancer treatments. They bind to various nucleophilic targets such as DNA, and form covalent bonds without distinction between normal and cancerous tissue. Therefore, thiol-containing compounds in healthy tissue provide an alternative nucleophilic target to reactive intermediates generated by radiation or chemotherapy (Links and Lewis 1999; van der Vijgh and Korst 1996).

To date, three cytoprotective agents have been approved by international regulatory agencies. Mesna is administered to protect the bladder from toxicity associated with ifosfamide and high-dose cyclophosphamide whereas dexrazoxane protects the heart from cumulative cardiotoxic effects associated with doxorubicin (Capizzi 1999a). Both of these agents are drug-specific and their protective effects are limited to specific organs. A third agent, amifostine, is the first selective *broad-spectrum* cytoprotective agent approved by international regulatory

agencies. In this context, broad-spectrum refers to the protection of multiple organ systems from a broad selection of cytotoxic agents (see Table 1) (Capizzi 1999a).

Table 1: Broad-spectrum cytoprotection by amifostine. Various organs are protected from many different cytotoxic drugs (Capizzi 1999a).

Organs	Drugs
Kidney	Cisplatin
Auditory	Carboplatin
Peripheral nerves	Cyclophosphamide
Bone marrow	Nitrogen mustard
Salivary glands	Melphalan
Oral mucosa	Mitomycin-C
Heart	Carmustine
Lung	5-fluoruracil
Esophagus	ionizing radiation
Small intestine	...others
Colon	
Immune system	
Testes	
Genome	

1.4 Amifostine

1.4.1 Development

The Walter Reed Army Institute of Research originally developed amifostine during the Cold War as part of the anti-radiation drug development program of the US Army. The goal of this program was to identify compounds that were capable of protecting the body against the adverse effects of radiation in order to safeguard the military personnel in event of nuclear war. Amifostine was selected from 4 400 compounds that were screened since it offered the most effective radioprotection and it had the safest toxicity profile as evidenced by its high MTD relative to other compounds that were tested. Mice, dogs and monkeys that were given amifostine were protected from lethal doses of whole body irradiation (Capizzi 1999a).

Subsequently, Yuhas and Storer demonstrated that with amifostine pretreatment, normal tissues were protected from the non-specific toxicities of therapeutic doses of radiation while tumors were not (Yuhas and Storer 1969). Thereafter, studies demonstrated that amifostine was effective in protecting normal tissue from the toxic effects of alkylating agents, cisplatin, anthracyclines and taxanes (Capizzi 1999a). To date, it is the broad-spectrum cytoprotective agent with the largest preclinical and clinical data base (Capizzi 1999a).

1.4.2 Metabolism

Amifostine (WR-2721: Ethyol: S-2 (3-aminopropylamino) ethylphosphoro-thioic acid) is a phosphorylated amino thiol prodrug. It is dephosphorylated by membrane-bound alkaline phosphatase at the tissue site to the free thiol WR1065 (the active metabolite and major cytoprotective species). WR1065 is rapidly taken up into cells where it exerts its cytoprotective effects. It can be further oxidized to disulfides, either symmetrical (WR33278: may also be cytoprotective (Shigematsu *et al* 1994)) or mixed disulfides with cysteine, glutathione or thiol containing proteins (Figure 1) (Capizzi 1999a).

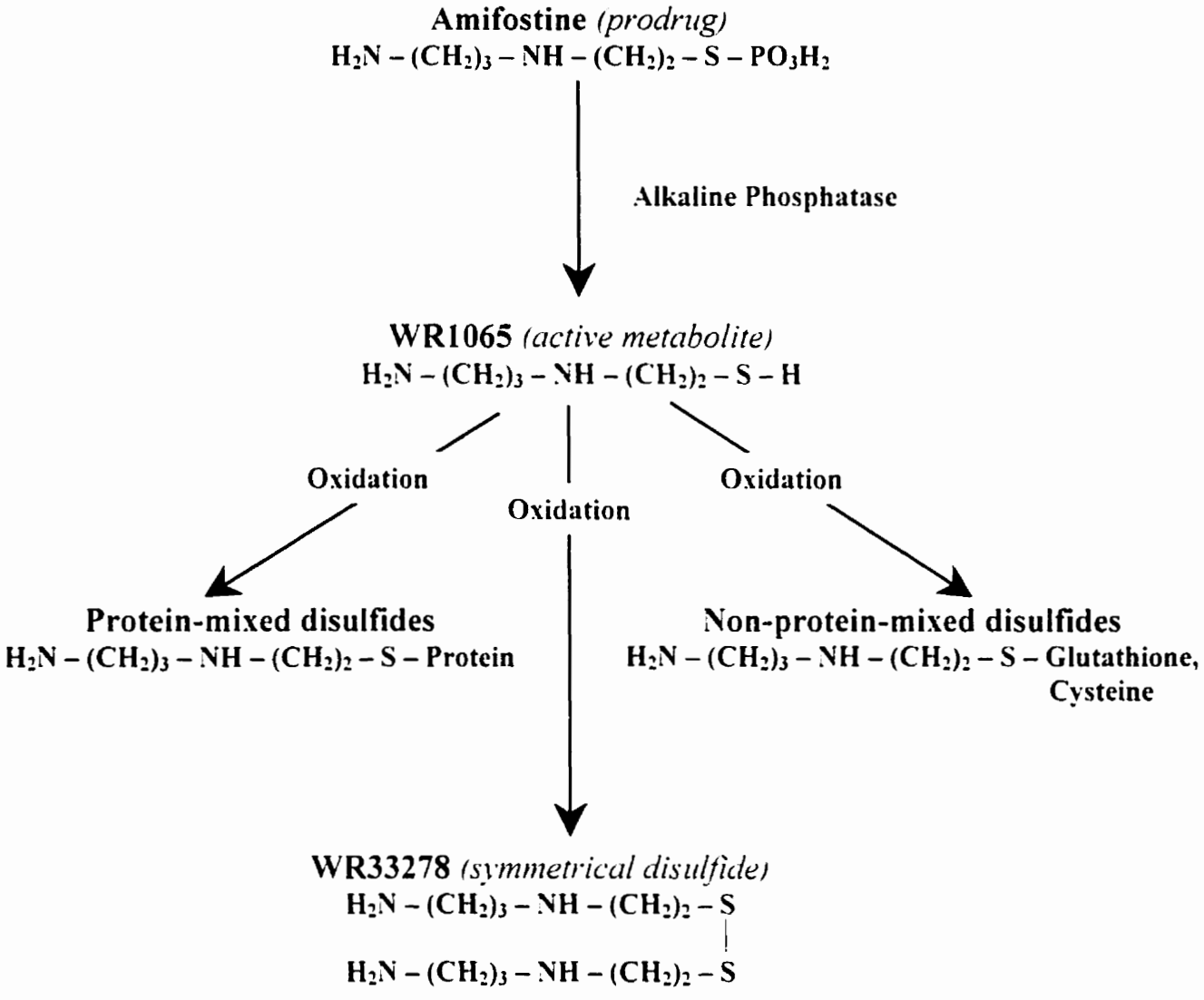


Figure 1: Amifostine metabolism (taken from (Shaw *et al* 1994))

1.4.3 Preclinical Studies

The preferential uptake and distribution of amifostine and its metabolites into normal tissue (except for brain tissue) has been demonstrated in several animal models using mice and rats (Tanaka 1984; Utley *et al* 1976; Yuhas 1980). Maximal concentrations of WR1065 as well as the disulfides are achieved between 10 and 30 minutes after administration providing useful information for defining the time at which the maximal protective effect may be observed for translation to dosing regimens to be used in clinical studies. For example, Yuhas and Storer (1969) showed that amifostine administered 15 minutes prior to nitrogen mustard doubled the LD_{50} dose (*i.e.*, the dose required to kill 50% of the animals) without altering the effect of the chemotherapeutic drug on lung carcinoma in C57BL/6 mice.

In vitro work has been carried out to investigate the effect of amifostine pretreatment on a variety of bone marrow progenitor cells, as these are especially sensitive to the toxic effects of chemotherapy. It has been demonstrated that these cells are protected from the damaging effects of carboplatin (Doz *et al* 1991), cyclophosphamide derivatives (4-hydroperoxy-cyclophosphamide, mafosfamide) (Douay *et al* 1995; Shpall *et al* 1994), paclitaxel, doxorubicin, daunorubicin, mitoxantrone (List *et al* 1996) and photodynamic therapy (Meagher *et al* 1989) with amifostine pretreatment. However, there are many published accounts of amifostine's protection of other organs. These include protection against cisplatin-induced nephrotoxicity (Capizzi 1999c), melphalan toxicity (Millar *et al* 1982) and radiation damage (Sigdestad *et al* 1975) in intestinal crypt cells, cisplatin-induced damage in neural tissue (Mollman 1991) and aminoglycoside-induced hearing loss (Pierson and Moller 1981). In addition to amifostine's reduction in acute, often-reversible radiation induced toxicities, it may also decrease permanent radiation-induced damage of some tissues such as salivary glands (Sodicoff *et al* 1978) or the soft tissue in the limbs, which can cause limb contractures (Milas *et al* 1982). It is therefore

evident that amifostine is indeed a broad-spectrum cytoprotective agent with potential applications in a wide variety of settings.

1.4.4 Mechanism of Action

The phosphorylation of amifostine appears to be a significant contributor to the selective uptake of WR1065 by normal tissue in contrast to the insignificant uptake by tumor tissue. In fact, normal tissue can accumulate 50 to 100 times more WR1065 than tumor tissue (Capizzi 1996; Links and Lewis 1999; Nici and Calabresi 1999) and this is thought to be due to certain characteristic differences between the two types of tissue.

In general, tumor tissue tends to be hypovascular relative to normal tissue (Nici and Calabresi 1999). Moreover, normal tissues have higher concentrations of alkaline phosphatase, which is located on the plasma membrane of endothelial cells, especially at the capillary level. This localization of alkaline phosphatase increases the conversion of amifostine to the free thiol, thus allowing its rapid uptake into normal tissue (Capizzi 1999a; Shaw *et al* 1996a). Furthermore, studies have demonstrated that the specific activity of membrane-bound alkaline phosphatase is 275-fold higher in normal lung cells than in non-small cell lung cancer cells (Yang *et al* 1995). The neutral environment of normal tissue compared to the acidic environment of tumor tissue also favours the action of alkaline phosphatase (Capizzi 1999a; Nici and Calabresi 1999) and the rate constant for WR1065 uptake increases with increasing pH with a general preference to a normal tissue pH of 7.4 (Calabro-Jones *et al* 1988). Finally, normal tissue has a temperature-dependent, non-adenosine triphosphate dependent and non-sodium dependent transport system consistent with a carrier mediated facilitated diffusion transport process. In contrast, tumor tissues rely on passive non-facilitated uptake of WR1065 (Yang *et al* 1995; Yuhas 1980).

All these factors combined allow for the selective uptake and concentration of WR1065 in normal cells, which contributes to a temporary state of acquired resistance to the effects of chemotherapy and radiation in normal tissue. This concentration of WR1065 is analogous to a heightened intracellular concentration of the endogenous protector, glutathione. Tumor tissue, however, remains vulnerable to the cytotoxic effects of chemotherapy and radiation (Capizzi 1999a).

Although the exact mechanism by which WR1065 protects normal tissue has not been clearly delineated, several mechanisms have been proposed:

1. The free thiol can act as a nucleophile that binds directly to and detoxifies the active species of alkylating and platinum agents. This reduces the formation of DNA-DNA interstrand crosslinks induced by the alkylating agent nitrogen mustard as well as the formation of platinum-DNA adducts (Capizzi 1999a). WR1065 can also partially reverse preformed platimum-DNA adducts when administered after agents such as cisplatin although it is less effective (Capizzi 1999a; Nici and Calabresi 1999).
2. WR1065 can act as a scavenger of oxygen free radicals formed after chemo- or radiation therapy (Capizzi 1999a; Nici and Calabresi 1999) by donating a hydrogen to repair damaged target molecules such as DNA (Grdina *et al* 1999; Nici and Calabresi 1999). This suggests that amifostine administration after chemotherapy can enhance its cytoprotective effect.
3. Treating normal cells with WR1065 *after treatment* has been shown to reduce radiation- and chemical induced apoptosis. It is hypothesized that either WR1065 or WR33278 (symmetrical disulfide) bind to DNA and nuclear proteins, altering the structures of the internucleosomal region of the chromatin thus making them less vulnerable to degradation. Further work is still required in this area to explore the cytoprotective implications of this observation (Capizzi 1999a).

4. The symmetrical disulfide metabolite of amifostine, WR33278, also demonstrates a cytoprotective effect (Grdina *et al* 1995). It can bind to DNA and enhance relaxation of DNA supercoils that are mediated by topoisomerase I. It has also been shown that this disulfide metabolite can protect cells from radiation-induced cytotoxicity and mutations (Capizzi 1999a).
5. Recently, selective modulation of intracellular glutathione levels by amifostine, or more specifically, WR1065 has been proposed as a possible mechanism of action of the cytoprotective effect. This is hypothesized to occur via enhanced cysteine delivery into normal cells (Issels and Nagele 1989; Souid *et al* 1999).

Given that WR1065 demonstrates more efficient tissue protection than rescue and based on its selective uptake and retention, it must be present in tissue when protection from cytotoxic treatment is required. Since peak levels of WR1065 are achieved shortly after amifostine administration, laboratory and clinical protocols generally administer the drug as a 15-minute infusion (van der Vijgh and Korst 1996) 5 to 30 minutes prior to cytotoxic therapy (Capizzi 1999a). Although many studies have used single doses, there is emerging laboratory (Green *et al* 1994) and clinical evidence (Betticher *et al* 1995; Budd *et al* 1994) that support the administration of multiple doses of amifostine to maximize cytoprotection and protocols using two or three doses suggest that such a dosing schedule is safe and does not interfere with anti-tumor efficacy. Furthermore, administering amifostine after chemotherapy can be especially beneficial for drugs that have long half-lives (Capizzi 1999a; van der Vijgh and Korst 1996).

1.4.5 Adult Clinical Trials

1.4.5.1 Pharmacodynamics

The primary goal of including amifostine in conventional dosing regimens of cytotoxic compounds is to reduce toxicity. The majority of recent trials have examined the efficacy of amifostine in conjunction with platinum-based regimens. Betticher *et al.* (1995) performed a randomized Phase II trial comparing amifostine at 910 mg/m^2 dose with carboplatin at 600 mg/m^2 to carboplatin alone in patients with non-small cell lung carcinoma. Amifostine was administered 20 minutes before and again 2 and 4 hours after carboplatin administration. There was no significant difference in neutrophil nadir or recovery between the two groups. However, the time to platelet recovery and the need for hospitalization favoured the amifostine arm. Budd *et al.* (1997) carried out a larger study in which 55 patients with a variety of tumors were randomized to carboplatin (500 mg/m^2) with or without amifostine (910 mg/m^2 dose) given 15 minutes before and 2 hours after carboplatin. They observed that the median platelet nadir for all cycles was higher in the amifostine arm and there was no significant difference in the incidence of neutropenia.

The best evidence of amifostine's efficacy in reducing chemotherapy-induced toxicity was provided by Kemp *et al.* (1996) in a large-scale randomized study of 242 stage III and IV ovarian cancer patients. These patients were treated with 100 mg/m^2 of cisplatin and 1000 mg/m^2 of cyclophosphamide every three weeks. Patients were randomized into an amifostine group (910 mg/m^2 as a 15-minute infusion before cyclophosphamide) or no amifostine. Dose reductions were scheduled for hematological toxicity. Significantly fewer patients discontinued treatment in the amifostine arm and there were significant reductions in the incidence of neutropenia with fever, days in the hospital and grade 2/3 neurotoxicity. Most importantly, there was no compromise in the pathological response rate or median survival time. In summary,

there is clinical evidence that amifostine can effectively reduce acute and cumulative toxicity such as neutropenia, thrombocytopenia, nephrotoxicity, neurotoxicity (including ototoxicity and peripheral neuropathy), musculoskeletal toxicity, cardiotoxicity and mutagenicity caused by anticancer drugs (Rose 1996).

1.4.5.2 Pharmacokinetics

There are few published reports of amifostine pharmacokinetics in humans. The majority of data exists in adults with extremely limited data available in children. After a 10-second bolus injection of 150 mg/m^2 amifostine to 13 cancer patients, maximal peak concentrations were reached within 2 minutes (Shaw *et al* 1986). The same was noted in patients treated with a 15-minute infusion of 740 or 910 mg/m^2 of amifostine (Korst *et al* 1997a). In the patients who were given the bolus injection of amifostine, it was noted that the prodrug had a small volume of distribution (6.4 L) indicating that it was primarily confined to the intravascular space (mainly plasma) (Shaw *et al* 1986). Furthermore, amifostine is very rapidly cleared from the plasma compartment (clearance = 2.17 L/minute; (Shaw *et al* 1986) as indicated by its short half-life (distribution half-life of 0.88 minutes; elimination half-life of 8.76 minutes; (Shaw *et al* 1986)). This rapid clearance is further supported by the observation that 90% of the drug has disappeared from the plasma 6 minutes after administration (Shaw *et al* 1986). Again, similar results were noted in patients who received a 15-minute infusion of 740 mg/m^2 (Shaw *et al* 1988). Only about 1% of the dose appeared in cancer ascites (Korst *et al* 1996), suggesting that the presence of ascites or other third-space fluid accumulations would not impact plasma or tissue pharmacokinetics of amifostine.

It is not entirely clear whether infusion of multiple doses causes increases in peak plasma concentrations of the prodrug and its active metabolite, WR1065. When patients were treated with 15-minute i.v. infusions of amifostine (740 mg/m^2) 15 minutes before as well as 2 and 4

hours after a carboplatin dose, mean peak plasma concentrations of 240 μM amifostine, 48 μM WR1065 and 184 μM mixed disulfides were obtained immediately after the first infusion (Korst *et al* 1997a). Similar levels were also seen in a study by Shaw *et al.* (Shaw *et al* 1996b). Peak plasma amifostine concentrations after the second and third infusions were similar to the peak after the first dose (Korst *et al* 1997a; van der Vijgh and Korst 1996) while some reported a very slight increase in the peak levels of WR1065 (Korst *et al* 1996; Korst *et al* 1997a).

Most of the pharmacokinetic studies have focussed on the behavior of the parent drug due to a lack of reliable methods for the quantification of the metabolites. Consequently, only a few reports could be located that described metabolite pharmacokinetics. After administration of a bolus of 150 mg/m^2 to 6 patients, only very small amounts of amifostine and its metabolites were excreted in the urine (0.69% of the administered dose was excreted as amifostine, 2.64% as WR1065 and 2.22% as WR33278) (Shaw *et al* 1986). Similar results were observed following administration of 740 mg/m^2 as a 15-minute infusion (Shaw *et al* 1988). Maximal concentrations of WR1065 were observed shortly after bolus injections and infusions of the prodrugs (Korst *et al* 1997a; Shaw *et al* 1986; Shaw *et al* 1996b). Following this, plasma WR1065 concentrations rapidly declined (with an initial half-life of 10.8 minutes (Korst *et al* 1997a)). This short half-life can be explained by fast uptake into tissues and rapid oxidation into disulfides (Korst *et al* 1997a).

After a single dose of amifostine, the total disulfides were still detectable after 24 hours (~3 μM) (Korst *et al* 1997a; van der Vijgh and Korst 1996). In one patient receiving three 740 mg/m^2 doses of amifostine, the final plasma half-life of the disulfides was 13.6 hours indicating that disulfides are cleared much slower than amifostine and WR1065 (Korst *et al* 1996).

In summary, all the pharmacokinetic studies carried out to date confirm the rapid clearance of the drug, the minimal elimination phase, small volume of distribution of the parent drug and that maximal concentrations of amifostine and WR1065 are achieved at the end of the

infusion (Korst *et al* 1996; Korst *et al* 1997a; Shaw *et al* 1986; Shaw *et al* 1996a; van der Vijgh and Korst 1996). Furthermore, studies with radiolabeled amifostine indicate that the drug does not bind to plasma proteins, therefore, the entire administered dose is free to interact with alkaline phosphatase (Shaw *et al* 1996a). This supports the hypothesis that the parent drug leaves the bloodstream rapidly and enters normal tissue where it exerts its protective effect (Shaw *et al* 1986). The amount of amifostine converted to WR1065 in the systemic circulation is small relative to the amount dephosphorylated in normal tissue (Capizzi 1999a) and this supports that metabolism of the prodrug occurs at its site of action.

1.4.5.3 Toxicities

The principle side effects associated with amifostine treatment in adults are hypotension (Kemp *et al* 1996; Rose 1996), nausea and vomiting (Links and Lewis 1999; Rose 1996). In a study conducted by Rose (1996), addition of amifostine to cyclophosphamide and cisplatin treatment increased the incidence of nausea and vomiting although the difference was not statistically different from the chemotherapy-only arm. Hypotension is another relatively common side effect associated with amifostine. 61.5% of the patients in Rose's study (1996) required interruption of the amifostine infusion due to hypotension. Reductions in systolic blood pressure are generally noted near the end of the amifostine infusion but are generally transient in nature.

Other reported side effects include flushing, sneezing, dizziness, sleepiness, hiccups, chills, metallic taste, anxiety and urinary retention. All of these are generally transient in nature and do not interfere with therapy (Links and Lewis 1999; Rose 1996). Hypocalcemia may also occur, particularly with daily administration of amifostine. It is believed that this is caused by inhibition of parathyroid hormone activity, however, calcium supplementation may reverse this effect (Wadler *et al* 1993).

1.4.6 Pediatric Clinical Trials

The potential cytoprotective benefits offered by amifostine may be particularly important in pediatric oncology. Children treated for cancer tend to have lower granulocyte and platelet nadirs after chemotherapy than adults and consequently tend to require more frequent transfusions and treatment for fever neutropenia. Additionally, the duration of chemotherapy in children tends to be months longer than in adults and they often receive multimodal therapy so the cumulative effects of treatment can be of greater concern in this population. Since great proportions of children with cancer are cured, delayed and chronic toxicities are more troublesome and they have a greater risk of developing secondary malignancies and infertility. Furthermore, younger patients are more vulnerable than older patients to growth, CNS, cardiac and endocrine sequelae of chemo- and radiotherapy. Since amifostine is associated with broad-spectrum protection from cytotoxic therapy, it may have a potentially greater impact in young patients (Alberts and Bleyer 1996).

Trials of amifostine use in pediatric patients are extremely limited. Only a few studies have been reported. A Phase I trial (Adamson *et al* 1995) studied 19 patients ranging from 3 to 25 years of age with refractory tumors. They were treated with a single dose amifostine 15 to 30 minutes prior to melphalan. The amifostine dose was escalated in groups of three patients at a time from 740 mg/m^2 to 1 650 mg/m^2 while the melphalan dose was held constant at 35 mg/m^2. Hematologic DLTs from melphalan were observed in all patients treated with amifostine doses between 740 and 1 300 mg/m^2 but in none of the patients treated with 1 650 mg/m^2. Subsequently, the amifostine dose was increased to 2 100 and 2 700 mg/m^2 while melphalan was increased to 45 mg/m^2 but these higher doses did not prevent melphalan-induced hematologic DLTs. In addition, these amifostine doses were associated with reversible anxiety and urinary retention. The investigators of this study concluded that a dose of 1 650 mg/m^2 should be used for pediatric Phase II studies even though DLTs were not observed until 2 700 mg/m^2 (three

times the adult MTD). Unfortunately, they also concluded that amifostine was ineffective in preventing melphalan-induced myelosuppression.

In a critical analysis of the above study, Alberts and Bleyer (1996) suggested that the conclusion that amifostine is not efficacious in preventing melphalan toxicity is limited for several reasons. Firstly, there was no control arm in the study so amifostine and melphalan treatment was not compared to mephalan treatment alone. Secondly, the lack of cytoprotection conclusion was drawn on a very small group of patients (n = 3). Finally, cytoprotection from melphalan-related toxicity may require extended exposure to amifostine rather than the exposure resulting from a single dose. Hence a second amifostine dose after melphalan administration may have made the protective effect clearer.

In a single case study (Borsi *et al* 1996a), a 17 year old boy with heavily pretreated recurrent medulloblastoma had received five courses of carboplatin on 2 consecutive days (600 mg/m^2/day) over 8 months. The first four doses caused increasing hematologic toxicity and hence with the fifth dose, he was given a 740 mg/m^2 dose of amifostine twice. One dose was administered 15 minutes before carboplatin and the second was given 2 hours after each carboplatin dose. The patient served as his own control and there was evidence that amifostine reduced the duration of both neutropenia and thrombocytopenia along with the number of platelet transfusions and the time to complete hematological recovery. Of the side effects experienced, all were transient and included hypotension, hypothermia, nausea, flushing, chills, dry mouth and blurred vision.

This case report resulted in a Phase I/II trial (Borsi *et al* 1996b) in which amifostine was used with high-dose carboplatin in children with relapsed or refractory malignancies. The goals of this study were to examine the safety and tolerability of amifostine in children and to investigate the efficacy of amifostine in preventing or decreasing the myelotoxicity associated with high dose carboplatin. Seven patients were given 740 mg/m^2 of amifostine prior to and 2

hours after the administration of 600 mg/m^2 of carboplatin on two consecutive days. Again, all side effects of amifostine were reversible. In the six evaluable patients, four showed reduced carboplatin related hematopoietic toxicity, two had complete hematopoietic recovery by day 21 post treatment, reduced bone marrow toxicity and a decrease in need for supportive treatment.

The studies reported here provide preliminary data regarding the safety and tolerability of amifostine in pediatric patients as well as somewhat limited evidence of cytoprotection. However, data regarding pharmacokinetics of amifostine in children are lacking but would be useful to help determine appropriate dosing schedules for this population as it is becoming evident that multiple doses of amifostine may be more effective than single doses.

1.5 Glutathione

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine; GSH; mol. wt. 307) that is the major low-molecular-mass thiol compound in plants and animals (Sies 1999) (Figure 2). It acts as a "redox buffer" by maintaining a given thiol/disulfide redox potential in cells. Its peptidic γ-linkage is thought to protect it from degradation by aminopeptidases (Sies 1999). Glutathione is found in many forms in cells, tissues and plasma. Glutathione disulfide (GSSG) arises when GSH is oxidized, however, oxidation of GSH can also lead to formation of GSSR (mixed disulfides e.g., glutathione-cysteinyl disulfides on proteins) (Sies 1999).

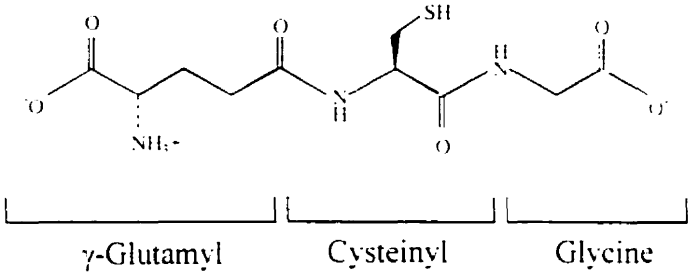


Figure 2: Glutathione structure

Glutathione is synthesized from L-glutamate, L-cysteine and glycine in two consecutive steps catalyzed by γ-glutamyl-cyteine synthase and glutathione synthase (Sies 1999). Cysteine is the rate-limiting amino acid in this reaction scheme (Baruchel *et al* 1995; Baruchel and Viau 1996; Wang *et al* 1996). Degradation to the constituent amino acids occurs via γ-glutamyl-transpeptidase and cysteinyl-glycine dipeptidase (Sies 1999).

The major fates of GSH include hydrolytic cleavage, disulfide formation and formation of thioethers and thiolesters. Glutathione S-transferases (GST) are enzymes that generate glutathione S-conjugates and use GSH in detoxification (phase II metabolism and elimination) (Sies 1999).

Once GSH is synthesized, it can be transported across biological membranes to take part in an elaborate inter-organ transport network. This applies to GSH export from the liver (a site of major GSH synthesis) to the bloodstream for supply to other tissues, as well as to GSSG release whereby it is transported into the bile for excretion (Sies 1999). Virtually all major biological processes involve the thiol redox state and so it is inevitable that GSH would play important biological and physiological roles.

Particularly, cells strive to maintain high levels of GSH. When these levels reach their minimum, GSH is usually regenerated from endogenous GSSG through the action of NADPH-dependent glutathione reductase. Large amounts of NADPH are supplied by the pentose-phosphate pathway through glucose-6-phosphate dehydrogenase (Figure 3). As a result of this reaction, endogenous GSH generally returns to its normal level (Usal *et al* 1996). Problems arise when there is a constant presence of ROS and the cells are in a constant state of GSH deficiency. If the GSH/GSSG ratio cannot be restored to its normal level, continued exposure to ROS results in enhanced release of GSSG and ultimately to cell lysis (Engin 1995).

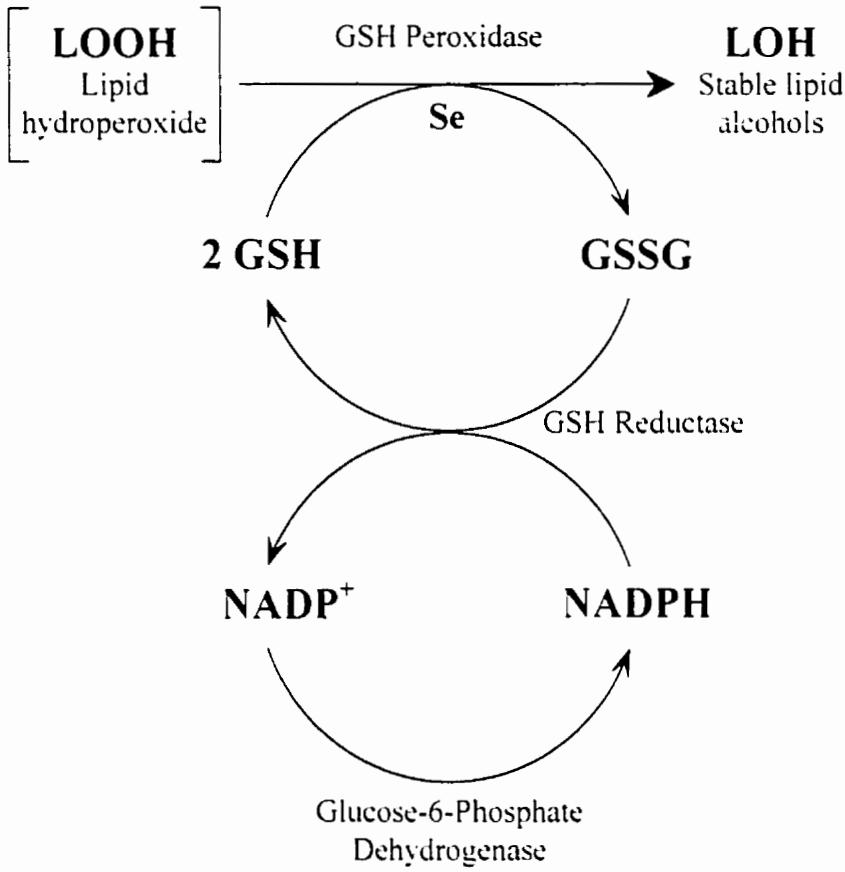


Figure 3: Glutathione homeostasis (Wells 1997)

1.5.1 Glutathione and Cytoprotection

Recently, the interest in GSH has shifted from its biochemical, physiological and toxicological functions to its medical and clinical aspects including the role it plays in carcinogenesis and drug resistance (Sies 1999). GSH exerts its cytoprotective properties via several different mechanisms. These include reaction of its nucleophilic sulfhydryl group with reactive oxygen species (ROS), restoration of damaged molecules via hydrogen donation, reduction of peroxides and maintaining protein thiols in their reduced state (Navarro *et al* 1997). Furthermore, GSH is an important cofactor or substrate for a number of protective enzymes such as GSH peroxidase and GSH S-transferases (Navarro *et al* 1997). Given GSH's key role in protecting cells from free radicals and electrophiles, it has been shown that cells containing high levels of GSH are more resistant to radiation or chemical-induced injury as compared to those containing lower levels (Navarro *et al* 1997).

1.5.2 Glutathione and Anticancer Drugs

Glutathione and its dependent enzymes provide the primary defense mechanism in cells against the cytotoxic effects of chemotherapeutic agents (Wolf *et al* 1987). The anticancer drugs in which glutathione may play an important role in detoxification can be divided into two categories:

1. Drugs which are detoxified by direct conjugation with glutathione (Figure 4)
2. Drugs that form ROS, which are subsequently detoxified by the oxidation of GSH to GSSG catalyzed by glutathione peroxidases (Wolf *et al* 1987) (Figure 5).

The relative abundance of glutathione in normal and tumor cells is an important factor in determining the therapeutic index of chemotherapy. Work *in vitro* and *in vivo* has shown that the

total glutathione concentration is positively correlated with resistance to chemotherapy and radiation therapy (Bump *et al* 1990).

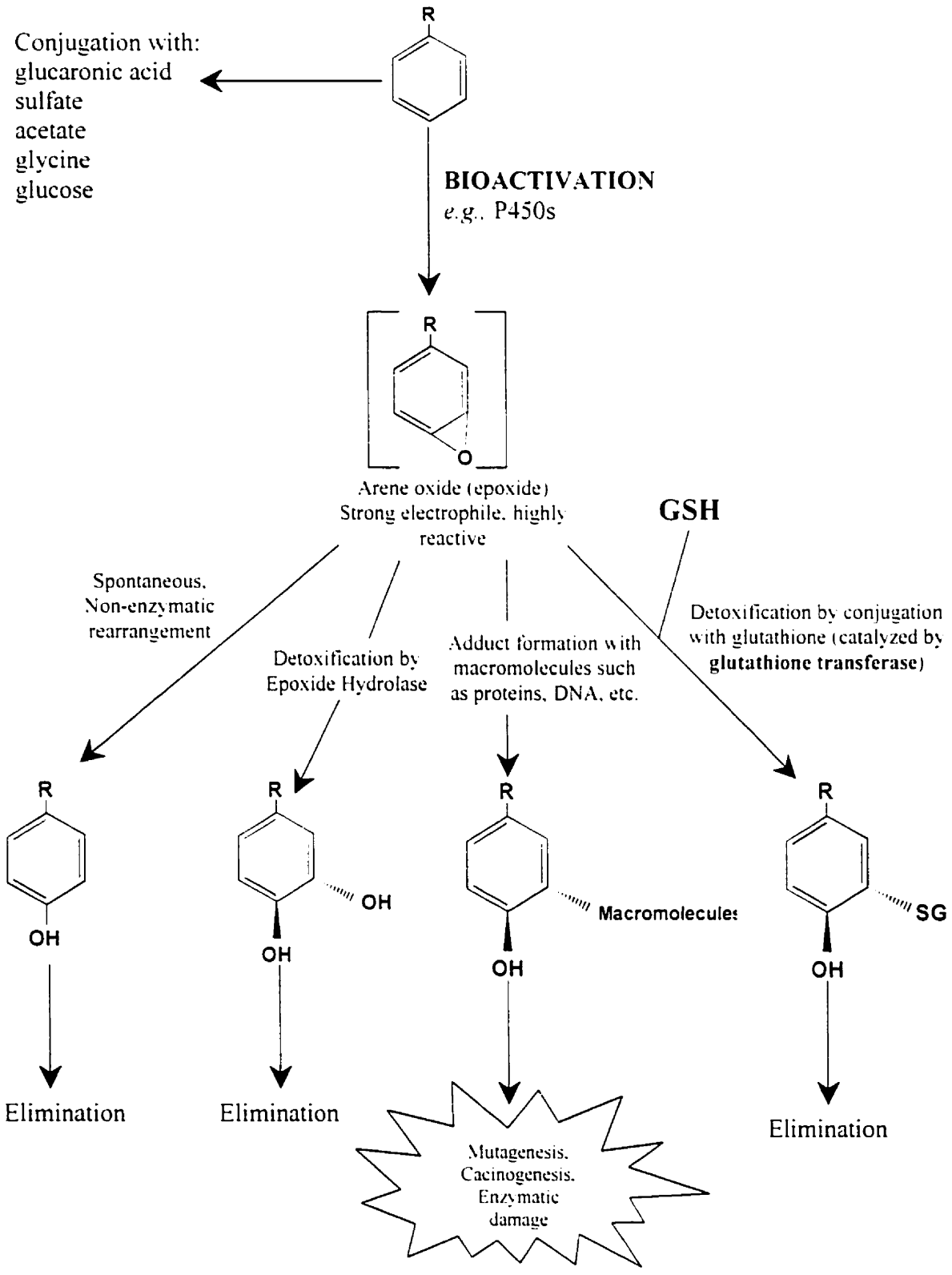


Figure 4: Detoxification of reactive metabolites by glutathione (Wells 1997)

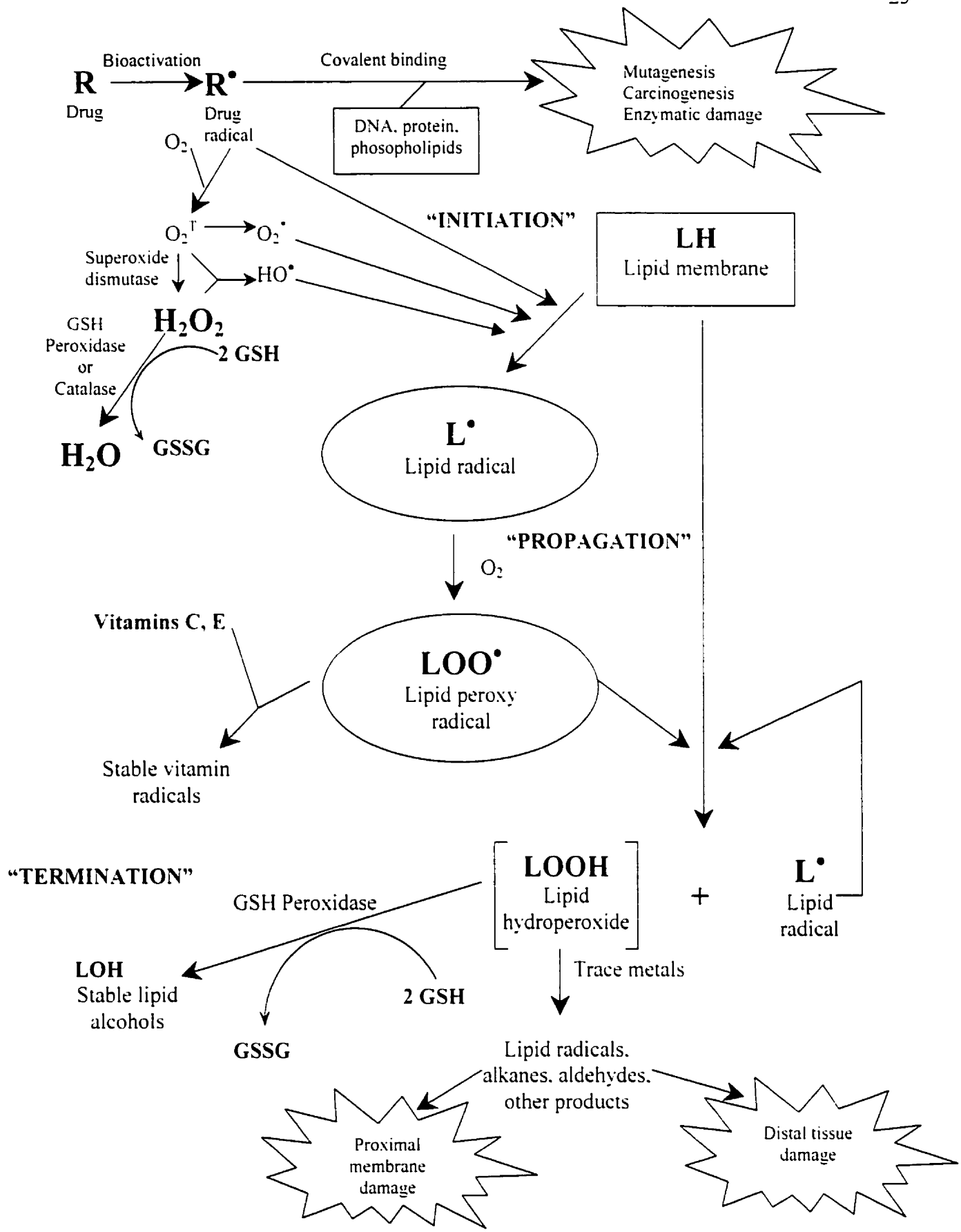


Figure 5: Free radical toxicity and detoxification by glutathione (Wells 1997)

1.5.3 Modulating Glutathione Levels as an Adjunct to Cancer Therapy

In the past two decades, a great deal of effort has been put into identifying agents that can reduce the toxicity of anticancer drugs to normal tissue by elevating glutathione levels in normal tissue. It was found that compounds containing sulfhydryl groups could achieve this goal. One specific example is Mesna (Wolf *et al* 1987). The main concern with the use of such agents is that reduction in toxicity to normal tissue may be accompanied by a compromised antitumor effect. Ultimately, it would be desirable to identify an agent that can be used to modulate glutathione levels in all healthy tissues, offering protection from a variety of anticancer treatments.

1.5.3.1 Recent Advances in Modulating Cellular Glutathione Levels

Recent work has focussed on various cysteine-delivery systems as one approach to increase intracellular glutathione levels since cysteine is the rate limiting amino acid in GSH synthesis (Baruchel *et al* 1995; Baruchel and Viau 1996; Wang *et al* 1996). However, supplying cysteine directly to cells has proven to be quite difficult because of its toxicity, inefficient transport into cells and spontaneous oxidation at neutral pH (Meister *et al* 1986).

Several cysteine-delivery vehicles have shown efficacy in selectively altering glutathione levels in normal cells compared to cancer cells. The first is the cysteine prodrug, OTZ (L-2-oxothiazolidine-4-carboxylate). *In vitro* studies have shown this drug to have selectivity for normal cells as opposed to tumor cells (Russo *et al* 1986a) and *in vivo* work has shown that it is effective in increasing GSH levels in normal mouse tissue (Robert and Francetic 1991). Furthermore, it was found to paradoxically reduce tumor GSH (Baruchel *et al* 1995).

A second cysteine delivery system whose role in selective glutathione modulation has been demonstrated is humanized whey protein concentrate (HWPC), which is rich in cystine and glutamyl-cysteine residues (Baruchel and Viau 1996). In normal cells, HWPC stimulates cell

proliferation, which is followed by a parallel increase in intracellular GSH. In contrast, cell proliferation was inhibited in cancer cells treated with HWPC and this was accompanied by a reduction in intracellular GSH (Baruchel and Viau 1996).

The observations described here have great implications for cancer therapy. If one of the most important cellular defense mechanisms can be enhanced in normal tissue and weakened in tumor tissue, a greater therapeutic index for anticancer treatments should theoretically be achievable.

1.5.3.2 Amifostine and its Role in Modulating Glutathione Levels

As mentioned earlier, there are several proposed mechanisms for amifostine's protective effect against radiation- and chemotherapy-induced toxicity, however, the exact mechanism remains to be delineated. Issles *et al.* (1988) demonstrated that exposing cells to aminothiols such as cysteamine and N-acetylcysteine could promote cellular cystine uptake, which could lead to an increase in glutathione by *de novo* synthesis. The same investigators then examined whether amifostine or WR1065 could lead to the same effects. They showed that amifostine exposure alone did not increase cystine uptake relative to control cells, however, if the cells were pretreated with amifostine in the presence of alkaline phosphatase (the enzyme responsible for dephosphorylating amifostine to its active metabolite, WR1065), cystine uptake was enhanced more than two-fold. Since precursor availability was enhanced, glutathione biosynthesis also increased (Issels and Nagele 1989).

The mechanism for this enhanced cysteine delivery to the cells appears to be a two-step process. First, the cysteine mixed disulfide with the added aminothiol is formed from cystine (*i.e.* Cys-SS-WR1065). This is accompanied by a simultaneous generation of equimolar amounts of cysteine in the medium. The second step is the uptake of both cysteine and the cysteine-mixed disulfide across the cell membrane (Issels and Nagele 1989). This promotion of

cystine uptake was strictly dependent on the addition of alkaline phosphatase to the medium. This further supports the proposed mechanism of cysteine-mixed disulfide formation because this reaction requires a free SH group (available in WR1065 but not in amifostine). Since drug hydrophilicity is a major factor in a drug's ability to cross biological membranes, the less hydrophilic thiol derivative such as the cysteine-mixed disulfide would very readily cross the cell membrane (Issels and Nagele 1989). This study also verified that cysteine delivered by WR1065 was indeed utilized in new GSH synthesis.

1.5.4 Blood Glutathione as a Marker of Oxidative Stress

Based on this newly proposed mechanism of action of amifostine, it can be inferred that cellular fluctuations in GSH, due to changes in oxidative profiles, represent an indirect measure of amifostine's cytoprotective capacity. Thus, it would be reasonable to use blood GSH as a potential marker of oxidative stress caused by anti-cancer therapy.

Red blood cells are frequently used in studies of oxidative stress (Giulivi *et al* 1994). Since oxidative stress alters the redox state of various different tissues and interorgan flow of glutathione has been demonstrated, it is reasonable to hypothesize that GSH and GSSG levels in blood would reflect changes in glutathione status in other less accessible tissues (Navarro *et al* 1997). Furthermore, cells tend to maintain low levels of GSSG. A rise in cellular GSSG levels is often accompanied by its release into extracellular space (Navarro *et al* 1997). Therefore, the radiation-, chemical- or disease-induced increase in blood GSSG levels may be due, at least in part, to its export from other organs (Navarro *et al* 1997). Table 2 shows baseline GSH and GSSG levels as well as the GSH/GSSG ratios of various tissues including blood in mice. This illustrates the correlation between blood and tissue glutathione levels.

Table 2: Baseline GSH and GSSG concentrations in various mouse tissues (from (Navarro *et al* 1997))

Tissue	GSH *	GSSG *
Blood	1240 ± 140	4.3 ± 1.2
Brain	1554 ± 226	19 ± 6
Lung	1672 ± 441	30 ± 17
Heart	719 ± 207	15 ± 7
Liver	6790 ± 859	21 ± 10
Pancreas	592 ± 133	12 ± 5
Spleen	3017 ± 359	31 ± 19
Kidney	2468 ± 404	28 ± 9
Skeletal Muscle	525 ± 117	16 ± 8
Bone Marrow	347 ± 52	17 ± 6

* Blood concentrations are expressed in μM units (n = 10)
Tissue concentrations have units of nmol/g of tissue (n = 6)

All results are expressed as mean ± S. D.

Several studies have demonstrated the usefulness and practicality of measuring blood glutathione for various pathological and physiological purposes. Herebergs *et al.* (1992) attempted to correlate erythrocyte glutathione levels of cancer patients receiving their first course of chemotherapy and response to treatment. It was hypothesized that erythrocyte glutathione would reflect the glutathione status of the less accessible tumor tissues. This was based on the observation that patients with lower pretreatment GSH levels tend to have complete or partial response to chemotherapy while those who had higher GSH levels tended to have stable or progressive disease. Since higher GSH levels represent a mechanism for drug resistance (Navarro *et al* 1997), this inference may be true.

In another study, Navarro *et al.* (1997) (1997) demonstrated how a single dose of irradiation could significantly decrease the glutathione redox ratio in the whole blood of mice.

This was mainly due to increases in GSSG, as GSH levels did not change significantly. The change was rapid and detectable as early as 10 minutes after radiation with a maximal increase in GSSG observed 2 hours after radiation. Similar findings were observed in humans. The GSH:GSSG ratio decreased in a dose-dependant manner and this was mainly due to an increase in GSSG with decreases in GSH playing only a minor role (Navarro *et al* 1997).

Blood glutathione has also been used as a marker of oxidative stress in many other pathological and physiological conditions including acute myocaridal infarction (Usal *et al* 1996), HIV infection (Rodriguez *et al* 1998), cystic fibrosis (Lands *et al* 1999), inflammatory bowel disease (Sido *et al* 1998), diabetes (Sharma *et al* 2000), physical exercise (Laaksonen *et al* 1999; Sen 1999; Vina *et al* 1995) and during that process of aging (Michelet *et al* 1995). These examples clearly demonstrate the application of blood glutathione as a relatively non-invasive marker of oxidative stress in a variety of conditions.

2.0 STATEMENT OF PROBLEM and RATIONALE

Despite amifostine's long history, its clinical use has been limited to a few Phase I/II studies. Research to date has demonstrated its promise in reducing chemotherapy-induced toxicity in normal tissues yet amifostine has not advanced into standard care protocols. This may be explained partially by several gaps that exist in the current knowledge of amifostine's pharmacokinetics and pharmacodynamics. Amifostine pharmacokinetic data are extremely limited in humans. Information is largely confined to adult studies whereas pediatric data are lacking. Since children may benefit particularly from the cytoprotective effects of this drug, independent studies in this population are required to determine how children handle the drug and to translate these findings into clinical practice. In addition, although there are several proposed mechanisms of action for amifostine, however, the exact mechanism has not been completely delineated. *In vitro* research has shown that WR1065, the active metabolite of amifostine, can actually increase *de novo* GSH synthesis via enhanced cysteine uptake into normal cells (Issels and Nagele 1989). Given that the patients eligible for this study had solid tumors, blood represents a healthy tissue that can be obtained in a minimally invasive manner. Therefore, whole blood glutathione may be a suitable biological marker of amifostine's cytoprotective effects. The Phase I study presented herein represents an attempt to investigate amifostine's pharmacokinetics and cytoprotective mechanisms in the pediatric population.

3.0 HYPOTHESES

3.1 Initial Hypotheses

Two hypotheses were originally to be tested in this study:

1. Children and adults differ significantly with respect to the pharmacokinetics of amifostine.
2. Amifostine's cytoprotective effect is related to its selective modulation of glutathione levels in healthy tissues.

3.2 Modified Hypotheses

Hypothesis 2 could not be adequately addressed in this study due to identification of deficiencies in the original sample preparation method for glutathione analysis. Therefore the hypotheses were modified to reflect the true nature of the project. The hypotheses that were actually addressed in this thesis were:

1. Children and adults differ significantly with respect to the pharmacokinetics of amifostine.
2. The choice of acid and concentration selected for preparation of biological samples for glutathione analysis greatly impacts sample stability even when the samples are stored at ultra-cold temperatures (-80°C) prior to analysis.

4.0 RESEARCH OBJECTIVES

1. To establish the pharmacokinetics of amifostine, WR1065 and the disulfide metabolites in pediatric cancer patients and to compare these findings to published data obtained in similar adult studies to identify any differences that exist between the two populations.

2. To evaluate whether whole blood glutathione levels are modulated following amifostine administration relative to pretreatment levels.

As a result of having identified problems with the original method for sample preparation for glutathione analysis, a third objective was added to the study:

3. To evaluate the effects of various concentrations of perchloric acid, trichloroacetic acid, metaphosphoric acid and sulfosalicylic acid on glutathione concentrations in samples that were stored prior to analysis, on glutathione concentrations in freshly drawn and immediately analyzed samples, on efficiency of deproteinization and on chromatographic characteristics.

5.0 MATERIALS

Reduced and oxidized glutathione (GSH and GSSG, respectively), penicillamine, ethylene diamine tetracetic acid (disodium salt, dihydrate) (EDTA), metaphosphoric acid (MPA), 5-sulfosalicylic acid (SSA), octane sulfonic acid and HPLC grade water were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Perchloric acid (PCA) stock solution and acetonitrile were obtained from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada). Trichloroacetic acid (TCA) stock solution was purchased from Fisher Scientific Co. (Fair Lawn, New Jersey, USA) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) was obtained from ACP Chemicals Inc. (Montreal, Quebec, Canada).

Amifostine and WR1065 standards were obtained from U.S. Bioscience (West Conshohocken, Pennsylvania, USA). Methanol, monochloroacetic acid, sodium octyl sulfate and ethylamine were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) and sodium hydroxide was purchased from Fisher Scientific (Nepean, Ontario). Dithiothreitol (DTT) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

6.0 METHODOLOGY

6.1 Ethics and Patient Consent

This study was approved by the Research Ethics Board at the Hospital for Sick Children on April 16, 1997. A copy of the approval is included as Appendix 2. Before participating in the study, the goals, potential benefits and hazards were explained to the parents and patients and written consent was obtained. Copies of the information and consent forms are included as Appendix 3.

6.2 Patient Enrollment and Eligibility Criteria

Patients who had a confirmed diagnosis of recurrent or refractory solid malignancies were eligible for this study. The eligibility criteria are listed in Table 3.

Table 3: Eligibility Criteria

- Less than 21 years of age
- Karnofsky score greater than 50%
- Life expectancy greater than 3 months
- Nutritional status: must be greater than 3rd percentile for weight and height, and albumin concentration greater than 3 g/dL
- Evidence of recovery from previous chemotherapy
- No prior radiation to the pelvis or spine for at least six weeks
- No total-body irradiation for six months
- Adequate hematopoietic function (absolute neutrophil count [ANC] >1,500/μl, platelet count >100/μL)
- Adequate renal function (normal creatinine for age or glomerular filtration rate >70 ml/min per 1.73 m^2)
- Adequate hepatic function (bilirubin <1.5 mg/dL, aspartate transaminase (AST) <2 × upper limit of normal for age)
- Adequate pulmonary function (no abnormality on pulmonary function tests)
- Adequate cardiac function (>27% shortening fraction, >50% ejection fraction)

6.3 Study Design: Establishing the Maximum Tolerated Dose of Amifostine

In Phase I pediatric trials, the initial experimental dose of the new agent is generally 80% of the adult MTD (Baruchel and Rowell 1998). Three patients are enrolled into each dose level initially and the study temporarily closes while data are assessed. If two of these three patients experience dose-limiting toxicity (DLT), it will be concluded that the MTD has been exceeded. If none of these three patients experience DLT, the study will reopen at the next higher dose level (usually a 20% increase) (Baruchel and Rowell 1998). If one patient experiences DLT, three more patients will be accrued at this dose level and the study will proceed to the next higher dose level if none of these additional patients experience DLT. If one or more of these additional patients experience DLT, it will be concluded that the MTD has been exceeded. In summary, three to six patients will be accrued at each dose level. Dose escalation will occur if zero of three or one of six patients experience DLT. If two of three to six patients show evidence of DLT, it shall be concluded that the MTD has been exceeded and the study will continue at the previous lower dose level or at a dose that is 20% lower than the starting dose if the initial dose is deemed to exceed the MTD. The MTD is defined as the dose level immediately below which two of three to six patients experience DLT (Figure 6). Dose-limiting toxicity is defined as any grade 3 or grade 4 non-hematologic toxicity (excluding nausea or vomiting, or asymptomatic hypocalcemia) that occur at a particular dose level. Toxicities are evaluated according to the National Cancer Institute Common Toxicity Criteria (version 2.0). The maximum tolerated dose to be recommended for subsequent Phase II studies is defined as the highest dose level at which none or one of six patients experienced DLT.

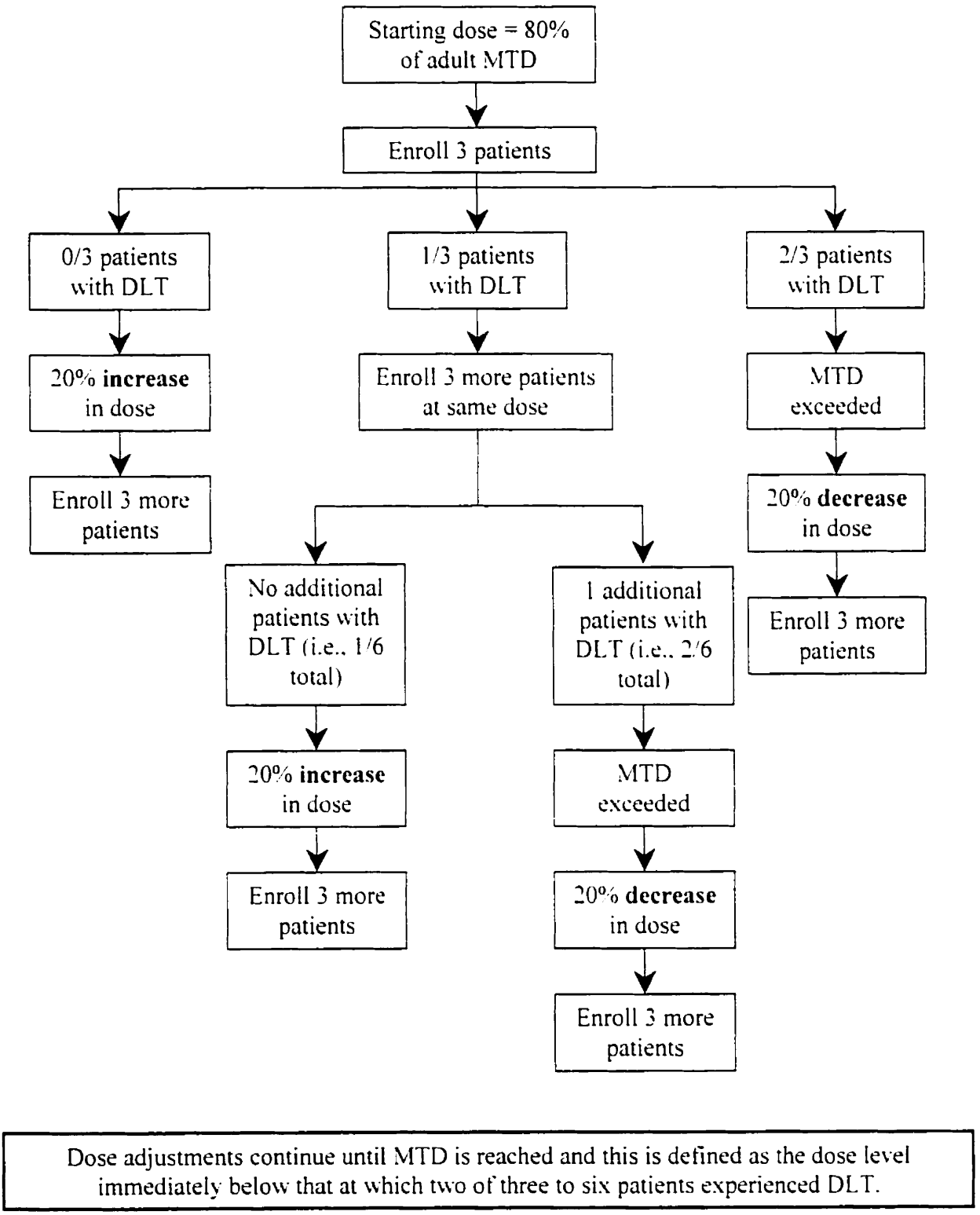
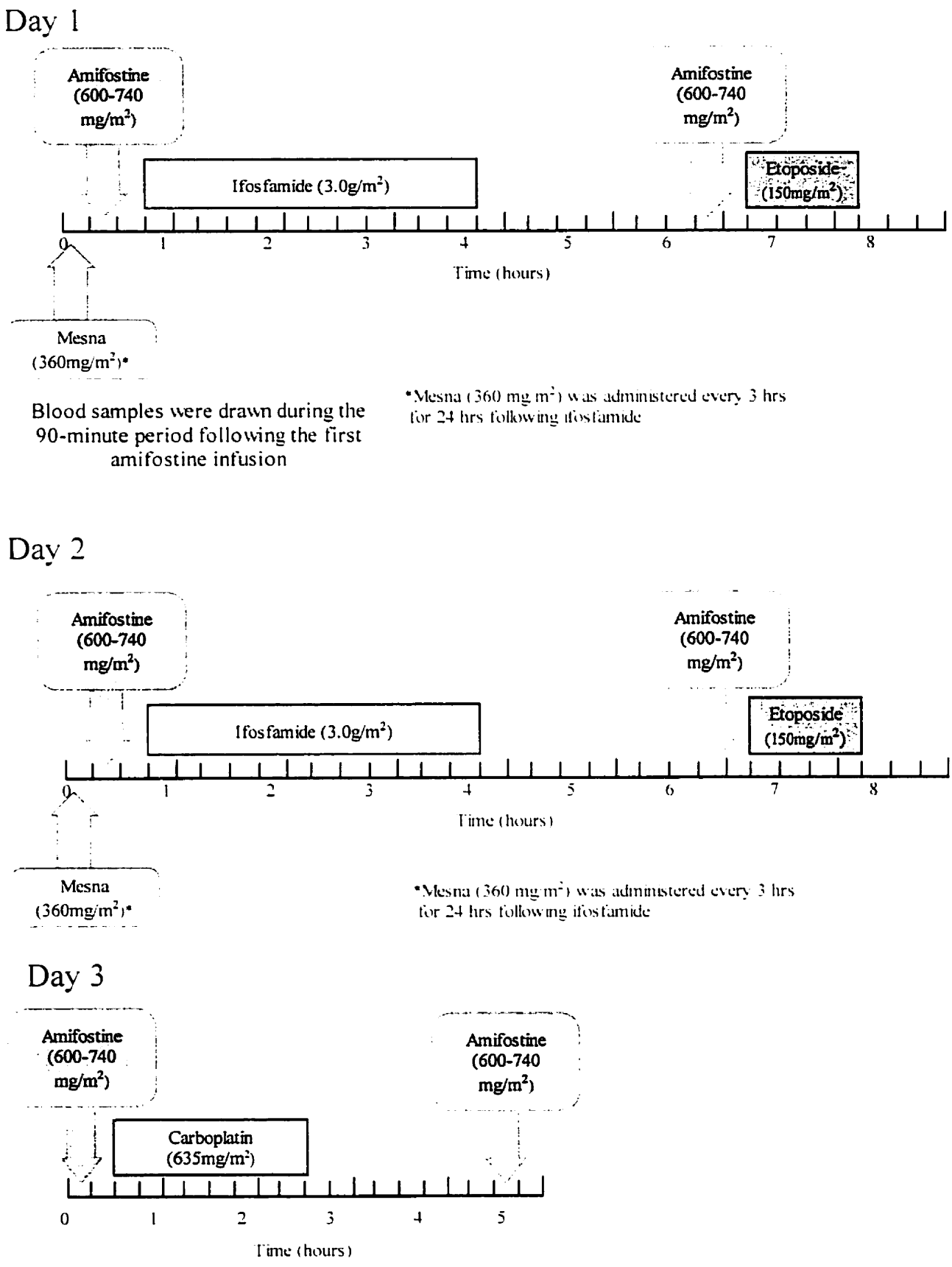


Figure 6: Flow chart for establishing the maximum tolerated dose (MTD) and dose limiting toxicities (DLT) in pediatric Phase I clinical trials.

6.4 Treatment Regimen

The starting dose of amifostine was 740 mg/m^2. This is 80% of the adult single dose MTD (910 mg/m^2) (Dorr and Holmes 1999; Kemp *et al* 1996), and slightly higher than the MTD of 683 mg/m^2 for triple daily doses of amifostine in adults (Adamson *et al* 1995). Amifostine was administered intravenously over a period of 15 minutes, starting 30 minutes before and again, 2 hours after the infusions of ifosfamide and carboplatin. (Figure 7). Ifosfamide was administered intravenously at a dose of 3.0 g/m^2 as an infusion over 3 hours on days 1 and 2; etoposide infused at a dose of 150 mg/m^2 over one hour on days 1 and 2 (following the second amifostine dose); and carboplatin, infused at a dose of 635 mg/m^2 over 2 hours on day 3. Mesna (360 mg/m^2) was administered before each ifosfamide infusion and was repeated every 3 hours until 24 hours after the end of the ifosfamide infusion. Granulocyte colony-stimulating factor (G-CSF) was given at the discretion of the primary oncologist at a daily dose of 5 μg/kg, starting 24 hours after the completion of chemotherapy. This continued until an ANC of 1500/μl was documented for two consecutive days after the nadir. All patients received the same antiemetic regimen. This consisted of ondansetron (3 to 5 mg/m^2) administered intravenously before chemotherapy and every eight hours thereafter, and dexamethasone (10 mg/m^2) administered intravenously before chemotherapy and every 12 hours thereafter until completion of the chemotherapy cycle.

Figure 7: Drug Administration Schema for ICE-Amifostine Regimen



6.5 High Performance Liquid Chromatography

6.5.1 Apparatus

There were two Shimadzu HPLC systems available for use in the laboratory (Shimadzu Corporation: Kyoto, Japan). The work described here was carried out on both systems depending on availability. The first system consisted of an SCL-6B system controller, a LC-10ATvp pump and an SIL-6B autoinjector. A Shimadzu C-R4A recording integrator collected the output from the detector. The second system consisted of an SCL-10A system controller, a LC-10AT pump and an SIL-10A autoinjector. The current generated at the detector was integrated with the Shimadzu Class-VP Chromatography Data System software (version 4.2) installed on a PC.

A Prodigy C-18 ODS column (5 μm particles; 150 x 4.6 mm) (Phenomenex; Torrance, CA, USA) was used for all assays. It was protected by placing a C-18 (ODS, octadecyl) SecurityGuard™ cartridge (Phenomenex, Torrance, CA, USA) in front of it, just after the autoinjector. Isocratic elution with a flow rate of 1 ml/min. was used. Details of mobile phase composition will be described in forthcoming sections but in all cases it was passed through a 0.2 μm filter (Scientific Products and Equipment, Caledon, Ontario, Canada) and degassed prior to use.

6.5.2 Electrochemical Detection

A Coulochem II electrochemical detector was used for all HPLC experiments. The analytical component of the detector consisted of a porous graphite, dual-electrode cell (model 5010; Environmental Sciences Associates (ESA), Bedford, Massachusetts, USA), which contains two electrodes in series. Applying a lower potential to the upstream electrode (E1; channel 1)

rather than to the downstream electrode (E2: channel 2) allowed the removal of compounds eluting from the HPLC column with oxidation potentials lower than those of the compounds under study. A porous graphite guard cell (ESA model 5020) was connected between the pump and the autoinjector to remove contaminants from the mobile phase. The current generated at the analytical cell electrode (E2) was measured with a recording integrator.

As recommended by the manufacturer, the same applied potentials and flow rate were maintained during times of nonuse as during analysis and the eluate from the analytical cell was returned to the mobile phase reservoir. The EC detector was cleaned electrochemically after analyzing 30-50 samples by setting all three electrodes to +1000 mV for 15 minutes followed by −300 mV for 15 minutes. A monthly cleaning procedure included removal of lipophylic residues from the cell by flushing the analytical cell with water, methanol, acetonitrile, tetrahydrofuran, acetonitrile, methanol and water each for about 15 minutes while maintaining a flow rate of 1 ml/min.

6.5.3 Detector Sensitivity

A common problem experienced with electrochemical detection is that the use of high potential settings and the analysis of particularly "sticky" substances such as glutathione can cause a loss in detector sensitivity. The most appropriate way to determine the detector response is to frequently analyze standards (*i.e.*, daily, as well as intermittently during the day when heavy use is required). Regular cleaning procedures, as described above, can help overcome sensitivity issues.

The detector sensitivity can also be adjusted by changing the gain range (R) on E2. This ensures the chromatographic peaks on the output device are sufficiently large. The appropriate value for the gain range is generally determined experimentally but it can be adjusted as the

analytical cell begins to lose sensitivity after extended use and when cleaning procedures fail to rectify the problem. The gain range can be set from 100 pA to 100 μA. The increase in sensitivity is linear across this spectrum of gain range values such that a setting of 100 pA is 10^6-fold more sensitive than a setting of 100 μA. It is important to keep in mind that an increase in sensitivity of this magnitude also significantly increases baseline noise as well as the size of all peaks on the chromatogram (including those of the solvent front) so it is possible that peaks of interest may become obscured by interferences. We generally found that changing the range of 1 μA to 100μA (*i.e.*, a 100-fold range in sensitivity) worked well with our chromatographic conditions.

Yet another way to overcome problems with detector sensitivity is to inject a larger volume of analyte, if possible. As the size of the solvent front will also increase in response, a peak of interest that elutes relatively close to this area may be obstructed. Once all of these maintenance strategies fail, replacing the cell is likely necessary.

6.5.4 Assay Validation

The intraday variability, interday variability and limit of detection were determined for each compound studied. In order to define the intraday variation, a fixed concentration of a standard solution of the compound of interest was injected and analyzed repeatedly throughout the day. The standard solutions were kept on ice between analyses to ensure stability. To obtain the interday variability, the same fixed concentration solutions were injected daily for five consecutive days. The data were collected and the mean, standard deviation and percent coefficient of variation (%CV) were calculated. The limit of detection for the specific settings described for each assay (i.e., the R2 value as well as the volume injected) was determined by injecting decreasing concentrations of a solution containing the compound of interest.

Specifically, the concentration of the solution was diluted 2-fold with each injection. This process was continued until a peak could no longer be detected.

6.6 WR1065 Analysis

After injection of 15 µl of standard or sample, WR1065 was separated using the Prodigy column described earlier. The mobile phase for the WR1065 assay consisted of 30:70 (v/v) methanol-water containing 0.1 M monochloroacetic acid (adjusted to pH 3.0 with sodium hydroxide), 0.5 M sodium octyl sulfate and 10 mM ethylamine. With a flow rate of 1 ml/min., the retention time for WR1065 was approximately 4.2 minutes. The applied electrode potentials for the guard cell, E1 and E2 were set at 650, 200 and 600 mV respectively with the gain range for the second electrode (R2: sensitivity level) set at 10 µA.

6.6.1 Standards

A stock solution of 10 mM WR1065 was prepared by dissolving the drug in 0.5 M PCA-2 mM EDTA. The solution was aliquoted and stored at -80°C. WR 1065 standards ranging from 0 to 200 µM were prepared on each day of sample analysis in blank deproteinized plasma (plasma after removal of proteins with 0.5 M PCA-2 mM EDTA). The standard curve was constructed by linear regression of WR1065 peak heights plotted against WR1065 concentration.

6.6.2 Sample Collection

Blood samples for pharmacokinetic analyses were obtained during the first dose of amifostine. Blood was drawn from a vein in the arm opposite to that used for drug infusion and collected in 2-ml tubes containing EDTA as an anticoagulant. As the kinetics in children were not yet defined, 2 ml samples were obtained at the following time points: pre infusion, 1, 2, 5,

10. 15. 20. 30. 40. 60. 90 minutes after drug infusion for plasma determination of amifostine and its metabolites. The blood samples were immediately placed on ice and plasma was separated by centrifugation at 4°C for 10 minutes at 2000 rpm.

6.6.3 Sample Pretreatment

The samples were processed and analyzed using modified procedures previously reported by Korst *et al.* (Korst *et al* 1997b). Three different pretreatment procedures were performed on the plasma samples, as shown in Figure 8. This allowed for quantification of amifostine (procedure B), WR 1065 (procedure A) and the disulfides (procedure C). WR1065 was the compound that was measurable by HPLC with electrochemical detection. The concentrations of the parent drug (amifostine) as well as the disulfide metabolites were determined indirectly by subtraction. All procedures were carried out at 0°C unless otherwise stated.

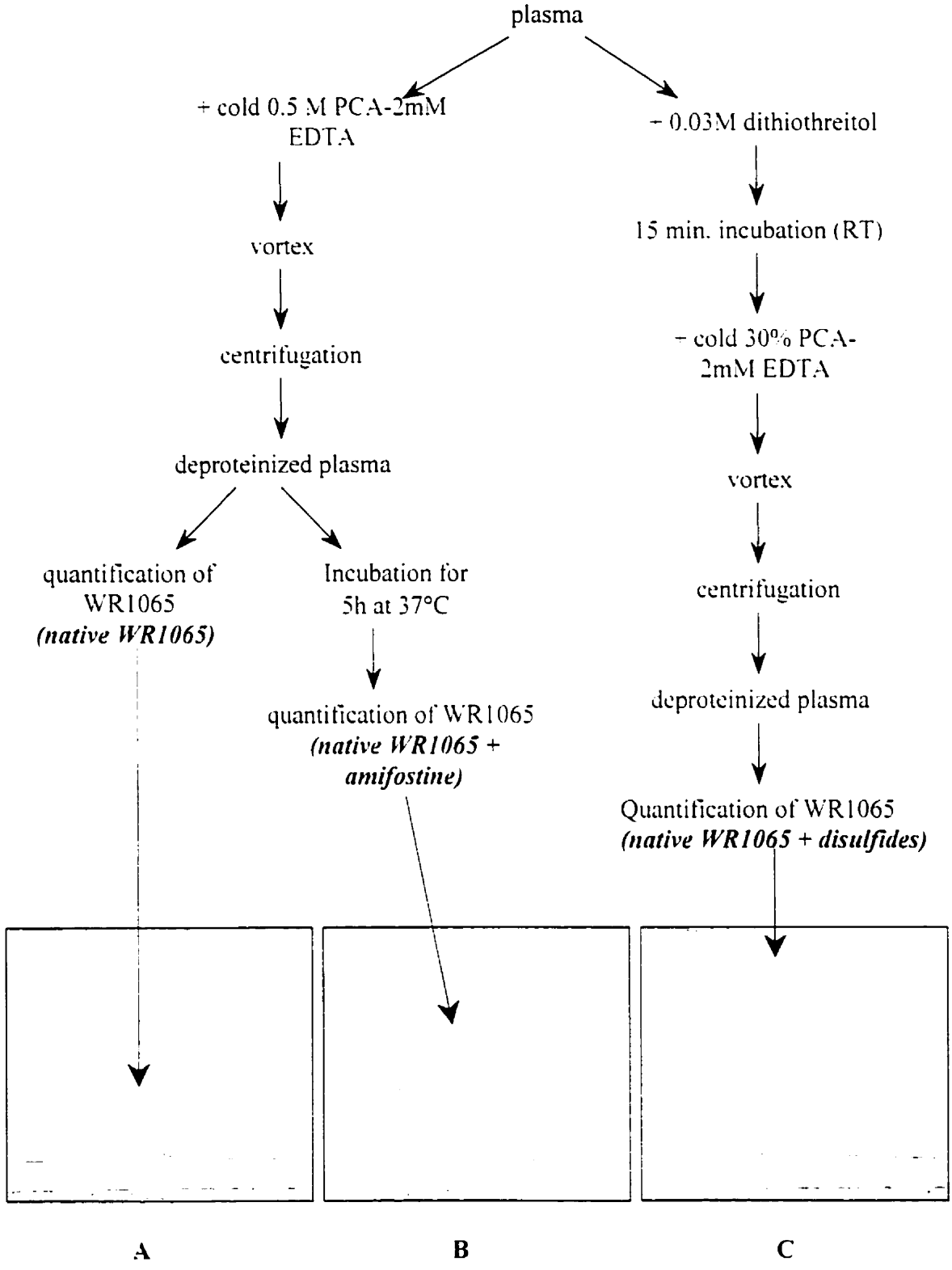


Figure 8: Sample pretreatment procedures for amifostine, WR1065 and disulfide determination

(PCA = perchloric acid: EDTA = ethylene diamine tetracetic acid: RT = room temperature)

6.6.3.1 WR1065 Quantification (Procedure B)

For the analysis of WR 1065, 250 µl of plasma was added to 250 µl of 0.5 M PCA solution containing 2 mM EDTA in polypropylene tubes to precipitate the proteins. The samples were vortex mixed, incubated on ice for 10 minutes (to completely precipitate proteins) and then centrifuged (13 000 rpm, 15 min., 4°C). After centrifugation, the clear supernatant was transferred to 1.5-ml polypropylene tubes, aliquoted and stored at -80°C until analysis. The concentration of WR1065 in this sample represents native WR1065.

6.6.3.2 Amifostine Quantification (Procedure A)

In order to determine the amifostine concentration, the plasma sample was deproteinized with 0.5 M PCA-2 mM EDTA as described above. The supernatant was then incubated at 37°C for 5 hours to convert amifostine to WR1065. Analysis of these samples and quantitation of WR 1065 represents the concentration of the native WR1065 plus amifostine. The concentration of amifostine was determined by subtracting the concentration obtained in procedure B from the one obtained here.

6.6.3.3 Disulfide Quantification (Procedure C)

For the analysis of the disulfides, 250 µl of plasma was added to 25 µl of 0.03 M dithiothreitol (DTT; a reducing agent). The samples were incubated at room temperature for 15 minutes to convert the disulfides to the free thiol, WR1065. Afterwards, the samples were treated as described for the analysis of WR1065. The final concentration of WR1065 determined in these samples represents the concentration of the disulfides plus native WR1065. The concentration of disulfides was determined by subtracting the concentration obtained in procedure B from this one.

6.6.4 Validation of Sample Pretreatment Procedures

Although some of the sample processing methods reported by Korst *et al.* (Korst *et al* 1997b) have been validated, a series of experiments were conducted to verify that the procedures described above efficiently converted amifostine and the disulfides to WR1065.

6.6.4.1 Conversion of Amifostine to WR1065

In order to quantify amifostine, it was converted to WR1065 during the pretreatment procedure. This takes place in an acidic environment in deproteinized plasma and it is temperature dependent. To test the efficiency of this conversion, blank human plasma was spiked with a known concentration of amifostine, deproteinized as described earlier, aliquoted and incubated at 37°C for 7 hours. A parallel experiment was set up where amifostine was spiked into deproteinized plasma and incubated in ice for the same length of time. At several time points throughout the 7-hour incubation period, the samples were analyzed for WR1065.

6.6.4.2 Stability of WR1065 at 37°C

In parallel to the above experiment, the stability of WR1065 in deproteinized, acidic plasma was tested at 37°C over 7 hours. Blank plasma was spiked with WR1065, deproteinized, aliquoted and incubated at 37°C for 7 hours. At various time points over this period, the samples were removed from the incubator and WR1065 was quantified.

6.6.4.3 Oxidation of WR1065 and Reduction of Disulfides

Blank plasma (not deproteinized) was spiked with WR1065 and incubated at room temperature for 90 minutes to deplete the free thiol. During this time interval, depletion of the free thiol (due to oxidation into mixed and symmetrical disulfides) was monitored by drawing

aliquots of plasma (at 0, 1, 2, 3, 5, 10, 15, 20, 30, 45, 60 and 90 minutes), deproteinizing them and quantifying WR1065. In order to reverse this depletion of WR1065, a 10% volume of 0.03 M DTT was added to a volume of plasma, which was then incubated at room temperature for an additional 90-minute period. Aliquots of plasma were drawn and deproteinized as described above and WR1065 was quantified.

Since sample pretreatment procedure C (described earlier) requires the incubation of plasma samples at room temperature, the above experiment was designed to verify the efficiency of disulfide conversion to WR1065. However, the patient samples that we receive from the clinical trial contain amifostine as well. The effect of incubating plasma at room temperature for 15 minutes on amifostine concentrations is unknown. We wanted to investigate whether this incubation would cause spontaneous dephosphorylation of amifostine to plasma. To resolve this, blank plasma was spiked with amifostine and incubated at room temperature over a 20-minute interval. Aliquots were drawn at 0, 1, 3, 5, 10, 15 and 20 minutes, deproteinized and analyzed for WR1065 levels.

6.7 Glutathione Analysis

A 10 µl injection of standard or sample was separated using the previously described column. Isocratic elution was achieved using a mobile phase containing a 2:98 (v/v) mixture of acetonitrile-water containing 10 mM sodium phosphate, monobasic, monohydrate (adjusted to pH 2.7 with 50% phosphoric acid) and 0.03 M octane sulfonic acid at a flow rate of 1 ml/min. The retention time for GSH, penicillamine and GSSG were approximately 4.1, 5.4 and 10.5 minutes respectively. The potential applied to the guard cell and the E1 and E2 electrodes were +850, 400 and 800 mV, respectively. The R2 was set at 100 µA for GSH and penicillamine and at 1 to 10 µA for GSSG.

6.7.1 Detector Specificity for GSH and GSSG

In order to determine how specific the electrochemical detector was to GSH and GSSG, 1 mM solutions of each of cyteine, cystine, N-acetyl cysteine, homocysteine, penicillamine, GSH and GSSG were prepared. These were then mixed together resulting in a final concentration of 166.67 μM for each species. 10 μl was injected, separated and analyzed using the same conditions described for glutathione analysis.

6.7.2 Standards

A stock solution containing 1 mM GSH and GSSG was prepared by dissolving in HPLC grade water. This solution was aliquoted and stored at -80°C. Working standard solutions covering the concentration range of 0 to 1000 μM for GSH and 0 to 500 μM for GSSG were prepared on each day of sample analysis by dissolving the stock with HPLC grade water. The internal standard penicillamine was then added to the standard solutions to achieve a concentration of 50 μM and the solutions were further diluted by adding an equal volume of 2mM EDTA in water (to mimic the dilution introduced by the acid when blood samples are deproteinized with acid). The standard curves were constructed by linear regression of the peak height ratio of GSH or GSSG to the internal standard versus the GSH or GSSG concentrations.

6.7.3 Patient Blood Samples

GSH and GSSG were quantified in the whole blood of the same patients enrolled in the pharmacokinetic study. Blood was collected at the same time points as in the 90-minute pharmacokinetic study period *i.e.*, pre amifostine infusion, 1, 2, 5, 10, 15, 20, 30, 40, 60, 90 minutes after drug infusion.

6.7.4 Sample Pretreatment

In order to minimize the oxidation of thiols or the enzymatic reduction of the disulfides, the samples were placed on ice and processed rapidly using a modified methodology of Bonner and Shaw (Bonner and Shaw 1991) (Figure 9). Just prior to separating the plasma, 100 µl of whole blood was removed and deproteinized by adding an equal volume of 0.5 M (2.5%) PCA-2 mM EDTA solution containing 50 µM penicillamine. The samples were vortexed for 1 minute and incubated on ice for 10 minutes to completely precipitate proteins. The acidic suspensions were centrifuged at 13 000 rpm for 15 minutes at 4°C and the clear supernatants collected, aliquoted and stored at -80°C until analysis. This sample processing procedure was followed for the first four out of six patients.

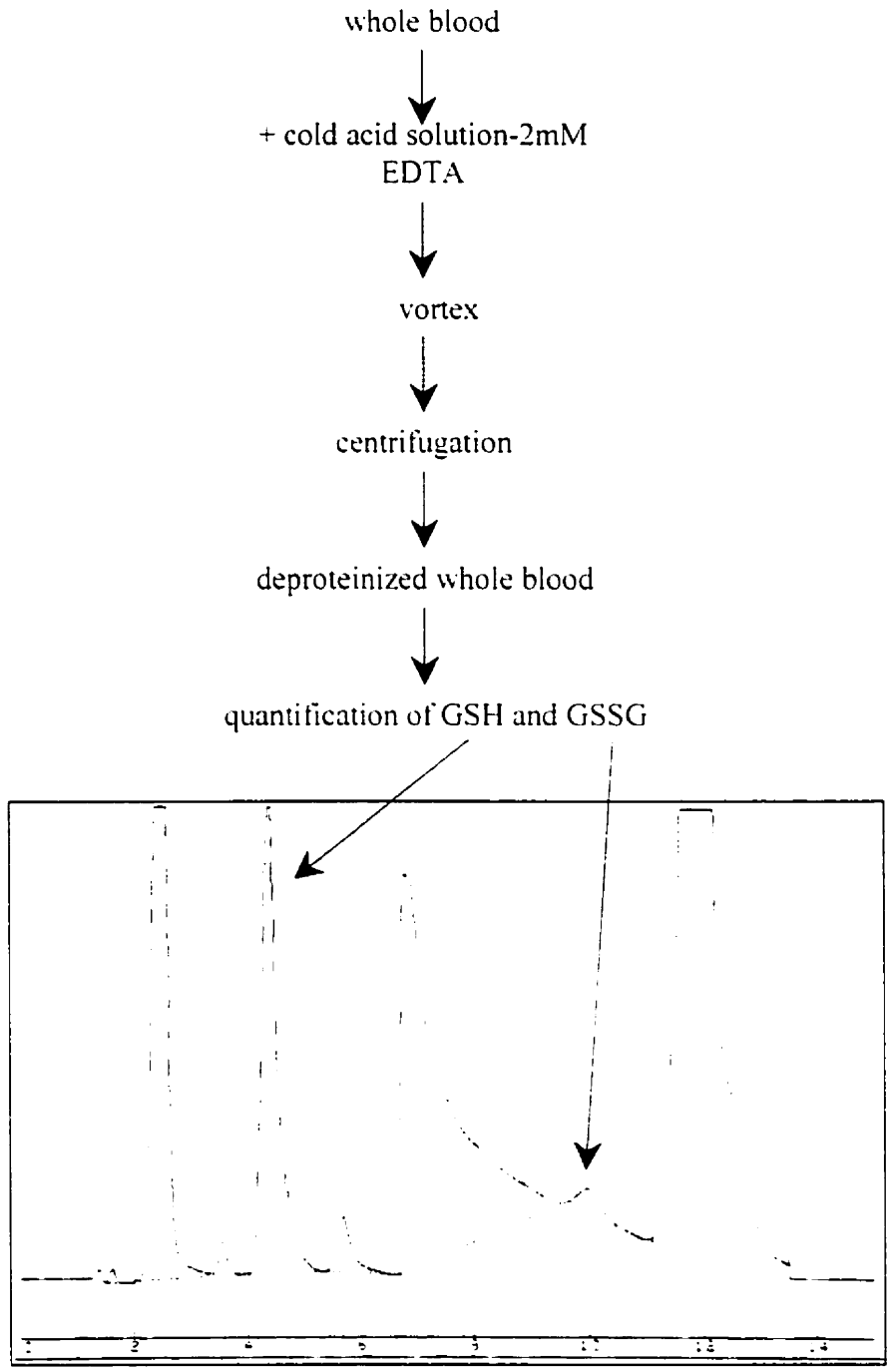


Figure 9: Sample pretreatment procedure for glutathione determination

(EDTA = ethylene diamine tetracetic acid: GSH = reduced glutathione; GSSG = oxidized glutathione)

6.7.5 Identification of a Sample Stability Issue

Much to our surprise, partway through this study, we realized that the method we were using to prepare our samples for glutathione analysis was completely unacceptable. We noticed that with repeated analyses, the composition of the samples (with respect to GSH and GSSG) was changing over time despite storing them at -80°C. This observation prompted a thorough evaluation of the literature regarding glutathione analysis and an attempt to clarify inconsistencies that arose.

6.7.6 Quantification of Glutathione in Biological Specimens

Thiols, such as GSH, can be determined by a variety of analytical means including enzymatic (Akerboom and Sies 1981; Anderson 1985; Tietze 1969), fluorometric (Cohn and Lyle 1966; Hissin and Hilf 1976) and liquid chromatography methods with various detection system including fluorometric, ultraviolet and electrochemical detection (Akerboom and Sies 1981; Anderson 1985; Slordal *et al* 1993). Each of these methods exhibits its own limitations and is generally useful in measuring GSH.

It is often desirable to measure both the thiol and disulfide concentrations together. While HPLC methods have been described for the simultaneous determination of both GSH and GSSG with ultraviolet (UV) detection (with (Reed *et al* 1980) and without (Liu *et al* 1996) derivatization), there are disadvantages. In methods requiring derivatization, factor and error is introduced due to a more complicated sample processing procedure. Methods not requiring derivatization are inappropriate for the quantification of glutathione in erythrocytes and whole blood due to an interference with heme. In contrast, electrochemical detection of GSH and GSSG is preferential as it involves simple and rapid sample preparation rather than lengthy

derivatization techniques, thereby decreasing the likelihood of altering sample composition (Rose 1996; Rose and Bode 1995).

It is generally agreed upon that high GSSG levels may be obtained during biological sample preparation for glutathione determination. However, there is little consistency in the literature regarding the appropriate methodology. There appears to be a common consensus that samples need to be rapidly acidified as this prevents spontaneous, non-enzymatic conversion (by oxidation) of GSH to GSSG and GSSR (mixed disulfides) (Anderson 1985; Asensi *et al* 1994; Carro-Ciampi *et al* 1988; Giulivi *et al* 1994; Harvey *et al* 1989; Anderson 1985). Acidification also inactivates γ-glutamyl transferase (GGT), an enzyme known to catalyze GSH and GSSG degradation reactions (Anderson 1985) (Figure 10).

GSH + amino acid → γ-Glu-amino acid + Cys-H-Gly (transpeptidation)

GSH + H_2O → Glu + Cys-H-Gly (hydrolysis)

GSH + GSH → γ-Glu-GSH + Cys-H-Gly (autotranspeptidation)

Figure 10: Glutathione degradation reactions catalyzed by γ-glutamyl transferase (GGT). Acidification of biological samples inactivates GGT. (From Anderson 1985)
(GSH = reduced glutathione; H_2O = water; Glu = glutamate; Cys = cysteine; Gly = glycine)

A variety of acids have been used to acidify and deproteinize biological samples prior to glutathione analysis. The most commonly used acids are PCA (Akerboom and Sies 1981; Allison and Shoup 1983; Asensi *et al* 1994; Chakrapani *et al* 1995; Rodriguez-Ariza *et al* 1994; Stein *et al* 1986; Akerboom and Sies 1981; Akerboom and Sies 1981; Akerboom and Sies 1981), TCA (Asensi *et al* 1994; Carro-Ciampi *et al* 1988; Krien *et al* 1992; Mitton and Trevithick 1994;

Asensi *et al* 1994), SSA (Anderson 1985; Roberts and Francetic 1993; Slordal *et al* 1993; Vina *et al* 1995; Anderson 1985) and MPA (Mills *et al* 1990; Mills *et al* 1994; Richie, Jr. and Lang 1987). Despite some suggestions that the selection of acid may affect glutathione stability in biological samples, it has not been formally studied whether or not samples can be stored prior to analysis, and for what length of time. Furthermore, a gap remains in the knowledge of how various acids and concentrations affect sample stability over time prior to analysis. Hence, the goals of this work were modified to include an evaluation of current sample preparation procedures. A side-by-side comparison study was designed and carried out to identify and validate an improved sample handling procedure that would conserve sample integrity while allowing samples to be stored prior to analysis without affecting their integrity.

6.7.7 Glutathione Assay Modification: Identification and Validation

6.7.7.1 Blood Samples

Venous blood samples (~10 ml) from healthy adult volunteers were collected in heparinized syringes and immediately placed on ice. The samples were processed as described for the patient samples only using various concentrations of different acids. Briefly, 500 µl of ice- cold 5, 10 and 15% PCA, TCA, MPA and SSA solutions containing 2 mM EDTA were prepared for each subject. In addition, a 2.5% PCA solution (with 2 mM EDTA) was also prepared. To each of these solutions, penicillamine (internal standard) was added to achieve a concentration of 50 µM. The clear supernatants were collected, aliquoted and stored at -80°C until analysis.

6.7.7.2 Effects of Various Acids on 4-week Stability

In order to evaluate the stability of GSH and GSSG in whole blood following deproteinization with various acids, 8 measurements were made on days 0, 1, 2, 3, 4,10, 15 and 28. The samples that were analyzed on day 0 were stored on ice until injection. On all other days, the samples were removed from the freezer individually and thawed rapidly in a 37°C water bath just prior to analysis.

6.7.7.3 Effects of Various Acids on Glutathione Levels in Freshly Drawn Samples

The effect of the various acids on glutathione levels in freshly drawn blood samples was investigated. The purpose of this set of experiments was to determine if the acids had an immediate effect on initial GSH levels. Blood was drawn and immediately deproteinized by treating it with an equal volume of acid containing 2 mM EDTA and 50 μM penicillamine. The same acids and concentrations described earlier were used. The deproteinized samples were then analyzed in triplicate immediately after processing. Only fresh blood was used. Each time a new acid was tested, a fresh sample of blood was drawn. All 13 acids were tested over a period of 4 days. The blood was drawn at the same time each day to minimize the contribution of diurnal variation in glutathione levels. Furthermore, a sample was treated with a 15% final concentration of PCA each day throughout this study period to serve as a control for intraday variability.

6.7.7.4 Efficiency of Protein Removal

To compare the amount of proteins remaining in the samples following acid treatment, samples that were prepared as described previously were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). 10% acrylamide gels were prepared on the day of use. 10μl of sample was loaded per lane and the gels were run at 60 volts for

approximately 18 hours. Proteins on the gels were stained with Coomassie Brilliant Blue R-250 (ICN Biomedical Inc., Cleveland, Ohio).

To quantify protein content in the samples, the Bio-Rad Protein Assay (Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario, Canada) was used. Based on the results obtained from the Coomassie stain of the gels, the standard procedure was used to quantify the samples that appeared to contain relatively large amounts of protein and the microassay was used for samples that contained relatively low amounts of protein.

6.7.7.5 Confirming Stability Results

It was found that a final concentration of 15% PCA was most suitable for use in determining GSH and GSSG in whole blood (see results and discussion). To validate our final conclusion, we tested this method in 10 *additional* subjects. Blood was drawn and processed immediately as described earlier. The samples were analyzed immediately after collection and aliquots were stored at -80°C and reanalyzed after 14 and 28 days.

6.8 Data Analysis

6.8.1 Pharmacokinetic Analysis

Plasma amifostine, WR1065 and disulfide concentration data were analyzed by standard non-compartmental pharmacokinetic methods using the program WinNonlin (version 1.0; Statistical Consultants, Inc., Apex, NC, USA). Pharmacokinetic parameters were calculated using the formulas presented in table 4.

Table 4: Formulas for the calculation of pharmacokinetic parameters.

Parameter	Formula/Description
t_{max}	Time of maximum observed concentration
C_{max}	Maximum observed concentration
k_e	Elimination rate constant: estimated via linear regression of time vs. log concentration curve (slope = - k_e/2.303)
$t_{1/2}$	Half-life: $t_{1/2} = -\ln(2)/ k_e$
AUC_1^2	Area under the concentration-time curve calculated using linear trapezoidal rule: $AUC_1^2 = \delta t[(C1 + C2)/k_e]$
$AUC_{0-\infty}$	AUC extrapolated to infinity: the portion extrapolated to infinity is calculated by C_n/ k_e: where C_n = observed concentration at last sample time

6.8.2 Amifostine Modulation of Glutathione

Patient glutathione levels are expressed in µmoles/L (µM). Glutathione levels after the amifostine infusions were compared to preinfusion (baseline) levels. Changes were generally reported as percent deviation from the baseline value.

6.8.3 Glutathione Stability Studies

For the glutathione stability studies, each subject served as his or her own control. Glutathione concentrations were expressed in µmoles/L. Total glutathione was expressed in GSH equivalents (GSH + 2 GSSG). Results were displayed graphically and summarized as a percent changed from day 0 to day 28.

7.0 RESULTS

Parts of this work have been submitted for publication as manuscripts entitled "Phase I Trial and Pharmacokinetic Study of a Twice-Daily-Dose Regimen of Amifostine With ICE Chemotherapy in Children With Refractory Cancer", authored by Maryam Fouladi, Diana Stempak, Janet Gammon, Julia Klein, Ping Li, Ron Grant, Mark L. Greenberg, Gideon Koren and Sylvain Baruchel (submitted to *Cancer*) and "Glutathione Stability in Whole Blood: Effects of Various Deproteinizing Acids", authored by Diana Stempak, Shannon Dallas, Julia Klein, Reina Bendayan, Gideon Koren, Sylvain Baruchel. (submitted to *Therapeutic Drug Monitoring*).

7.1 WR1065 Assay Validation

7.1.1 Intraday and Interday Variation, Limit of Detection, Standard Curves

The validation parameters for the measurement of WR1065 are shown in table 5. The intraday variability was approximately 3% and the interday variability was approximately 11%. In order to minimize the effect of interday variation of the assay, a new standard curve was generated each day samples were analyzed and quality controls were also analyzed daily. The lower limit of detection for WR1065 at the settings described herein was 0.78 μM (or 11.72 pmoles on the column). A representative standard curve is shown in figure 11. Linearity was observed within the selected range of concentrations (0 to 200 μM).

Table 5: Intra- and inter-day variability of WR1065

		Intraday Variation (n=5)			Interday Variation (n=5)		
		Mean (μM)	S. D. (μM)	%CV	Mean (μM)	S. D. (μM)	%CV
WR1065	6.25 μM	5.5	0.2	3.2	5.3	0.6	11.7
	25 μM	26.6	0.8	2.9	27.6	2.9	10.6
	100 μM	99.6	2.7	2.7	99.0	10.4	10.5

(S.D. = standard deviation: %CV = percent coefficient of variation)

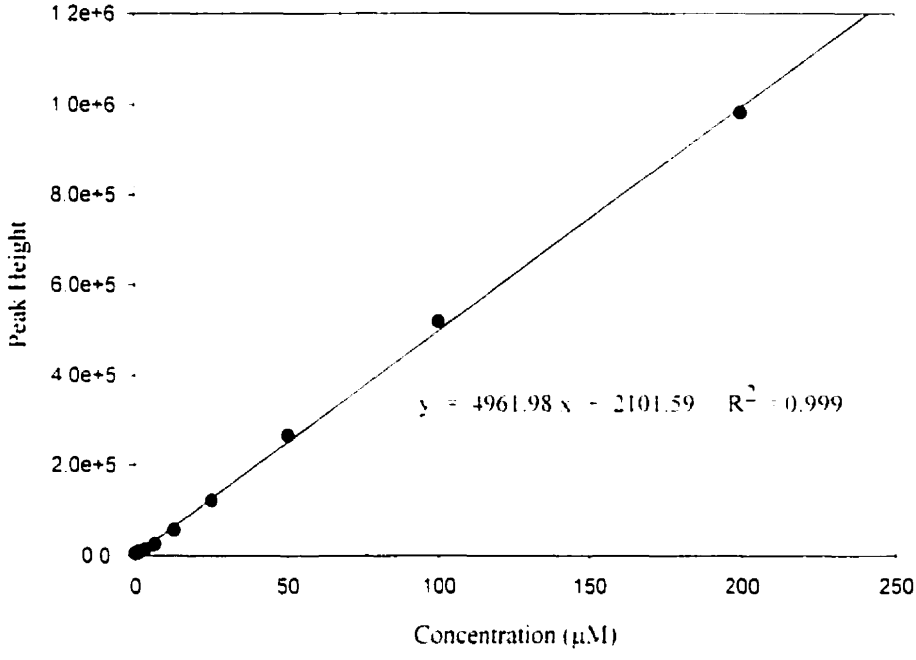


Figure 11: A typical WR1065 standard curve

7.1.2 Validation of Sample Pretreatment Procedures

7.1.2.1 Conversion of Amifostine to WR1065

The conversion of amifostine to WR1065 was carried out in the acid soluble fraction of plasma and was very strongly temperature dependent with an optimum rate of conversion occurring at 37°C (Korst *et al* 1997b). Figure 12A shows that the conversion of amifostine to WR1065 is approximately 90% complete by 4 hours and it is virtually complete by 5 hours. Incubation for 6 and 7 hours does not further increase WR1065 formation. In contrast, amifostine conversion proceeds very slowly at 0°C. At the end of the 7-hour incubation, only approximately 3% of amifostine had been hydrolyzed to WR1065.

7.1.2.2 Stability of WR1065 at 37°C

Due to the lengthy incubations at 37°C of samples containing WR1065, the stability of this compound under these conditions was tested. Figure 12A shows the loss of WR1065 during incubation at 37°C for 7 hours. Over a period of 7 hours an average of 5.3% of WR1065 is lost. During the typical 5 hour incubation, 4.7% of WR1065 is lost verifying that the oxidation reaction proceeds very slowly in acidic medium at this temperature.

7.1.2.3 Oxidation of WR1065 and Reduction of Disulfides

In contrast to the slow formation of disulfides in acidic medium, the oxidation of the free thiol (WR1065) proceeds very rapidly in a neutral environment at room temperature. Figure 12B summarizes these findings. Within 10 minutes, the concentration of WR1065 spiked into the plasma decreased by approximately 80%. At the end of a 90-minute incubation, only 8.5% of the original amount of WR1065 that was added to the plasma was found. These findings taken

together with the observation that WR1065 is relatively stable at 37°C in acidic medium suggest that two factors are likely to contribute to the rapid loss observed here. Firstly, neutral pH is a significant contributor. Oxidation of free thiols to their corresponding symmetrical disulfides or mixed disulfides is hindered at low pH. Secondly, whole plasma has a much higher abundance of thiol-containing compounds, therefore, WR1065 can form mixed disulfides with thiol groups found in various proteins as well as those found in compounds such as cysteine and GSH.

The second portion of this experiment was to evaluate the recovery of the free thiol, WR1065 by treating the sample with DTT. Immediately following the addition of DTT, approximately 50% of the WR1065 was recovered (figure 12B). The reduction reaction paralleled the oxidation reaction in that it occurred very rapidly. Within 10 minutes of adding the DTT, almost 90% of the WR1065 had been recovered. The protocol calls for a 15-minute incubation at room temperature in the presence of DTT. This experiment verifies that this is sufficient time to reduce virtually all of the disulfides (mixed and symmetrical) back to the free thiol, WR1065.

Finally, since the conversion of disulfides to WR1065 takes place in the presence of amifostine, we wanted to determine the rate of hydrolysis of the parent drug during the 15-minute incubation at room temperature. Assuming that the only fate of amifostine in plasma is dephosphorylation to WR1965, Figure 12C shows stability of amifostine at these conditions since the formation of WR1065 is relatively low. Within this 15-minute incubation, only approximately 4.5% of amifostine is converted to WR1065 indicating that this sample pretreatment procedure does not alter the overall drug and metabolite profile significantly.

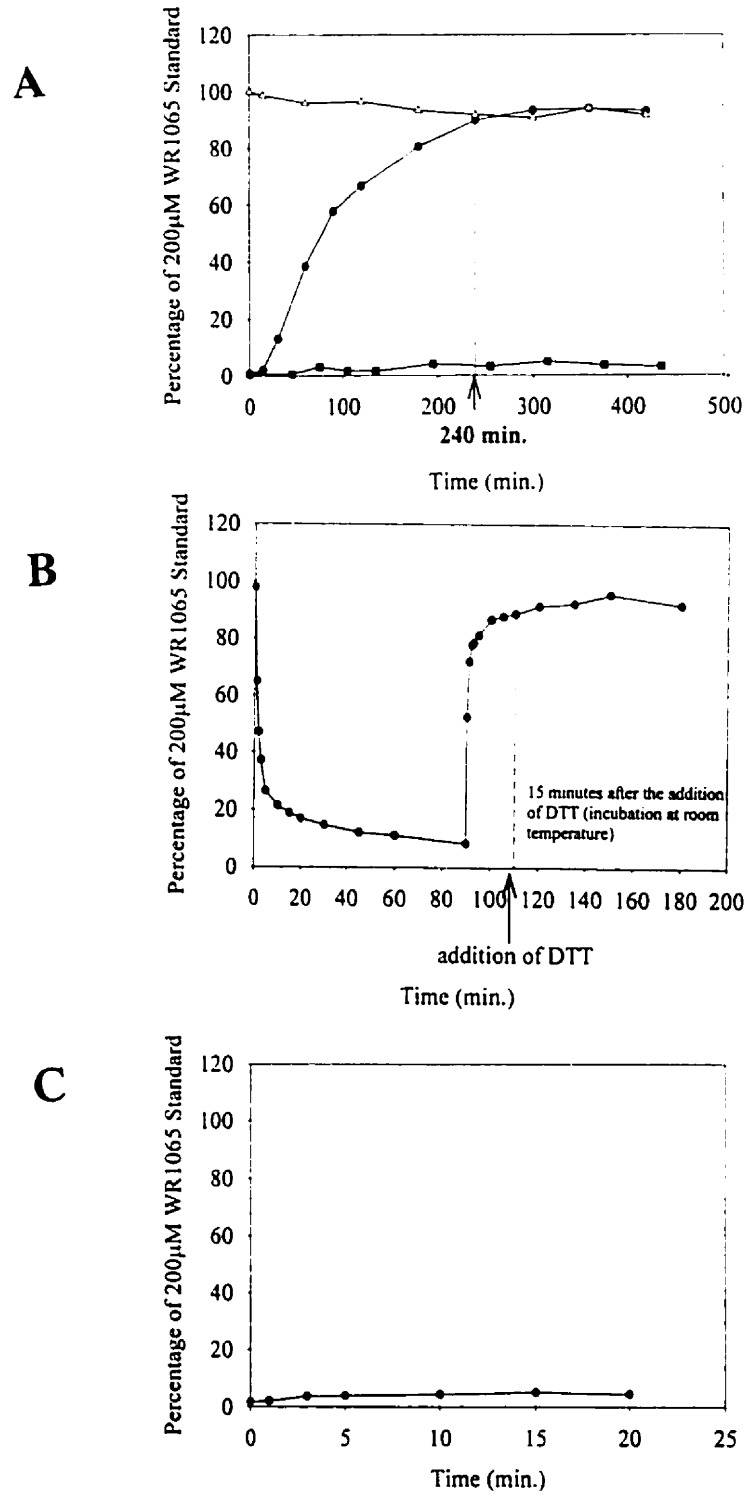


Figure 12: Validation of Amifostine Sample Pretreatment Procedures. (**A**) Amifostine (200μM) hydrolysis WR1065 is complete within 4 hours after incubation at 37°C in acidic deproteinized plasma (•). In contrast, hydrolysis proceeds very slowly at 0°C (□). WR1065 itself is stable at 37°C (△) indicating minimal loss of the amount originally present at the start of the incubation to convert amifostine to WR1065. (**B**) WR1065 is rapidly oxidized to disulfides in whole plasma at room temperature but it is rapidly recovered by reduction with DTT (dithiothreitol). (**C**) During the reduction of disulfides with DTT, the amifostine present in the sample is stable as indicated by the appearance of insignificant amounts of WR1065.

7.2 Glutathione Assay Validation

7.2.1 Intraday and Interday Variation, Limit of Detection, Standard Curves

The validation results for the electrochemical detection of GSH and GSSG are summarized in table 6. The intraday variability for GSH ranged from 3.0% to 4.5% and for GSSG it ranged from 1.5% to 1.9%. The interday coefficients of variation ranged from 5.1% to 6.4% for GSH and from 7.5% to 8.9% for GSSG. As for WR1065, in order to minimize the effect of interday variation, new standard curves were generated on each day that samples were analyzed and quality control samples were also analyzed. The lower limits of detection at the described settings were 1.25 μM (6.25 pmoles on the column) and 0.625 μM (3.125 pmoles on the column) for GSH and GSSG, respectively. A representative standard curve is shown in Figure 13. Linearity was observed in the selected range of concentrations (0 to 1000 μM).

Table 6: Intra- and inter-day variability of GSH and GSSG.

		Intraday Variation (n=5)			Interday Variation (n=5)		
		Mean (μM)	S.D. (μM)	%CV	Mean (μM)	S.D. (μM)	%CV
GSH	50 μM	50.3	1.5	3.0	52.7	3.4	6.4
	500 μM	478.5	21.5	4.5	508.6	25.9	5.1
GSSG	50 μM	50.3	0.8	1.5	49.7	3.8	7.5
	100 μM	102.0	1.9	1.9	95.4	8.5	8.9

(S.D. = standard deviation; %CV = percent coefficient of variation)

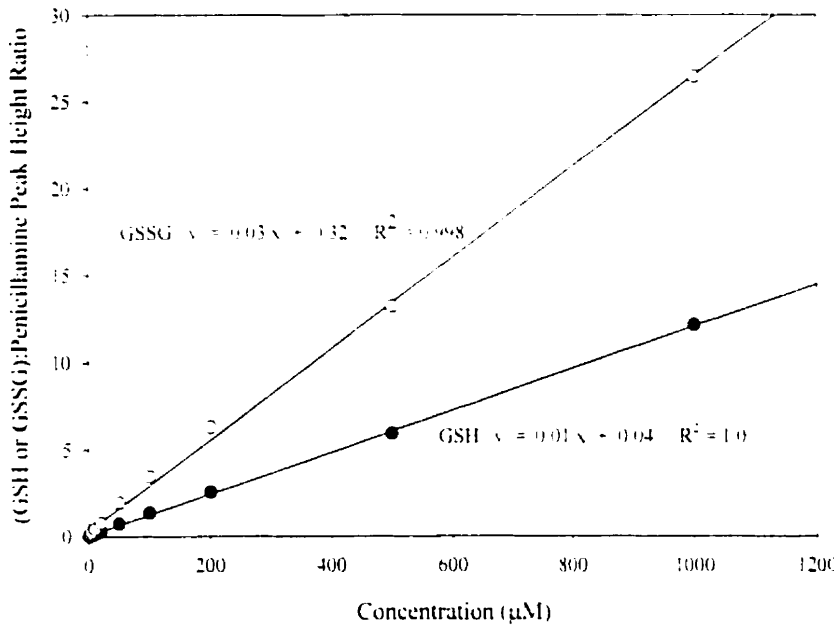


Figure 13: A typical glutathione standard curve
(GSH = reduced glutathione; GSSG = oxidized glutathione)

7.2.2 Specificity of the Electrochemical Detector for GSH and GSSG

The results of the chromatographic separation of cysteine, cystine, N-acetyl cysteine, homocysteine, GSH and GSSG are shown in Figure 14. Both GSH and GSSG are well separated from all of the other species. There is no evidence of interference from other thiols or disulfides, endogenous and exogenous. The only example of poor separation is seen with penicillamine and N-acetyl cysteine. Their retention times are very close (5.2 and 5.4 minutes, respectively). However, since neither of these is an endogenous compound, the suitability of penicillamine as the internal standard for this assay is unaffected. Should there be need to separate these two compounds, alterations to the chromatographic conditions could be made to facilitate this.

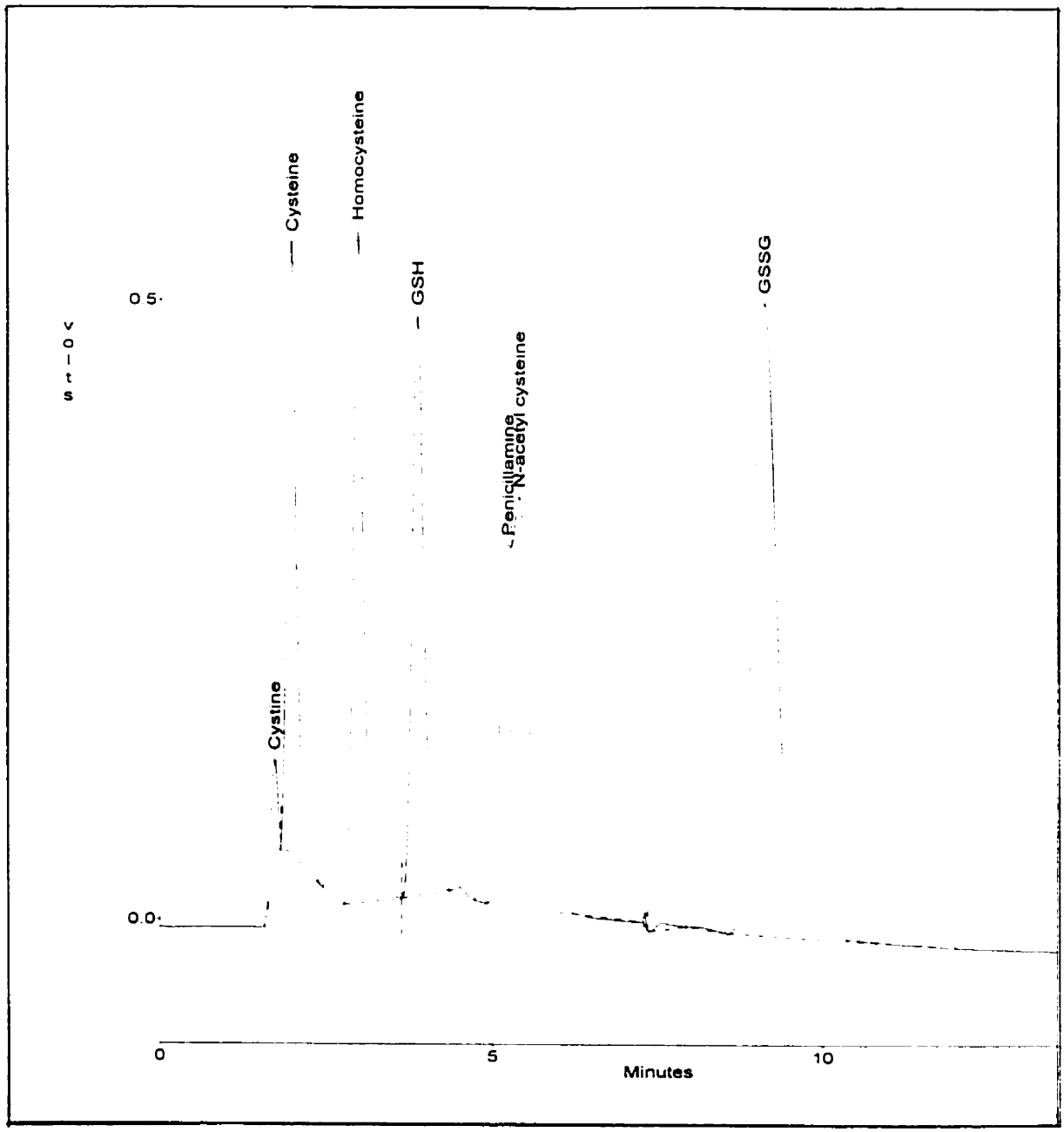


Figure 14: HPLC separation and electrochemical detection of various thiols and disulfides. A mixture containing 167 μM of each compound was analyzed and good separation was achieved.(GSH = reduced glutathione; GSSG = oxidized glutathione)

7.3 A Phase I Study of Amifostine in Children

7.3.1 Patient Characteristics

Twelve patients were enrolled in the Phase I study of amifostine with ICE (Ifosfamide, Carboplatin, Etoposide) chemotherapy between July 1997 and November 1999. However, given that my role in this clinical project commenced in September 1998, I was involved in the analysis of samples collected from the last six patients. Only these six patients will be included in the discussion of this thesis.

The patient characteristics are summarized in table 7. Within this group of six patients there were three males and three females. Their median age was 6.5 years (range 3 to 18 years). All of these patients were treated with a dose of 600 mg m^{-2} of amifostine. This is reduced from the study's starting dose of 740 mg m^{-2}. At this reduced dose level, no DLTs were observed. Four patients were diagnosed with neuroblastoma, one with Ewing's sarcoma and one with adnexal tumor of Wolffian origin.

Table 7: Patient Characteristics

Patient	Gender	Age	Diagnosis	Dose (mg/m^2)	Absolute Dose (mg)
1	male	7	Neuroblastoma	600	540
2	female	3	Neuroblastoma	600	342
3	male	6	Neuroblastoma	600	480
4	female	11	Ewing's sarcoma	600	696
5	female	18	Adnexal tumor of Wolffian origin	600	1040
6	male	6	Neuroblastoma	600	480
	mean	8.5			596.3
	S. D.	5.3			245.6

S.D. = standard deviation

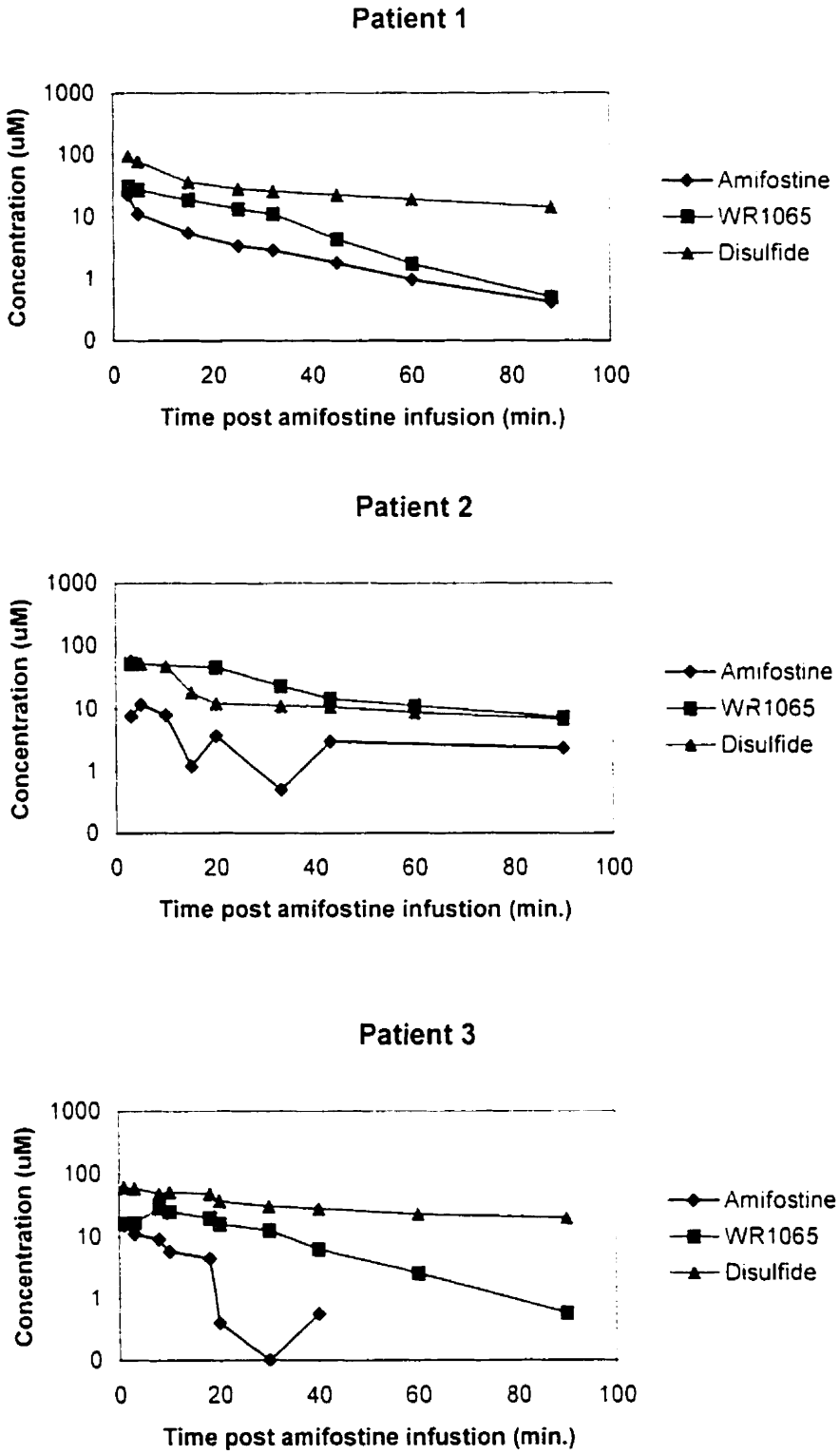
7.3.2 Defining the Maximum Tolerated Dose of Amifostine

The study opened with an initial dose of 740 mg/m^2. In the first group of three patients, one experienced DLT. Therefore, three more patients were enrolled at the same dose level. Upon review of this group of six patients, at least two were noted to have experienced severe toxicity. It was concluded that 740 mg/m^2 exceeded the MTD in children and the study continued at the lower dose level of 600 mg/m^2. Six patients were enrolled at this dose level and none had DLT. 600 mg/m^2 was concluded to be the MTD for amifostine in pediatric patients. (Fouladi 2001).

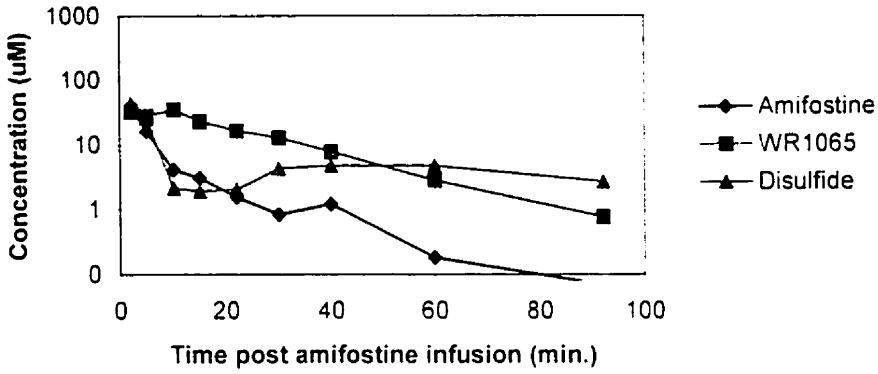
7.3.3 Amifostine Pharmacokinetics

All six of the patients enrolled in this portion of the Phase I study underwent complete plasma sampling, as described in the methods section, to determine the pharmacokinetics of amifostine, WR1065 and disulfides. Figure 15 displays the plasma concentration–time curves for each patient after their first dose of amifostine. The actual plasma concentration values of amifostine and its metabolites are included in appendix 5.

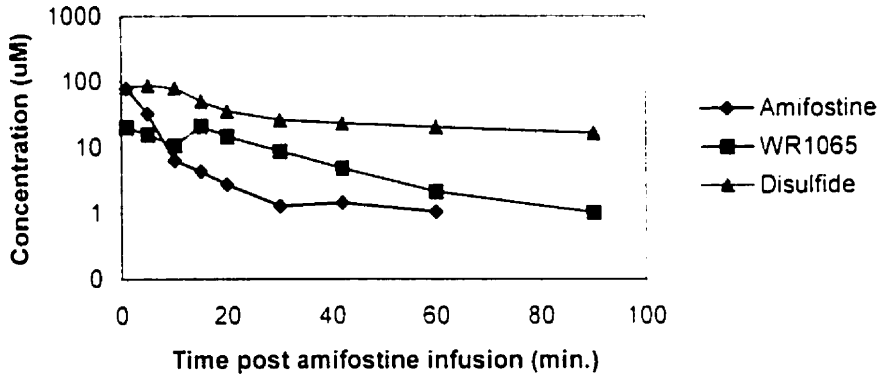
Figure 15: Plasma concentration-time profiles of amifostine and its metabolites in pediatric patients. Patients were treated with 600 mg/m^2 of amifostine administered as a 15-minute infusion. Samples were collected for 90 minutes following the end of the infusion.



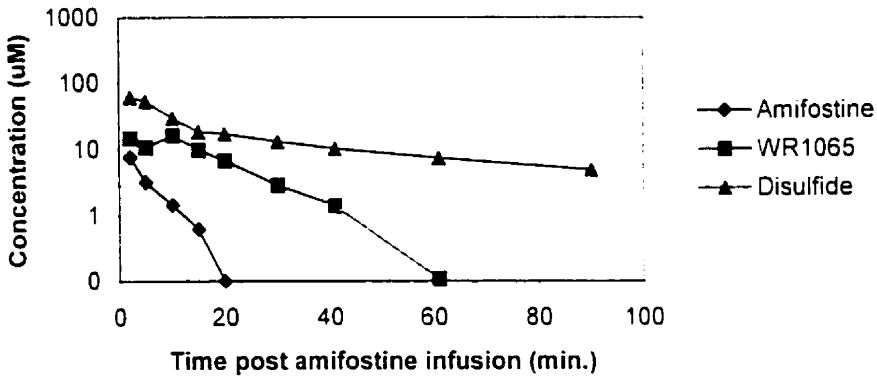
Patient 4



Patient 5



Patient 6



Peak values for amifostine, WR1065 and the disulfides were, in most cases, achieved at the end of the infusion, or shortly thereafter (Table 8), indicating that amifostine was rapidly converted to WR1065 and the disulfides. Mean peak concentrations (± S.D.) were 29.2 ± 26.5 μM for amifostine, 30.7 ± 12.4 μM for WR1065 and 67.8 ± 19.9 μM for the disulfides. These peak values were observed at 2.3 ± 1.5 minutes for amifostine, 3.2 ± 2.5 minutes for WR1065 and 2.7 ± 1.4 minutes for the disulfides. At 20 minutes after the end of the infusion, the amifostine concentrations had decreased to 1.3 to 30.7% of its post-infusion peak value and the WR1065 concentrations were 41.6 to 87.0% of their peak values.

Table 8: Peak concentration data for amifostine, WR1065 and the disulfide metabolite in patients treated with a dose of 600 mg/m^2

	Amifostine		WR1065		Disulfides	
Patient	t_{max} **(min.)**	C_{max} **(μM)**	t_{max} **(min.)**	C_{max} **(μM)**	t_{max} **(min.)**	C_{max} **(μM)**
1	3.00	22.31	3.00	31.16	3.00	96.28
2	5.00	11.70	3.00	51.63	3.00	58.24
3	1.00	15.23	8.00	28.85	1.00	60.55
4	2.00	40.29	2.00	35.48	2.00	43.04
5	1.00	77.95	1.00	21.16	5.00	87.01
6	2.00	7.59	2.00	16.13	2.00	61.61
Mean	2.33	29.18	3.17	30.74	2.67	67.79
S. D.	1.51	26.52	2.48	12.39	1.37	19.88

(t_{max} = time of maximal concentration: C_{max} = maximal concentration: S.D. = standard deviation)

The plasma concentration-time data were fitted to a non-compartmental model using the computer program, WinNonlin. The $AUC_{0-\infty}$ and $t_{1/2}$ were calculated for amifostine and its metabolites after a single dose and the results are shown in table 9. The results are highly variable as expected with such a small sample size (n = 6).

Table 9: Pharmacokinetic parameters for amifostine, WR1065 and the disulfide metabolites in patients treated with a dose of 600 mg/m^2

	Amifostine		WR1065		Disulfides	
Patient	**$AUC_{0-\infty}$ (μM·min.)**	**$t_{1/2}$ (min.)**	**$AUC_{0-\infty}$ (μM·min.)**	**$t_{1/2}$ (min.)**	**$AUC_{0-\infty}$ (μM·min.)**	**$t_{1/2}$ (min.)**
1	291.74	20.31	746.64	12.86	3796.09	65.41
2	132.68	111.30	2349.36	47.95	2101.77	80.23
3	144.08	8.47	795.61	14.46	5448.43	95.00
4	215.02	8.48	936.14	15.46	726.62	68.48
5	434.52	10.10	630.45	17.05	4951.61	90.27
6	40.41	3.10	307.18	7.35	1599.15	47.13
Mean	209.74	26.96	960.90	19.19	3103.95	74.42
S. D.	138.71	41.70	712.49	14.48	1913.63	17.72
Median	179.55	9.29	771.13	14.96	2948.93	74.36
Range	40.41 - 434.52	3.10 - 111.30	307.18 - 2349.35	7.35 - 47.95	726.62 - 5448.43	47.13 - 95.00

($AUC_{0-\infty}$ = area under the concentration curve extrapolated to infinity; $t_{1/2}$ = half-life;S.D. = standard deviation)

Amifostine's small AUC and short half-life support rapid removal of the parent drug from the plasma. From what is known about this drug, these observations are most likely due to rapid hydrolysis to WR1065 and uptake into normal tissue (Korst *et al* 1997a; Shaw *et al* 1986). The half-life of WR1065 was also very short (median = 15.0 min.; range = 7.4 to 48.0 min.) and this observation supports relatively rapid clearance and uptake into tissue sites as well as oxidation to the symmetrical and mixed disulfides (Korst *et al* 1997a). In comparison to the prodrug and active metabolite, the disulfides had a significantly longer half-life (median = 74.4 min.; range = 47.1 to 95.0 min.). It has been suggested that the disulfides may serve as an exchangeable pool for the free thiol (Korst *et al* 1997b). If this is true, the protective effect exhibited by WR1065 may extend longer than expected based simply on the WR1065 half-life.

7.4 Amifostine Modulation of Blood Glutathione

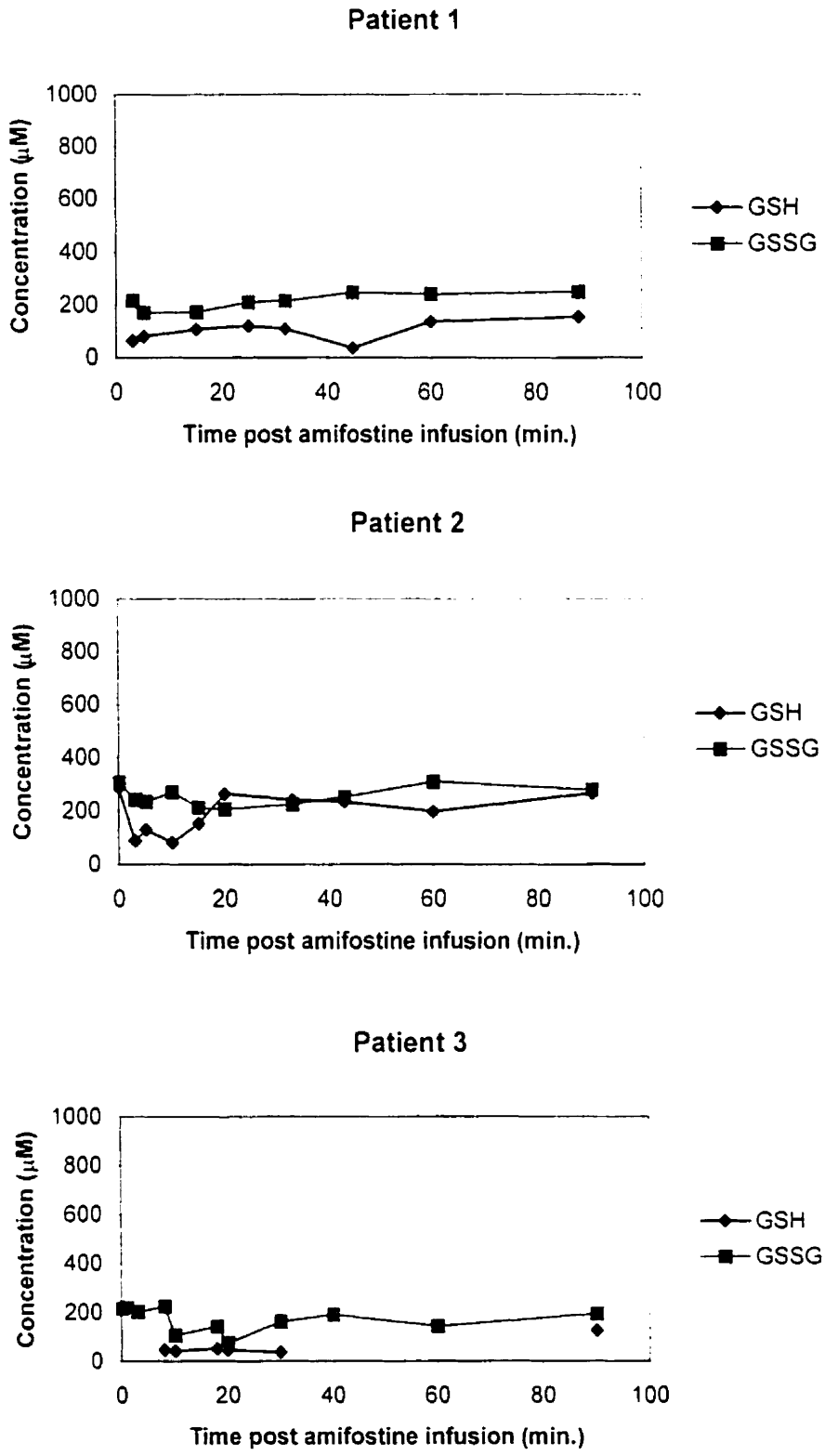
7.4.1 Preliminary Observations

To investigate whether amifostine's protective effect was related to its modulation of glutathione levels, GSH and GSSG levels were measured in the same blood samples which were drawn for pharmacokinetic analysis (*i.e.*, pre amifostine infusion and at various time points after the infusion as described in the Methods section). The samples were processed immediately as described by Bonner and Shaw (Bonner and Shaw 1991) and stored at -80°C until several sets of patient samples were accrued so that they could be analyzed as a batch. Figure 16 displays the whole blood concentration–time curves for each patient after their first dose of amifostine. The actual blood concentrations of GSH and GSSG are included in appendix 6.

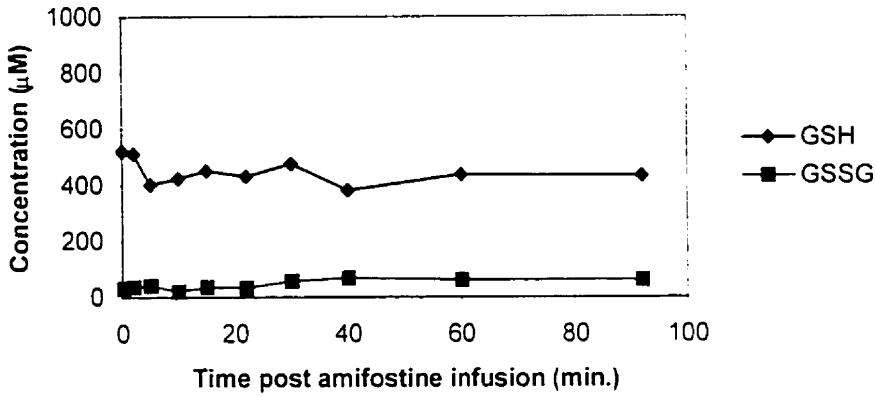
In samples that were stored for several weeks and sometimes months, we noticed that the GSSG levels were unexpectedly high and in almost all cases they were higher than GSH levels. This was contrary to what is expected under normal physiological conditions where GSSG

normally comprises less than 1% of total glutathione (Anderson 1985). Furthermore, the patients we were studying were cancer patients and there is some suggestion in the literature that cancer patients may have elevated GSH levels in blood (Engin 1995; Herebergs *et al* 1992). This is very clearly shown in patient 1 whose GSSG concentrations were 4 to 5 times higher than expected under physiological conditions and they were always higher than the GSH concentrations (ranging from 0.75 to 2.4-fold higher) throughout the 90-minute study period. These samples had been stored for 4 months prior to analysis. In patient 2, a similar trend was evident. In some instances GSH and GSSG concentrations were similar and in others, GSSG was over 2 times greater than GSH. Her samples were stored for 2 weeks prior to analysis. Finally, for patient 3, GSH was undetectable in approximately half of the samples and when it was detectable, it was very low (~5% of normal levels if 1 mM is used as the approximate normal physiological reference (Michelet *et al* 1995)). His samples were stored for 3 months prior to analysis.

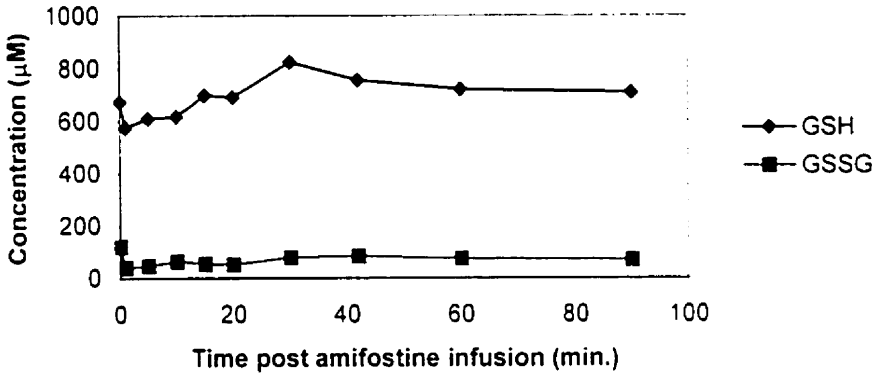
Figure 16: Whole blood concentration-time profiles of glutathione in patients treated with amifostine prior to ICE chemotherapy. Patients were treated with 600 mg/m^2 of amifostine administered as a 15-minute infusion. Samples were collected for 90 minutes following the end of the infusion. The values at time zero represent the pretreatment glutathione concentrations.



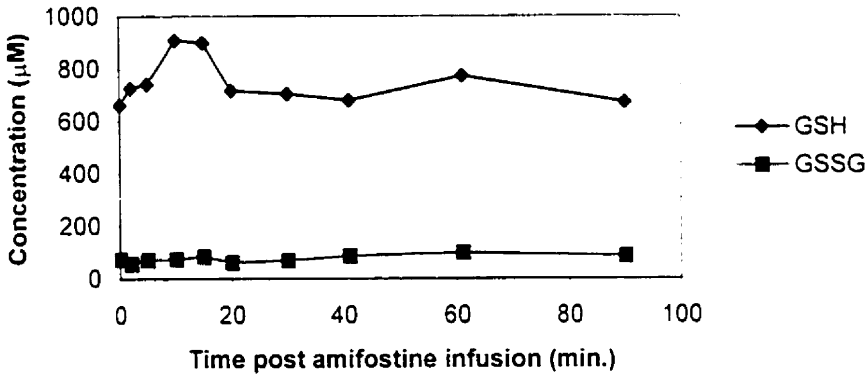
Patient 4



Patient 5



Patient 6



Subsequent reanalysis of some of these samples separated by several weeks gave rise to very inconsistent results. GSH levels decreased dramatically from their previous values and GSSG levels fluctuated randomly. An example of this is shown in Figure 17. This figure displays the results of glutathione analysis after 2 weeks and 21.5 weeks of sample storage at -80°C when the whole blood samples were deproteinized with 2.5% PCA. Although the GSH concentrations seemed low and the GSSG concentrations seemed high after the first analysis (2 weeks), we initially assumed this was either an effect of the drug or of the disease itself. Upon chance reanalysis several months later, we were alarmed at the results obtained. GSH levels had dropped to almost undetectable levels and GSSG levels had also decreased, although very slightly, when compared to the drop observed in GSH. A likely explanation of these results is that on the first analysis two weeks after the samples were obtained from the patient, a significant portion of the GSH had already oxidized to GSSG. Four and a half months later when the same samples were reanalyzed, GSSG levels were slightly lower than in the first analysis. Perhaps this was due to degradation caused by the long storage period. Most notable, however, was that GSH was almost undetectable in most of the samples. This could have been due in part to oxidation to GSSG, which subsequently degraded or it could have been due to degradation of the reduced glutathione itself.

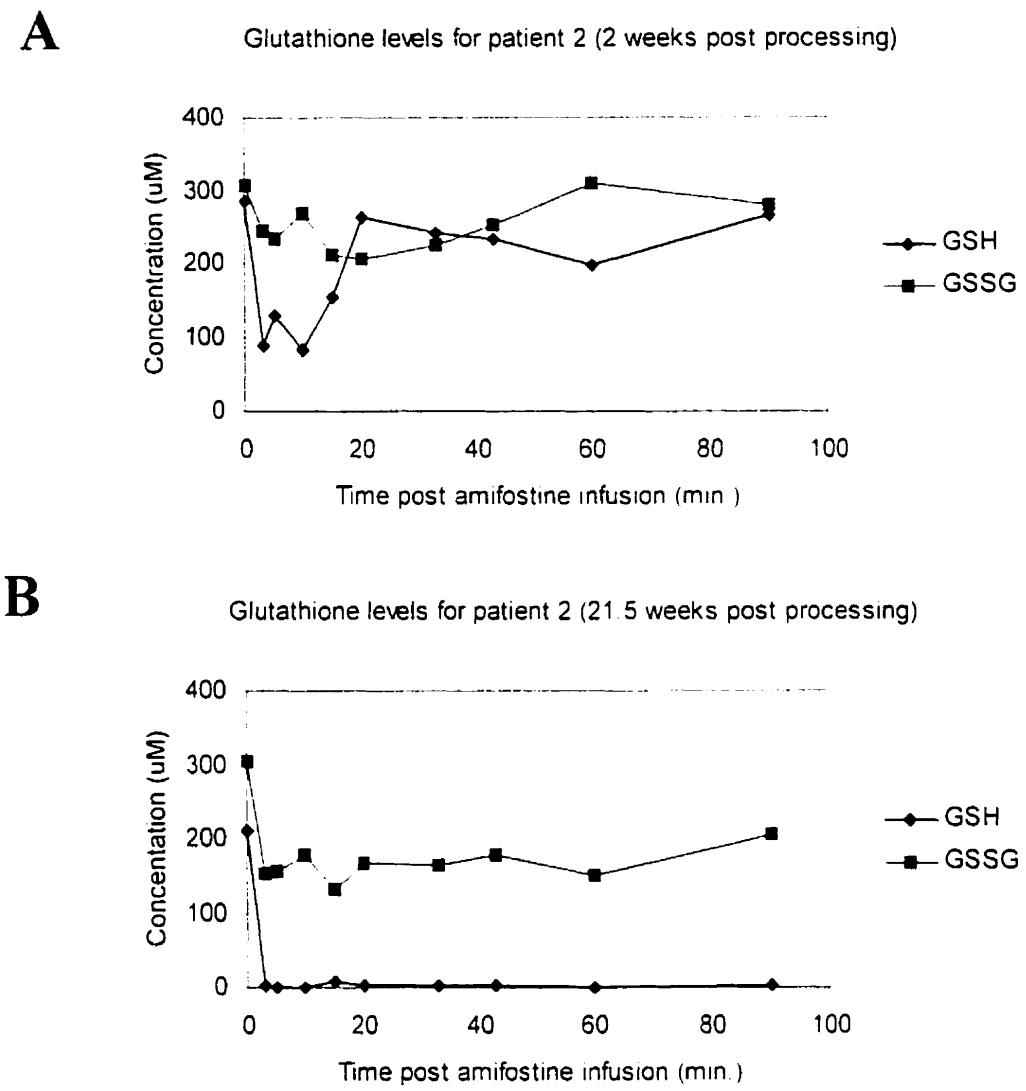


Figure 17: Comparing the effect of sample storage on glutathione levels in patients treated with amifostine. Whole blood samples were deproteinized with 2.5% perchloric acid (PCA) and the protein-free supernatants were stored at –80°C until analysis. In part A, the glutathione profile is shown after two weeks of storage. At this point in time, a significant portion of the reduced glutathione (GSH) has likely already been oxidized to GSSG as evidenced by the high GSSG levels and much lower than expected GSH levels. In part B, the same samples were reanalyzed after a total of approximately 21.5 weeks of storage. Here the GSH levels are alarmingly low and the GSSG levels have also decreased slightly from the values shown in part A. This observation is what initially alerted us to the problem with the method we were following for preparing glutathione samples for analysis.

Since this effect of sample oxidation/degradation was noticed after samples had been collected and processed for 4 out of the 6 patients enrolled in this phase of the study, we were only able to obtain reliable results for the last 2 patients. Patient 4 was enrolled after the problem was recognized but prior to a solution being found. Hence her samples were processed with 2.5% PCA and they were frozen only overnight. The results for this patient should, therefore, be interpreted with caution.

In patient 4, it appears that GSH levels decrease slightly after amifostine infusion and then fluctuate randomly within the 90-minute study period. Relative to preinfusion levels GSH remained lower than baseline by 17%. With respect to GSSG levels, there was also random fluctuation with no consistent trend evident. By the end of the study window it was almost 2-fold higher than it was before amifostine administration.

As mentioned previously, these results must be interpreted with extreme caution as the samples were processed with a concentration of PCA that was subsequently shown to cause sample oxidation over time when samples were frozen prior to analysis. Even though the samples were analyzed only one day after processing, significant oxidation may have occurred. Evidence of this is seen when this patient's GSH concentrations are compared to those of patients 5 and 6 (whose GSH concentrations were determined following pretreatment using the newly validated reliable method). Although a high degree of interpatient variability is expected, patient 4's GSH levels were 5.4% to 85.8% lower than patient 5's and 45.8% to 145.9% lower than patient 6's. While it is true that patient 6 had significantly higher levels than patient 5, the effect of the 2.5% PCA on sample oxidation which has been shown to occur cannot be disregarded.

Based on the results obtained for patients 5 and 6, it appears that amifostine *may* have some effect in raising GSH levels for a short time following the amifostine infusion, although this is very speculative. For patient 5, GSH increased by 22.3% 30 minutes after the amifostine

infusion relative to baseline. However, this effect was not sustained for a very long time, as the GSH concentration at 60 minutes was only 6.9% higher relative to baseline and only 5% higher at 90 minutes. For patient 6, similar results were observed. At 10 and 15 minutes, GSH levels were 37.4% and 36.1% higher when compared to baseline. Nevertheless, 90 minutes after the amifostine infusion the GSH concentration was a mere 1.8% higher than baseline.

In terms of GSSG concentrations, for patient 5, they actually decreased relative to baseline and remained low even after the 90-minute study period (a decrease of 42.5%). However, for patient 6, a similar trend was not observed. His GSSG levels were relatively constant throughout the study period.

7.5 Identifying and Validation of an Improved Sample Preparation Method for Glutathione Analysis

7.5.1 Effects of Various Acids on 4-week Stability

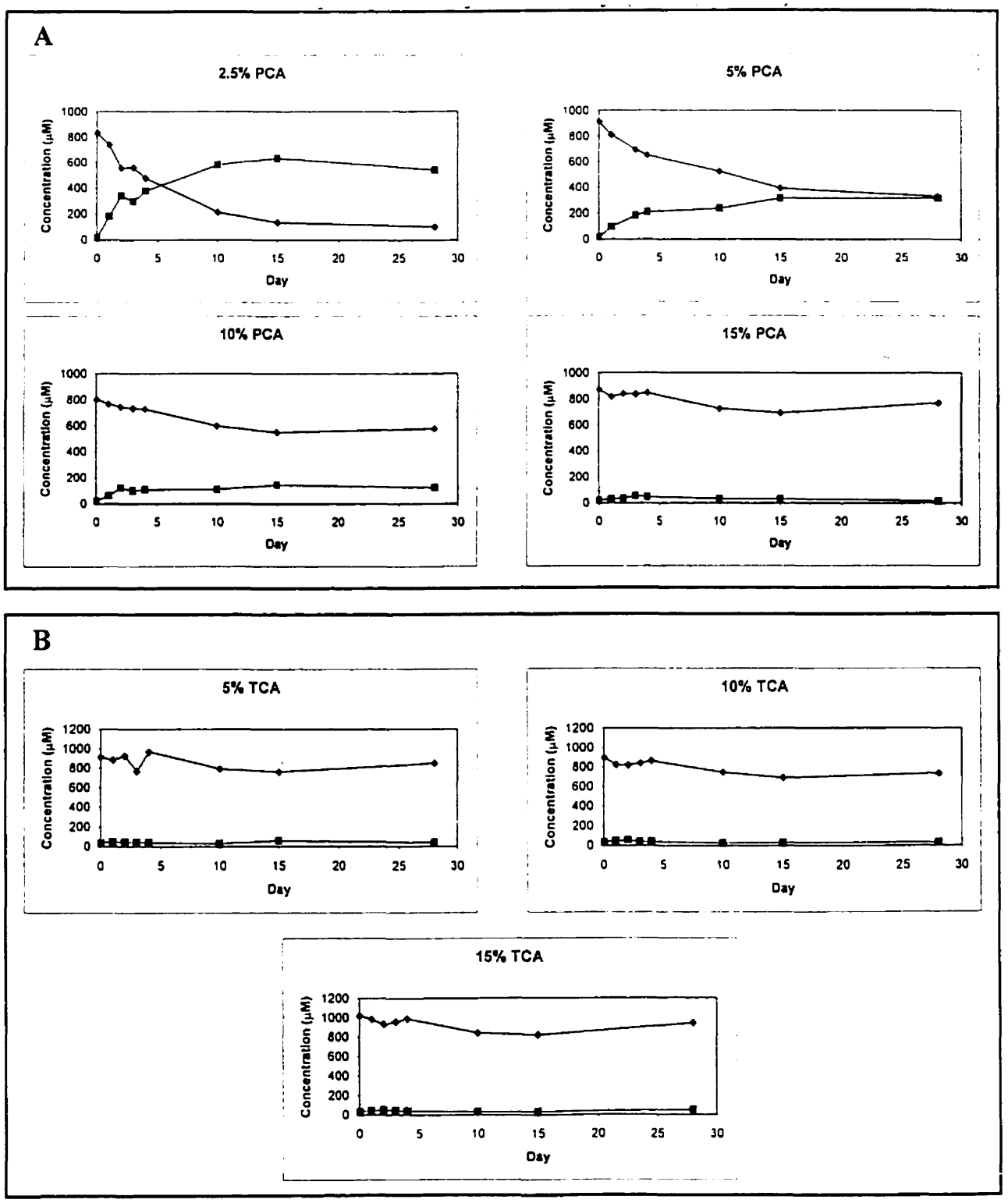
Table 10 summarizes the percent change (gain or loss) of GSH, GSSG and total glutathione (expressed as GSH equivalents: GSH + 2 GSSG) in two subjects. The mean baseline concentrations of GSH and GSSG (in subjects A and B for all acids tested) were 898.4 ± 140.7 and 63.7 ± 38.8 respectively. These are in agreement with values reported previously (Allison and Shoup 1983; Asensi *et al* 1994; Lang *et al* 1992; Michelet *et al* 1995; Mills *et al* 1994; Mills and Lang 1996; Navarro *et al* 1997; Richie, Jr. and Lang 1987; Vina *et al* 1995). In general, increasing the acid concentration allowed for greater GSH and GSSG stability prior to analysis. This was most evident for perchloric acid. Whole blood GSH and GSSG concentrations over 28 days from Subject A are shown in Figure 18A.

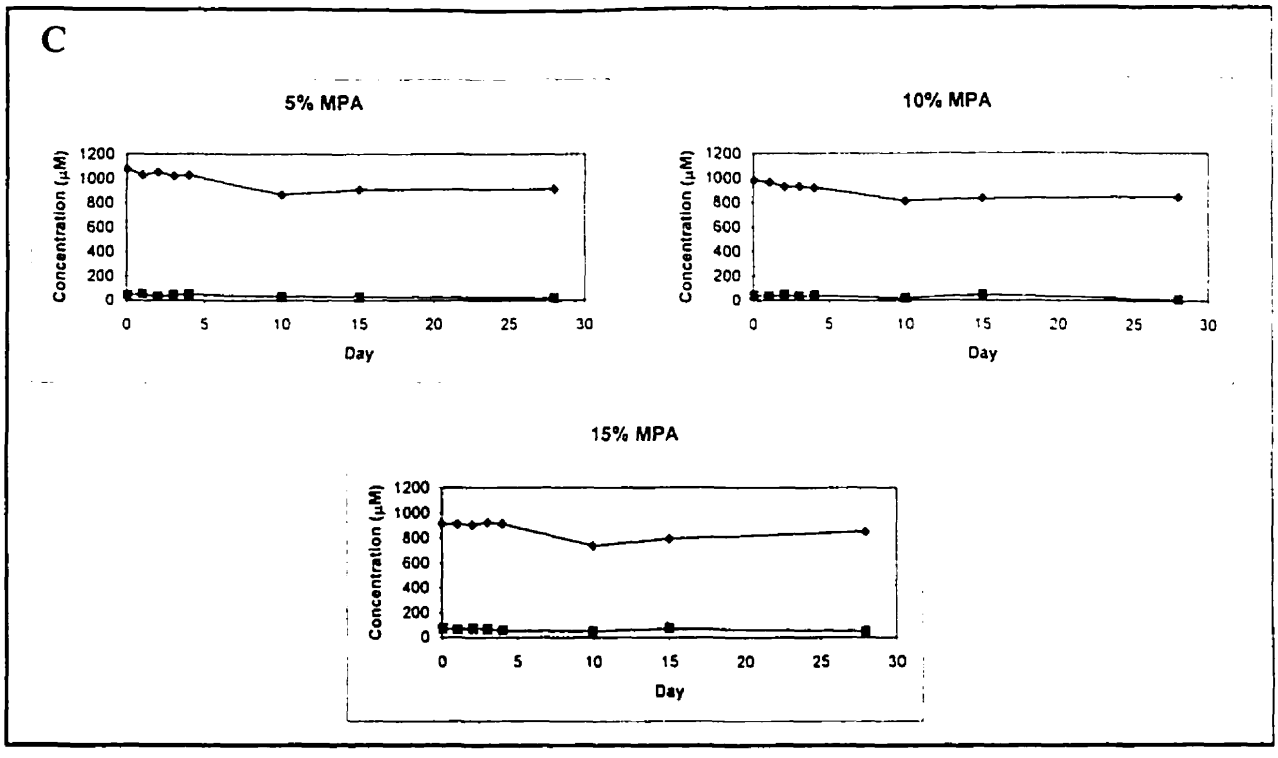
Table 10: Percent change in GSH, GSSG and total glutathione concentrations in the whole blood of 2 Healthy volunteers between day 0 to day 28 when samples were deproteinized with various acids and concentrations and stored at -80°C

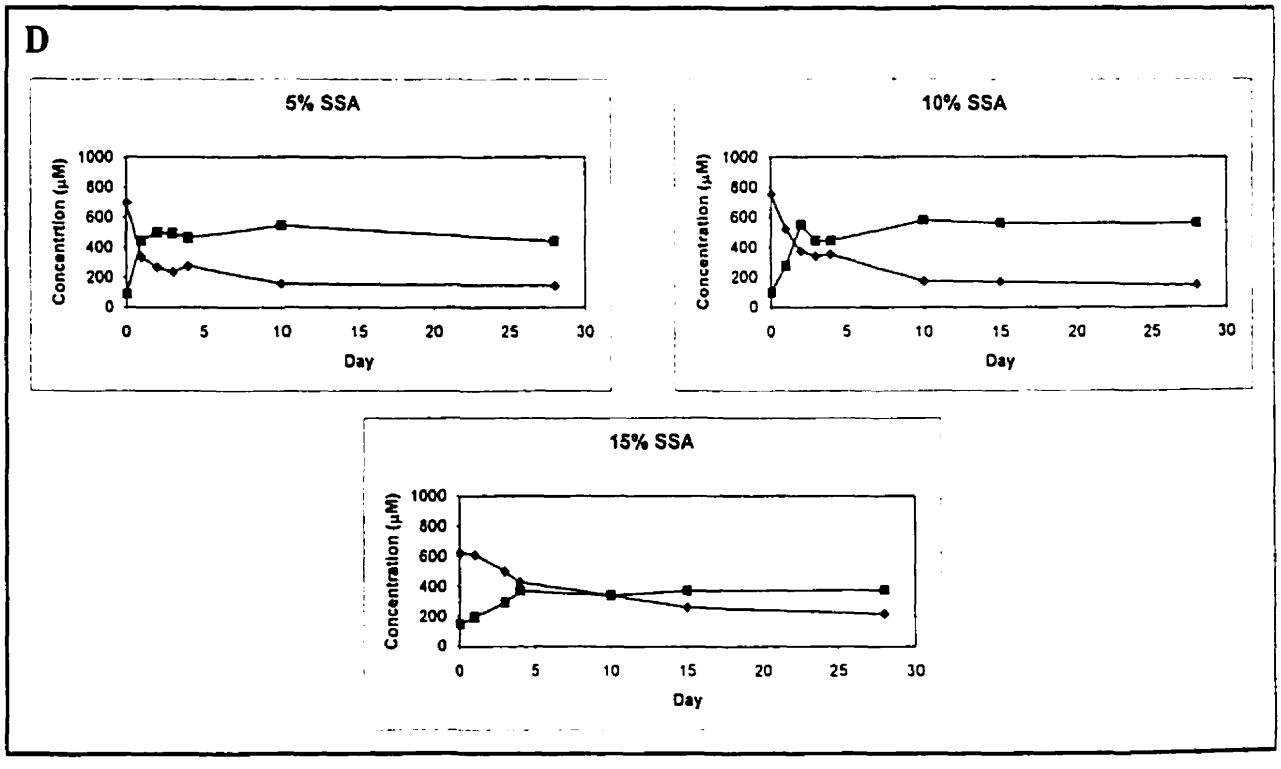
Acid and Concentration	GSH (% change)		GSSG (% change)		Total Glutathione (% change)	
	Subject A	Subject B	Subject A	Subject B	Subject A	Subject B
2.5% PCA	-87.8	-90.7	2852.5	771.3	35.9	38.4
5% PCA	-64.0	-86.6	610.7	864.2	-39.7	23.7
10% PCA	-27.8	-25.9	440.0	219.7	-2.5	1.2
15% PCA	-11.7	-10.7	-32.5	2.6	-12.7	-9.6
5% TCA	-6.7	-10.2	13.3	-53.1	-5.3	-20.2
10% TCA	-18.0	-0.1	-11.3	-14.6	-17.5	-2.7
15% TCA	-7.9	-15.2	75.9	-36.1	-3.5	-18.2
5% MPA	-15.9	-18.8	-63.4	-65.3	-19.4	-25.5
10% MPA	-13.8	-5.3	8.3	-75.2	-18.4	-17.4
15% MPA	-6.7	-3.3	-33.6	-33.0	-10.4	-8.7
5% SSA	-70.1	-79.2	1064.2	399.3	-5.6	17.8
10% SSA	-58.6	-80.8	1052.0	230.8	8.8	-17.7
15% SSA	-21.9	-65.1	135.9	154.0	-0.8	5.4

(GSH = reduced glutathione; GSSG = oxidized glutathione)

Figure 18: Whole blood GSH and GSSG concentrations after processing with various acids and concentrations in a representative subject over 28 days (♦GSH, ■GSSG).







(GSH = reduced glutathione; GSSG = oxidized glutathione)

The use of 2.5% PCA demonstrated a very rapid sample oxidation. There was an 11% decrease in GSH concentration one-day following sample collection and by day 15, 84% of the GSH was lost in subject A. Four weeks following sample collection, GSH was reduced by almost 90%. A similar pattern of sample degradation was seen in subject B with an 89% and 91% decrease at days 15 and 28, respectively. In contrast, GSSG concentrations within the samples increased sharply. One day after sample collection and processing a 10-fold increase in GSSG was observed in subject A; on day 28, GSSG had increased 30-fold. Similarly in subject B, a 14-fold increase was observed by day 28.

Sample integrity was preserved to a greater degree at the highest concentration of PCA (i.e., 15% PCA). In subject B, a loss of 5% in GSH was observed at the end of the first week and GSSG increased only 1.2-fold. Similar results were seen in subject A.

All concentrations of TCA and MPA tested demonstrated reasonable sample stability over the four-week period similar to that observed with 15% PCA (as illustrated in Figure 18B and C). In contrast, SSA showed extremely poor sample stability at all concentrations tested. Although increasing the SSA concentration from 5 to 15% did improve sample stability significantly, even at the highest concentration, SSA could not display stability that was comparable to a similar concentration of PCA (Figure 18D). Interestingly, all conditions tested showed little change in total glutathione concentrations over the course of the study suggesting that alterations in GSH and GSSG concentrations over time were mainly due to sample oxidation with degradation playing a less important role.

7.5.2 Effects on Various Acids on Glutathione Levels in Freshly Drawn Blood

The effect of the various acids on glutathione levels in freshly drawn blood was to determine whether different acids caused immediate oxidative or degradation effects on blood glutathione. 15% PCA was used to monitor interday variability and the results are shown in Figure 19. Remarkable stablity was noted and the degree of variation was within what is expected from the assay itself (see table 6).

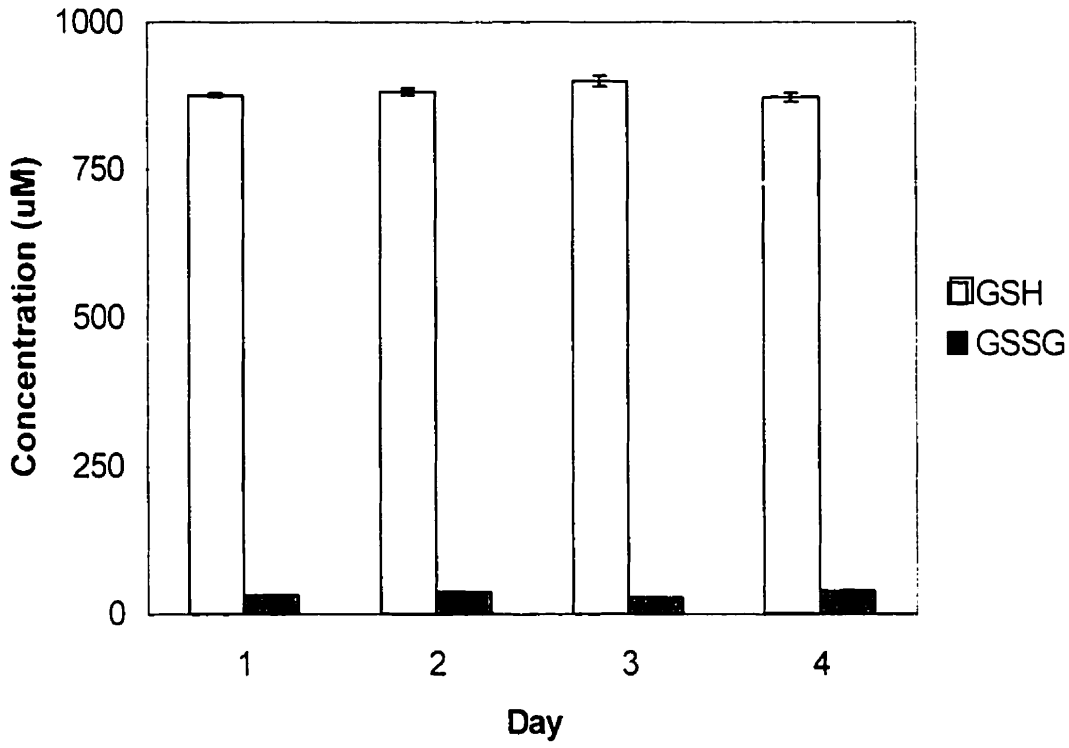


Figure 19: Interday variability of GSH and GSSG concentrations after deproteinization of fresh blood samples with 15% PCA (perchloric acid). This procedure was carried out daily for 4 days. Samples on each day were processed and analyzed immediately after they were drawn. (GSH = reduced glutathione: GSSG = oxidized glutathione)

In general, all concentrations of PCA, TCA and MPA gave similar results such that the initial GSH and GSSG levels were comparable (Figure 20). Blood samples deproteinized with TCA and MPA resulted in slightly higher GSH levels. However, this difference is no greater than the variation of the assay itself. To recapitulate, the interday percent coefficient of variation for GSH measurement is approximately 4.5%. The greatest difference in GSH concentrations when using the sample deproteinized with 15% PCA as a reference was 3.8%.

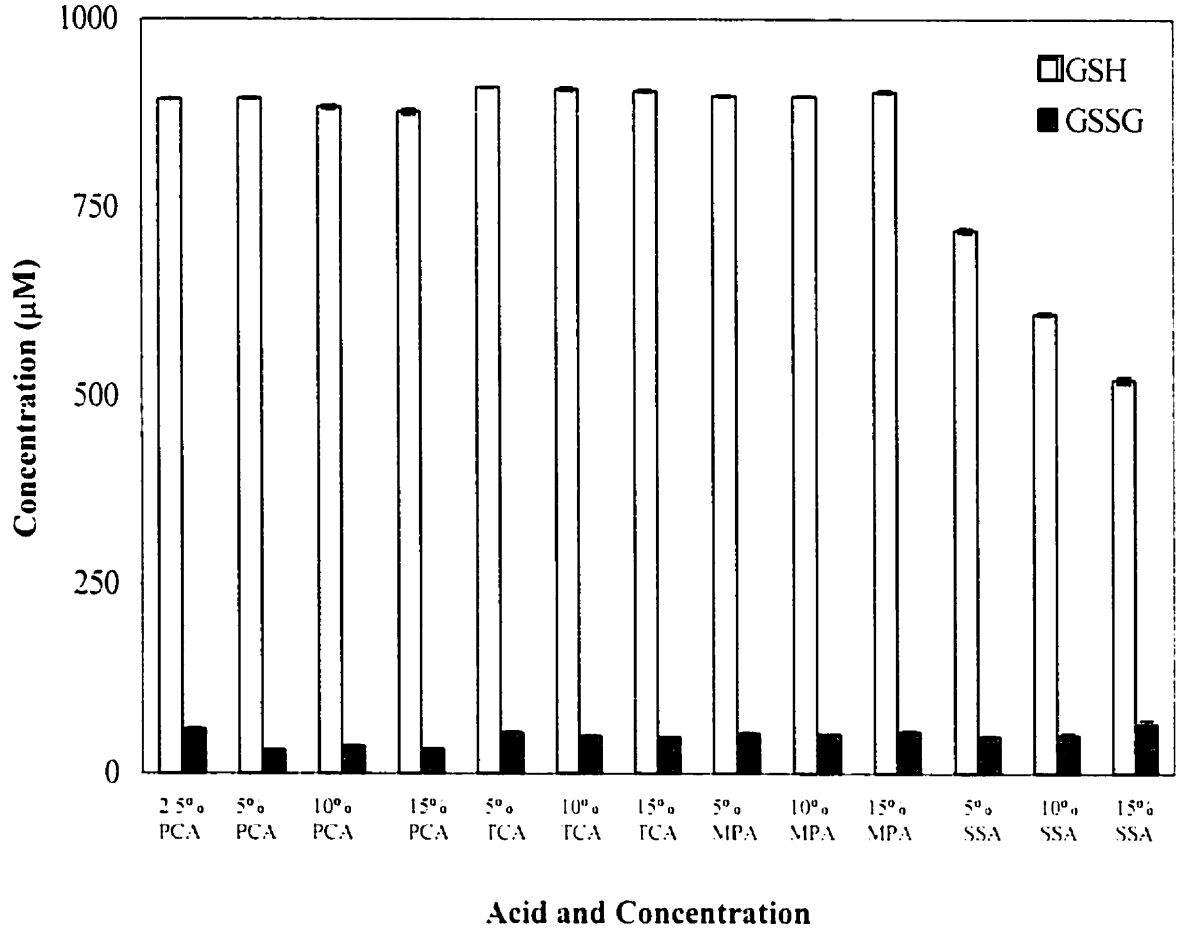


Figure 20: The effect of various acids and concentrations on initial GSH (reduced glutathione) and GSSG (oxidized glutathione) concentrations. (PCA = perchloric acid; TCA = trichloroacetic acid; MPA = metaphosphoric acid; SSA = sulfosalicylic acid)

The most striking difference is observed in the blood samples deproteinized with SSA, where the initial GSH levels decreased with increasing SSA concentration. GSH levels are approximately 17% to 40% lower when compared to 15% PCA. Of even greater interest is that the lower concentrations of GSH observed in this set of experiments do not correspond to equally higher concentrations of GSSG. This suggests that while SSA may play a role in causing GSH to oxidize to GSSG, it appears to play a greater role in degrading GSH and this is noticeable immediately.

These results show that if blood samples are processed and analyzed immediately, the choice of deproteinizing acid is less important except in the case of SSA. While immediate analysis is desirable, it is often impossible and the evidence presented in this study assists in the selection of an acid that will maintain sample integrity.

7.5.3 Efficiency of Protein Removal

The SDS-PAGE results of subject A are shown in Figure 21. Low concentrations of protein were visually detected in samples deproteinized with PCA (lanes 1 to 4), TCA (lanes 5 to 7) and SSA lanes (11 to 13). In contrast, samples treated with MPA contained relatively large amounts of protein as seen in lanes 8 to 10. Absolute protein concentrations, as determined by the Biorad microassay (PCA, TCA and SSA) or standard assay (MPA) are summarized in Table 11. Concentrations of protein in the PCA, SSA and TCA treated samples were negligible (with the exception of 5% TCA). The protein removal efficiency of MPA, on the other hand, was extremely poor, as evidenced by high residual levels of protein remaining within samples following MPA treatment (>100 μg/ml).

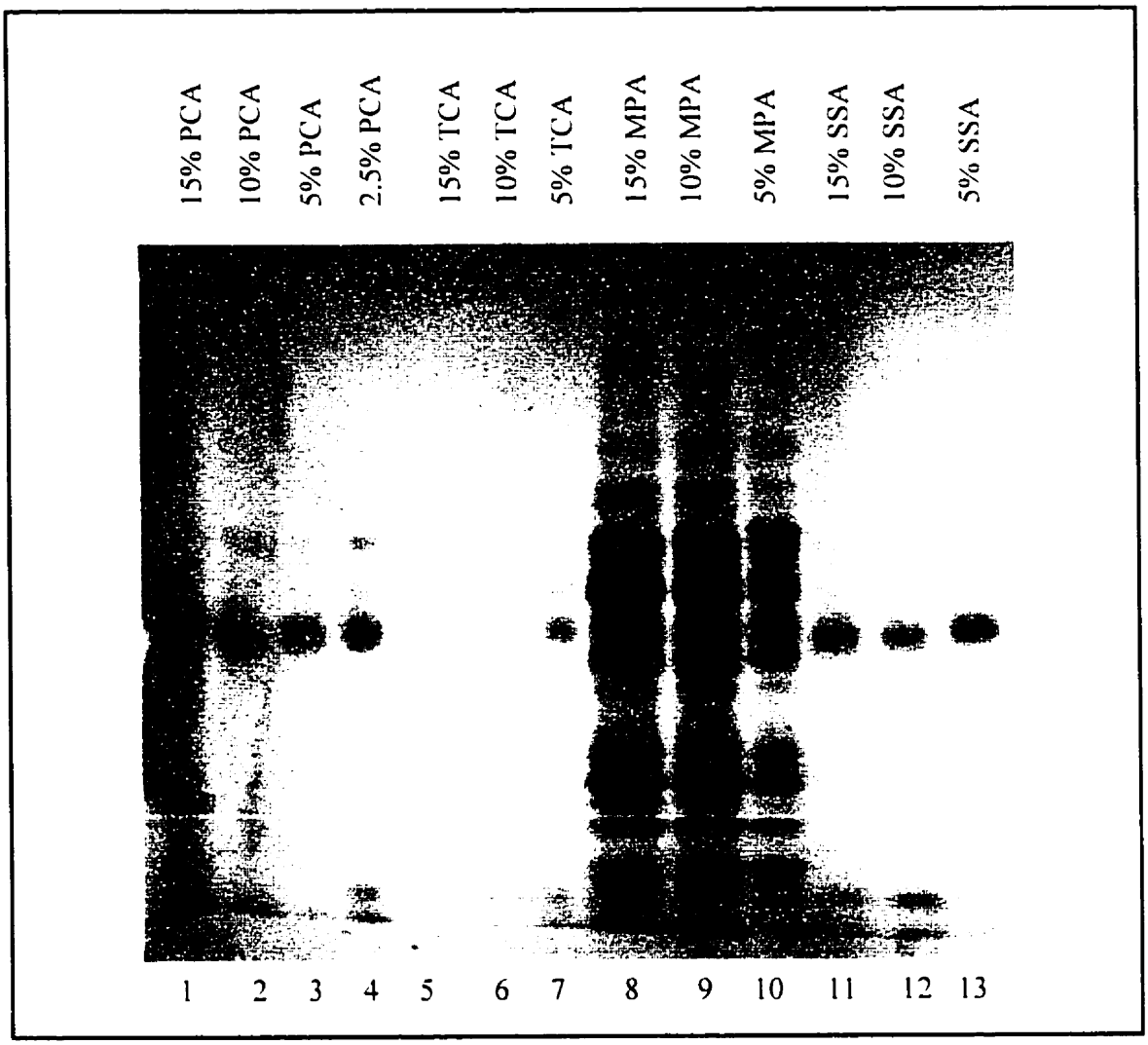


Figure 21: Efficiency of various acids at removing proteins from whole blood samples. 10µl of acidic supernatants resolved on a 10% poly-acrylamide gel and stained with Coomassie Brilliant blue to screen for protein content in the samples. (PCA = perchloric acid; TCA = trichloroacetic acid; MPA = metaphosphoric acid; SSA = sulfosalicylic acid)

Table 11: Protein concentrations in blood samples following acid precipitation.

Acid and Concentration	Protein concentration (μg/ml)	
	Subject A	Subject B
2.5% PCA	N/D	N/D
5% PCA	N/D	N/D
10% PCA	N/D	N/D
15% PCA	N/D	N/D
5% TCA	78.4 ± 0.08	76.1 ± 0.40
10% TCA	N/D	N/D
15% TCA	1.7 ± 0.20	11.9 ± 0.68
5% MPA	136 ± 0.01	494 ± 0.01
10% MPA	466 ± 0.01	839 ± 0.01
15% MPA	606 ± 0.01	940 ± 0.01
5% SSA	2.2 ± 0.20	20.9 ± 0.15
10% SSA	N/D	N/D
15% SSA	N/D	N/D

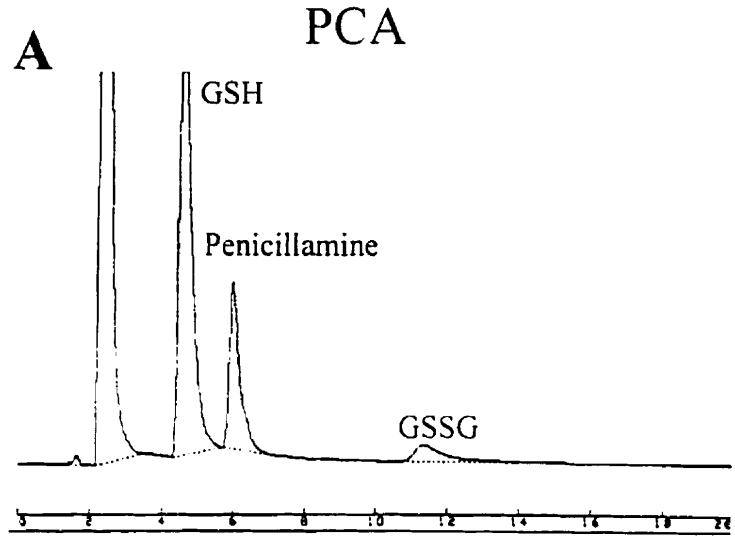
Results are expressed as mean ± S. D.

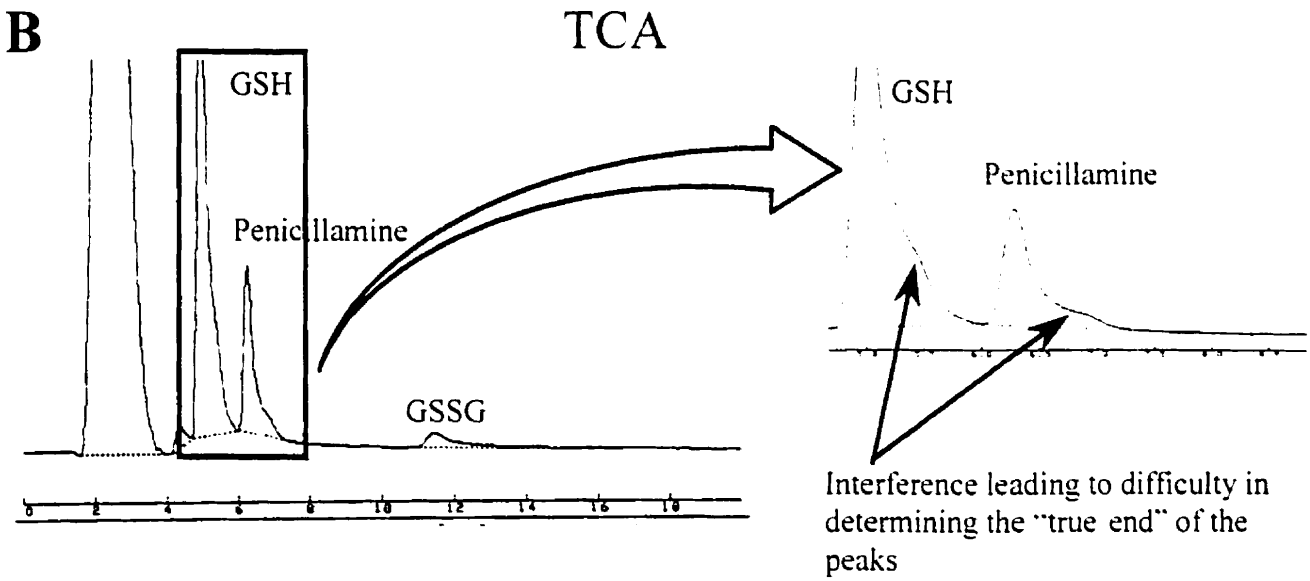
(PCA = perchloric acid; TCA = trichloroacetic acid; MPA = metaphosphoric acid; SSA = sulfosalicylic acid; N/D = below the limit of detection)

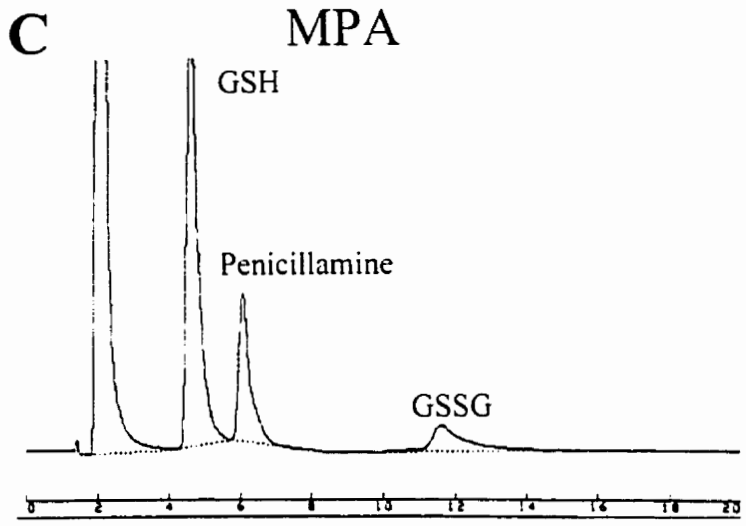
7.5.4 Effects on Chromatographic Characteristics

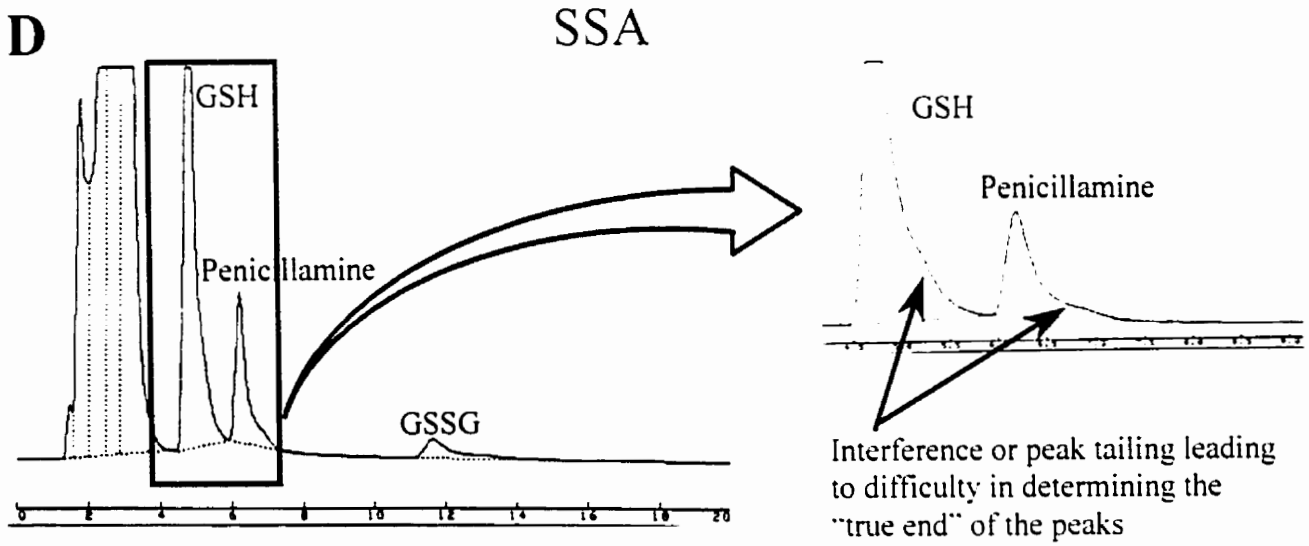
Whichever acid is selected as the protein-precipitating agent, it is important to verify that it is compatible with the system selected for analysis. In HPLC, there is the potential for the acid to alter the characteristics of the chromatogram. Examples of potential problems include peaks that interfere with the GSH and/or GSSG and large initial peaks (positive or negative) that may result in subsequent baseline drift (Harvey *et al* 1989). All of the acids tested in our study allowed for relatively good separation of the peaks of interest (GSH, GSSG and penicillamine) (Figure 22). However, TCA and SSA produced slight distortions at the end of the GSH and penicillamine peaks, thereby causing difficulty in determining the true peak endpoints (Figure 22B and D). SSA use also resulted in a broader than normal solvent front, which for the most part, did not interfere with the first peak of interest (Figure 22D). However, minute fluctuations in mobile phase composition would have the potential to result in GSH peak interference if GSH retention times shifted slightly to the left. In addition, SSA use resulted in baseline drift during GSSG elution following changes in detector sensitivity (after the elution of penicillamine and before GSSG) with the baseline falling steeply.

Figure 22: Chromatographic characteristics of various deproteinizing acids (GSH = reduced glutathione: GSSG = oxidized glutathione: PCA = perchloric acid: TCA = trichloroacetic acid: MPA = metaphosphoric acid: SSA = sulfosalicylic acid)






C
MPA
GSH
Penicillamine
GSSG


D
SSA
GSH
Penicillamine
GSSG
GSH
Penicillamine
Interference or peak tailing leading to difficulty in determining the "true end" of the peaks

7.5.5 Confirmation Studies

A final concentration of 15% PCA appeared to be most effective at preserving sample integrity when GSH and GSSG were measured in whole blood and it efficiently precipitated proteins. To further validate this conclusion, blood was drawn from 10 additional subjects and processed immediately as described previously. The samples were analyzed immediately following collection and aliquots were stored at -80°C and reanalyzed on days 14 and 28. Excellent stability was observed in all subjects as indicated by the minimal change in both GSH and GSSG concentrations between days 0 and 28 as shown in Table 12.

Table 12: Percent change in GSH, GSSG and total glutathione concentrations in the whole blood of 10 healthy volunteers from day 0 to day 28 when samples were deptroteinized with 15% PCA and stored at -80°C.

Subject	GSH (% change)	GSSG (% change)	Total Glutathione (% change)
1	1.1	15.6	2.0
2	0.2	16.5	1.4
3	1.1	12.6	0.2
4	-0.3	14.9	0.6
5	-0.8	-0.1	1.1
6	2.0	21.6	1.2
7	1.8	12.7	3.0
8	2.2	16.8	3.3
9	2.0	15.8	2.9
10	1.4	2.1	2.4
Mean	1.1	12.9	1.8
S. D.	1.0	6.7	1.1

GSH = reduced glutathione; GSSG = oxidized glutathione)

8.0 DISCUSSION

8.1 Pharmacokinetic Studies in Pediatrics

Given that treatment will fail in about 30% of all children diagnosed with cancer for various reasons, the urge to identify improved treatment modalities is of great importance. The potential cytoprotective effects offered by amifostine are of particular significance in this population for a number of reasons that have been described earlier. However, in order to use this broad-spectrum cytoprotective agent effectively in children, a Phase I study was needed to establish the safety of amifostine, to determine its maximum tolerated dose and to define the pharmacokinetics of the drug in this population. With this goal in mind, the first portion of this thesis focused on establishing the pharmacokinetics of amifostine in children.

It is a well-known fact that children handle drugs differently than adults. Infancy and childhood extend from about 2 months of age to the end of puberty (Kauffman 1992). Although growth and development are most rapid during the first several years of life, maturation continues at a slower rate during middle and later childhood. It is this dynamic process of growth, differentiation and maturation that sets children apart from adults both physiologically and pharmacologically. Therefore, important changes in both the disposition of drugs and response to medication occur in childhood. Some of these include changes in the proportions of body weight contributed by fat, protein, intracellular water and extracellular water (Kauffman 1992). Liver and kidney size (relative to body weight) also change during growth and development with the maximum relative weight reached within the first two years of life (Kauffman 1992). It is during this time when the capacity for drug metabolism and elimination tends to be the greatest. Furthermore, the body surface area is greatest relative to body weight in infants and younger children compared to older children and adults (Kauffman 1992). For these

reasons, independent pharmacokinetic studies need to be conducted in order to identify necessary alterations in dosing regimens for children.

8.2 Amifostine Pharmacokinetics in Children with Cancer

Studies of the pharmacokinetics of amifostine in humans are extremely limited. Only a few studies have been carried out and from these, the majority involved small numbers of adult subjects. There is one published study describing the pharmacokinetics of amifostine and its metabolites in children, however, the data presented are extremely limited (Souid *et al* 1999). Although the pediatric pharmacokinetic data we collected were highly variable, the degree of variability is consistent with that published in adult studies. This can be partially explained by the small number of subjects in all these studies. Some variation can be attributed to the wide range of ages in the group of patients we studied (*i.e.*, 3 to 18 years). In addition, age-related changes in alkaline phosphatase levels in children (reference range = 45 to 630 U/L for males and females aged 3 to 18 years) have been reported (Hospital for Sick Children 1995). To date, a large-scale study examining the pharmacokinetics of amifostine and its metabolites in human subjects with cancer has not been carried out. However, even with the limited scope of the available data, several inferences regarding the pharmacokinetics of this drug can be made and trends are evident when comparing children to adults.

Firstly, to compare most effectively the pharmacokinetics observed in children to those in adults, it was important to identify published studies in adults that used similar doses and modes of administration. This is extremely important because amifostine is a prodrug with a very short elimination half-life. A review of the literature revealed that most of the research in the area of human amifostine pharmacokinetics has been conducted mainly by two groups. The first group is led by Shaw (Shaw *et al* 1986; Shaw *et al* 1996a; Shaw *et al* 1999) and the second by van der

Vijgh (Korst *et al* 1996; Korst *et al* 1997a; Korst *et al* 1997b; van der Vijgh and Korst 1996). In general, the data presented in the studies by Shaw *et al.* are based on bolus injections or very brief infusions of comparatively low doses of amifostine relative to what was used in the study presented herein. However, van der Vijgh *et al.* described pharmacokinetic data following 15-minute infusions of amifostine doses that were only modestly higher than ours. One specific study conducted by this group will form the basis of the comparisons between pharmacokinetics of children and adults (Korst *et al* 1997a). One key assumption was made in making these comparisons. Adults were given 740 mg/m^2 of amifostine while our patients were given 600 mg/m^2. In order to correct for the differences due to dose, a linear relationship between dose and plasma concentrations of the drug and metabolites was assumed. In addition, we made several comparisons regarding the 90-minute plasma concentrations available to us. However, plasma concentrations were reported at 120 minutes after amifostine administration in the adult study. Therefore, after correcting for the dose difference, the 90-minute plasma concentration was estimated by back extrapolation using the mean reported half-life. Knowing this half-life, the slope of the line representing the elimination phase could be calculated and used to estimate the concentration at an earlier time point.

8.2.1 Pharmacokinetics of the Active Metabolite, WR1065

Our first and primary comparison of interest is the exposure of adults and children to WR1065, the active metabolite and essentially the "drug" of importance since it is responsible for the cytoprotective action of amifostine. When studying metabolites, the pharmacokinetic parameters that can be calculated are AUC, $t_{\frac{1}{2}}$, C_{max} and t_{max}. Despite the limited nature of the metabolite parameters, a great deal of information can be deduced regarding the exposure of the patient to the active cytoprotective species.

When initially comparing the AUCs extrapolated to infinity and correcting for dose (to 600 mg/m^2), it appears that the children in our study had an approximately 2-fold lower exposure to WR1065 than the adults (mean $AUC_{0-\infty}$ = 960.9 ± 712.5 μM·min. in children *vs.* 2213.5 ± 414 μM·min. in adults). However, when examining WR1065 peak concentrations, the values did not differ much between the two groups (mean peak concentration = 30.7 ± 12.4 μM in children *vs.* 36.6 ± 13.5 μM in adults). Similarly, the concentrations of the active metabolite 90-minutes following amifostine infusion were comparable (mean = 0.6 ± 0.4 μM in children *vs.* 0.6 ± 0.8 μM in adults) (Korst *et al* 1997a). These points taken together infer that systemic exposure to WR1065 as well as the AUC from 0 to 90 minutes were similar in the two studies and that amifostine is equipotent in adults and children at a dose of 600 mg/m^2.

The differences that were initially observed in the $AUC_{0-\infty}$ can be explained by the differences in the length of sampling between the two studies. We collected blood samples for 90-minutes while the Korst *et al.* (1997a) collected samples for 6 hours following drug administration. This difference resulted in the identification of a much longer terminal half-life in the adult study (7.3 ± 3.6 hours vs. 19.2 ± 14.5 minutes in our study). In sampling for only 90-minutes, it is likely that we were unable to characterize the true terminal elimination portion of the concentration-time curve. Hence extrapolation of the AUC to infinity would be based on a half-life that is not the true terminal half-life thus resulting in an underestimation of the total AUC.

With respect to other pharmacokinetic parameters of WR1065, peak concentrations observed at that end of infusion or very shortly thereafter in both studies suggest rapid conversion of the prodrug into its active metabolite. Other studies have made similar observations and drawn similar conclusions (Korst *et al* 1996; Korst *et al* 1997a; Shaw *et al* 1986; Shaw *et al* 1994; Souid *et al* 1999). The rapid uptake of WR1065 into tissues results in a

rapid disappearance of the metabolite from the plasma compartment. We observed a median half-life of 15 minutes for the metabolite, which is similar to that reported by Souid *et al.* (1998) in one pediatric patient and the initial half-life reported by Korst *et al.* (1997a) in adults. This short half-life is also suggestive of rapid conversion into the disulfide metabolites. Therefore, WR1065 in plasma has two possible fates: rapid uptake into tissues where it exerts its cytoprotective effect and rapid oxidation into disulfide metabolites.

8.2.2 Pharmacokinetics of the Parent Drug, Amifostine

There are many more studies describing the pharmacokinetics of the parent drug, amifostine (Korst *et al* 1996; Korst *et al* 1997a; Shaw *et al* 1986; Shaw *et al* 1999; Souid *et al* 1998; Souid *et al* 1999). Interesting differences and distinctions between children and adults are noted. Firstly, all investigators agree that the peak concentration of amifostine is achieved immediately at the end of the drug infusion. This was also the case in our study, however, a very significant difference was noticed. Using the same adult study that was previously used for the comparison of WR1065 kinetics, an amifostine peak concentration of 190.3 ± 45.2 μM (when adjusted for a 600 mg/m^2 dose) was observed in adults following a 15 minute infusion. In stark contrast, our peak concentrations were 29.2 ± 26.5 μM (range = 7.6 to 78.0 μM). A study conducted by Souid *et al.* (1999) reported relatively low and extremely variable maximal concentrations in children much like ours. Correcting to a 600 mg/m^2 dose, a mean peak of 62.5 μM (range = 7.3 to 172.7 μM) was reported in whole blood. Investigators at St. Jude's Children's Hospital in Memphis, Tennessee have also administered amifostine to pediatric patients and they have measured plasma and whole blood levels of amifostine. They have observed that whole blood and plasma levels of amifostine are similar (personal communication, Kirstein 2001). Therefore, the results reported by Souid *et al.* (1999) are quite similar to ours.

Furthermore, preliminary results from the group at St. Jude's further corroborate our findings and those of Souid *et al.* (1999) that peak amifostine levels in children appear to be significantly lower than in adults (personal communication, Kirstein 2001).

This suggests major differences in prodrug (amifostine) metabolism in children *versus* adults. One might speculate that the rate of hydrolysis to WR1065 is significantly enhanced in children. There are numerous other examples of enhanced metabolism in this population such as those mediated by the cytochrome P450 systems (Cresteil 1998; Tanaka 1998) and plasma cholinesterases (Ahlstrom *et al* 1999; Bevan *et al* 1998). Since higher plasma WR1065 concentrations were not observed in children, a proposed explanation for this observation would be a much faster and more efficient uptake mechanism of WR1065 into tissue. However, it has been suggested that tissue uptake of WR1065 is saturable (Korst *et al* 1997a), therefore oxidation of WR1065 to disulfides and their subsequent elimination may also be enhanced in children. Specific studies investigating this have not been carried out to date.

Despite much higher peak concentrations observed in adults, the final concentration (at 90 minutes) that we evaluated was similar in both groups (0.4 ± 0.9 μM in children vs. 0.3 ± 0.3 μM in adults; corrected for a dose of 600 mg/m^2) (Korst *et al* 1997a). Therefore, differences in the $AUC_{0-\infty}$ were expected and they were indeed observed ($AUC_{0-\infty}$ = 209.7 ± 138.7 μM·min. in children and 2967.6 ± 948 μM·min. in adults; corrected for a dose of 600 mg/m^2) (Korst *et al* 1997a). As with WR1065, a much longer terminal half-life of 48 minutes was reported in adults (Korst *et al* 1997a) than our median half-life of 9.3 minutes. Again, this was likely due to differences in the sampling protocol between the two studies. While the differences in the terminal portion of the curves could have contributed slightly to the differences in the areas, a greater contribution would be the discrepancies in the peak concentrations between adults and children.

The rapid conversion of the parent drug to WR1065 suggests a fast decrease in amifostine concentrations in plasma. This was indeed observed in our patients who had an amifostine half-life of about 9 minutes. This is similar to values noted by Shaw *et al.* (1986) and Souid *et al.* (1998) who reported half-lives of 8.8 and approximately 10 minutes respectively. The much longer terminal half-life of 48 minutes reported by Korst *et al.* (1997a) is attributable to the longer time for which samples were collected in that study (3 hours). Although both Korst *et al.* (1997a) and Shaw *et al.* (1986) described a biphasic plasma clearance of amifostine, both phases in Shaw's study were observed within 45 minutes. The sampling frequency used in Korst's study was clearly too low to distinguish between these two phases. Hence, their initial half-life of 2.4 minutes is likely a combination of the two phases described by Shaw and colleagues (1986). Similar to the observations made for WR1065, plasma levels during the final elimination phase were so low that they were unlikely of clinical relevance (Korst *et al* 1997a).

8.2.3 Pharmacokinetics of the Total Disulfides

Finally, the disulfide kinetics described in our study refer to total disulfides. Observing peak disulfide concentrations 5 minutes post amifostine infusion is indicative of very rapid oxidation of WR1065 to mixed and symmetrical disulfides (refer to Figure 1). In agreement with studies presented by Korst *et al.* (Korst *et al* 1996; Korst *et al* 1997a) we observed much higher peak levels of the total disulfides which contrasted with the very low peak levels of WR33278 observed by Shaw *et al.* (1986). This indicates that a large proportion of the total disulfides is composed of mixed disulfides. It has been documented that the symmetrical disulfide (WR33278) does have some cytoprotective properties although to a much lesser extent than WR1065 (Capizzi 1999a; Grdina *et al* 1995). While this is less likely for the mixed disulfides (Korst *et al* 1997a; Korst *et al* 1997b), they may still have clinical relevance if they serve as an exchangeable pool of WR1065 (Korst *et al* 1996; Korst *et al* 1997a; Shaw *et al* 1994). The

observed half-life of the mixed disulfides of approximately 74 minutes in our pediatric patients is indicative of sustained presence of this metabolite in plasma much longer than amifostine or WR1065. Therefore, should reduction of these disulfides occur, the cytoprotective effect of WR1065 may indeed be extended beyond that predicted by its pharmacokinetics. In comparing the half-lives of the disulfides in children *versus* adults, pediatric patients have a shorter half-life (74 minutes *vs.* 150 minutes (Korst *et al* 1997a)). However, as in previous discussions, this may be due to differences in the length of time during which pharmacokinetic samples were collected.

8.2.4 Pharmacokinetic Outliers

As with most studies involving human subjects, apparent outliers always exist. In this study, there were two such patients who appeared to fall at opposite ends of the spectrum with respect to amifostine and WR1065 kinetics. Patient 2 was a 3-year old girl who, despite showing a comparable AUC for amifostine with respect to the other patients in this group, had a much longer half-life. This prolonged amifostine half-life resulted in an AUC for WR1065 that was 2.5 to 7.6 times larger than those observed for the other patients and a half-life that was 2.8 to 6.5 times longer. While this would suggest that this patient would likely have experienced amifostine toxicity, she actually tolerated the drug well. This may be an age-related phenomenon as this was the youngest patient in the group and young children often tend to have higher thresholds for toxicity (Steen 2000).

The other patient whose pharmacokinetic parameters were notably different from the others in the group was Patient 6, a 6-year old boy. His amifostine AUC was 3.3 to 10.8 times smaller which corresponded to a clearance rate that was 3.3 to 12.4 times faster and a half-life that was 2.7 to 6.6 times shorter. In line with this trend, his WR1065 AUC was 2.1 to 7.6 times smaller and the WR1065 half-life calculated for this patient was 1.7 to 6.5 times shorter.

Reasons for these differences are unknown. Shaw *et al.* (1986) noticed a similar phenomenon with one of their patients but it was explained by the very high leukocyte count in this patient at the start of study. Leukocytes are known to contain considerable alkaline phosphatase activity. Since alkaline phosphatase catalyzes amifostine hydrolysis to WR1065, this suggests that the high clearance may be due to leukocyte mediated amifostine hydrolysis. However, this child's leukocyte counts were not significantly different from any other patient in the group.

8.3 Amifostine Doses in Pediatrics

Having identified several differences as well as similarities in the pharmacokinetics of amifostine and its metabolites amongst children and adults, the issue of dose adjustment needs to be addressed. The most meaningful finding in this analysis was that these two populations have a similar exposure to the active metabolite, WR1065. The differences observed with respect to the prodrug are much less important since exposure to the active metabolite is what confers the biological effect of the drug. Amifostine is generally administered to adults at higher doses than what was found to be the MTD for the children in this study. Specifically, 910 mg/m^2 is the MTD of amifostine (administered as a single dose) in adults (Dorr and Holmes 1999; Kemp *et al* 1996). Other studies have shown that 683 mg/m^2 is the MTD when using multiple doses (Betticher *et al* 1995), while up to 740 mg/m^2 (given three times daily) has been used (Korst *et al* 1997a). Cytoprotective efficacy has been demonstrated at these doses. Therefore, it may be tempting to speculate that the children in our study did not fully benefit from the cytoprotective effects of amifostine based on these findings. Should cytoprotection be limited by dose, a potential problem arises. This Phase I study had identified DLTs (grade 3 anxiety and somnolence) at a dose of 740 mg/m^2; therefore the MTD was defined as 600 mg/m^2 (Fouladi 2001). However, well-controlled studies to determine the minimum effective dose have not been

carried out to date. In general, it has been assumed that higher doses of amifostine would result in cytoprotective action. Whether our patients would benefit from amifostine's cytoprotection at a higher dose is unknown due to the lack of a "no amifostine" control arm. Unfortunately, this objective was not within the scope of this study, however, future experiments would be useful.

8.4 Amifostine and Glutathione

The second goal of this thesis was to identify a biological marker that could be used as a measure of amifostine's cytoprotective effects. Blood glutathione is a surrogate marker of oxidative stress in a variety of conditions as it changes in response to exposure to various drugs (Hogarth *et al* 1996; Malik *et al* 1997) and disease states (Lands *et al* 1999; Rodriguez *et al* 1998; Sharma *et al* 2000; Sido *et al* 1998; Usal *et al* 1996). Glutathione changes in less accessible tissue are reflected by fluctuations in blood glutathione (Navarro *et al* 1997). Evidence in *in vitro* work suggests that glutathione may increase in response to exposure to amifostine (Issels and Nagele 1989). It therefore may be an important marker to indicate the degree of protection offered by the drug.

8.4.1 Issues Associated with Glutathione Analysis

In order to establish the validity of this marker, a reliable analytical method needed to be identified. Effective analysis of GSH and GSSG requires a simple, rapid and sensitive analytical method. HPLC coupled with dual electrode electrochemical detection (HPLC-EC) represents one such method. It involves simple sample preparation without the need for derivatization, relatively short analysis times and has the ability to directly and simultaneously determine both compounds of interest. Prior to electrochemical analysis, whole blood samples must be deproteinized as proteins may interfere with the assay, and shorten the life of HPLC columns and

detectors. Furthermore, rapid acidification of sample is critical as it prevents both autooxidation and enzyme-catalyzed degradation (via γ-glutamyl transferase) of GSH to GSSG (Anderson 1985). Since there is an abundance of published methods using HPLC-EC (Bonner and Shaw 1991; Carro-Ciampi *et al* 1988; Harvey *et al* 1989; Krien *et al* 1992; Richie, Jr. and Lang 1987), we did not question the validity of the method we chose to follow. Unfortunately, serious problems with respect to sample stability arose and the primary objectives for this portion of the project were modified to include identification and validation of an improved method for sample preparation.

As described in the results section, we noticed a change in the GSH and GSSG content of whole blood samples. Despite using a published method for sample preparation (Bonner and Shaw 1991) that allowed sample storage prior to analysis, we found our samples to be unstable. Oxidation of GSH to GSSG was likely the major contributor to sample instability even though whole blood was immediately acidified with 0.5 M PCA and stored at -80°C upon receipt. Subsequent review of the literature regarding glutathione analysis revealed that (Hercbergs *et al* 1992; Navarro *et al* 1997) while numerous protein precipitating acids and concentrations have been reported in the literature, investigators disagree as to which acid is most appropriate and whether sample storage following acidification prior to analysis is acceptable (Anderson 1985; Bonner and Shaw 1991; Carro-Ciampi *et al* 1988; Krien *et al* 1992; Roberts and Francetic 1993; Slordal *et al* 1993). To resolve these questions, we systematically compared the effects of various concentrations of the most commonly used acids on the short-term stability of whole blood GSH and GSSG. Our results show that both the choice of acid and acid concentration are important determining factors in the feasibility of sample storage prior to analysis. To complete this portion of the study, we also examined the efficiency of these acids at removing protein from the blood samples and we critically inspected the resulting chromatograms to determine if they caused any significant alteration of chromatographic characteristics.

In general, increasing concentrations of the precipitating acid resulted in greater stability of blood GSH and GSSG. Nonetheless, differences were found in the efficacy of the various acids in maintaining sample stability. Following four weeks of sample storage, even the highest concentrations of SSA tested demonstrated poor stability of both GSH and GSSG within our samples. Furthermore, SSA appears to cause an immediate change in sample composition immediately after acidification and deproteinization as indicated in Figure 26. Our results contradict several studies which have reported minimal loss of glutathione over time following pretreatment with SSA (Anderson 1985; Roberts and Francetic 1993; Slordal *et al* 1993). The discrepancies observed within these studies may be explained by various methodological differences. Firstly, GSH was derivatized with either monobromobimane or 5,5'-dithiobis(2-nitrobenzoic acid) either prior to, or shortly after acidification with 5% SSA. This extra derivatization step may play a role in the stabilization of GSH irrespective of subsequent acidification although carrying out this additional reaction has the potential to introduce error in the determination of GSH concentrations. Secondly, total glutathione levels were measured in several studies rather than GSH and GSSG individually. Thus increases in GSSG due to oxidation may have been offset by decreases in GSH. This would then be consistent with our results. While total glutathione concentrations remained relatively constant in both subjects in the present study during all SSA treatments, individual GSH and GSSG concentrations were altered significantly.

In addition to problems with stability, SSA was found to produce broader than normal solvent fronts and baseline drift during GSSG elution. Liu *et al.* have (1996) also reported baseline drift following HPLC analysis of biological samples treated with SSA. While these changes did not interfere with GSH and GSSG separation for the most part, alteration in retention times due to minute fluctuations in mobile phase composition or changes in analytical

column effectiveness (i.e. decreased analyte retention due to age) have the potential to result in peak interference.

In contrast with SSA, MPA yielded acceptable GSH and GSSG stability during the 28-day study period. These results corroborate several previous studies where 5% MPA provided good short-term stability of GSH and GSSG within blood samples (Mills *et al* 1990; Mills and Lang 1996; Richie, Jr. and Lang 1987; Russo *et al* 1986b). Nevertheless, we found all MPA concentrations tested to be inefficient at removing protein from the samples, which can be problematic for electrochemical detectors (see below).

One specific disadvantage of electrochemical detection is loss of analyte signal following repeated use. Carro-Ciampi *et al.* (1988) noted a 50% loss in the GSSG signal after only 100 injections of sample. We observed a similar pattern of decreased analytical sensitivity with our electrochemical detector. Presumably signal loss is due to particulate accumulation on the surface of the graphite electrode, which necessitates frequent cleaning of the detection system. While electrode cleaning can often restore the analyte signal in the short-term, surface accumulation will lead to complete electrode failure over time. Thus, it is imperative that samples injected onto the chromatographic system contain as little electrochemically active components as possible, and it is on this basis we recommend that MPA not be routinely used as a precipitating agent.

As indicated in figure 18, PCA presented extremely divergent results with respect to sample stability over time. Concentrations of 2.5% to 10% were clearly pro-oxidant, whereas 15% attained acceptable sample stability. Controversy exists within the literature regarding the pro-oxidant versus anti-oxidant properties of PCA (Asensi *et al* 1994; Liu *et al* 1996; Michelet *et al* 1995; Ozcimder *et al* 1991; Stein *et al* 1986; Vina *et al* 1995). Similar to our results, Aseni *et al.* (Asensi *et al* 1994) observed GSH oxidation in blood samples following deproteinization with 6% PCA. However, this oxidation was eliminated in the presence of GSH derivatizing agents

such as N-ethylmaleimide (Asensi *et al* 1994; Vina *et al* 1995). Using a similar sample preparation method as ours, Michelet *et al.* (1995) reported effective GSH and GSSG sample storage for 15 days at -80°C. On the other hand, our results contradict studies by Özcimder *et al.* (1991) and Liu *et al.* (1996) who reported that high PCA concentrations (23%) resulted in GSH oxidation to GSSG within eye lens homogenates. Stein *et al.* (1986) also observed dissimilar results to ours showing that GSH and GSSG in biological fluids and tissues from rats including serum, bile, liver and kidney were stable at -80°C for 7 to 10 days following deproteinization using 1.25% PCA. Whether a tissue-specific difference exists with respect to the oxidizing properties of PCA remains to be investigated.

TCA, the final acid tested, demonstrated acceptable sample stability at all concentrations. Aseni *et al.* (1994) reported TCA to be pro-oxidative at concentrations below 15%. These findings were further corroborated by Viña *et al.* (1995) using spectrophotometric methods. The lack of an appreciable difference in sample stability in this study with TCA concentrations of 5 to 15% may be the result of methodological improvements in the present study such as flash freezing and storage of the samples at ultra low temperatures (-80°C). Despite acceptable sample stability at all concentrations tested, 5% TCA did not remove proteins efficiently from the sample and this is the reason to avoid using it in sample preparation for electrochemical detection.

A final consideration determining the optimal precipitating agent for electrochemical detection of GSH and GSSG involves assessment of chromatographic characteristic following sample acidification. Criteria examined included interfering and overlapping peaks, large initial peaks at the solvent front (negative or positive), baseline drift and peak tailing. Significant peak tailing and some interference were observed for GSH and penicillamine in the presence of TCA and SSA while these problems were not observed with PCA and MPA. Aside from SSA, the other acids did not cause appreciable baseline drift nor were the size of the peaks in the solvent

front a concern. All of the acids tested allowed for good separation of GSH, GSSG and penicillamine, contrary to previous reports (Richie, Jr. and Lang 1987; Stein *et al* 1986).

Considering sample stability, efficiency of protein removal, and chromatographic characteristics, deproteinization using 15% PCA appears to be most suitable for analysis of whole blood GSH and GSSG by HPLC coupled with electrochemical detection. While immediate analysis of GSH and GSSG is always preferable, sample storage with 15% PCA offers excellent stability up to 4 weeks, efficiently removes proteins from the samples, and achieves good peak separation with minimal baseline drift.

8.4.2 Glutathione Measurement in Pediatric Patients Treated with Amifostine

Unfortunately, the solution to the problem of sample stability came after samples for four patients had been obtained and processed using the original method. Hence, we could confidently analyze the glutathione levels in the blood of the remaining two patients receiving amifostine. Preliminary data shows that while a transient increase in GSH was observed, by the end of the 90-minute study window, GSH concentrations returned to their pre-amifostine levels. These findings are in agreement with those of Souid *et al.* (1999) who recently attempted to evaluate a similar hypothesis. They showed that GSH increased slightly during the first amifostine infusion. Based on a very limited amount of data, we may cautiously suggest that while we cannot be certain that the increase in glutathione is significant, amifostine may at least help prevent the expected decrease in glutathione that is associated with ifosfamide administration (15 minutes into the 90-minute study period). A decrease in glutathione is expected since part of ifosfamide's detoxification pathway is mediated by conjugation with glutathione. A larger group of patients with controls would be needed to verify this proposition and enable us to attribute these effects to amifostine treatment.

The major accomplishment in this portion of our project was that we were able to validate an improved method for glutathione sample preparation in biological specimens. Sample integrity can now be preserved such that the specimens can be stored prior to analysis, which is desirable in a clinical setting. As a result, we now have a reliable tool that will allow us to more accurately evaluate our initial hypothesis that amifostine's cytoprotective effect is mediated by modulation of glutathione levels.

9.0 LIMITATIONS and FUTURE STUDIES

Studies involving human subjects tend to have many limitations and this study was no exception. The use of terminally ill children in Phase I studies introduces further complications. Firstly, the eligibility criteria are very strict and therefore patient recruitment proceeded very slowly. Because Phase I studies differ significantly and are independent from standard care treatment, the principal investigator must rely on the patients' primary care physician to keep in mind that the study is open and to offer it as a treatment option to eligible patients. With the current demands placed on health care professionals, relying on them to remember a study in which they are not directly involved presents limitations in identifying possible candidates. The six patients discussed in this study were recruited over a period of 17 months spanning from July 1998 to November 1999. Difficulty in obtaining parental consent may also have contributed to this slow recruitment rate. The children involved in these studies were very ill and the primary concern for parents is the comfort of their children during treatment. Since a peripheral intravenous line must be started to facilitate blood collection, the children are exposed to an additional venipuncture. Furthermore, it is often difficult to obtain consent for frequent blood sampling especially within a short-time period such as 90-minutes.

There are ethical issues involved in blood sampling for pharmacokinetic studies. A limited number of blood samples can be obtained from children for research purposes. This makes characterizing the pharmacokinetics of the parent drug in this study particularly difficult. Amifostine was administered over a period of 15-minutes and given that its elimination half-life is extremely short (~10 minutes), sampling during the infusion period may have yielded useful information. However, this was not possible due to the ethical constraints and therefore, the first sample was obtained at the end of the infusion. On the other hand, sample collection occurred for much longer periods of time in some adult studies of amifostine pharmacokinetics. This

allowed better characterization of the terminal elimination phases of amifostine and its metabolites. The sampling time chosen in this study was based on published studies, both in humans and animals, that reported significantly shorter half-lives of amifostine and its active metabolite, WR1065. Pharmacokinetic studies generally require sample collection for five to seven half-lives, therefore our 90-minute study period appeared to be sufficient. Moreover, we would not have been able to collect more blood samples following the initial amifostine dose due to the terminal nature of the patients' illness and their treatment schedule since a second dose of amifostine was administered after the ifosfamide infusion.

A final point to consider with respect to the pharmacokinetic data is that we had a small sample size. While this was sufficient to close the Phase I study, whose primary objectives included defining dose-limiting toxicities and establishing the maximum tolerated dose of amifostine, more patients would be required to adequately define amifostine pharmacokinetics in this population. Kinetics studies should therefore be part of the objectives of future Phase II and III studies of this drug in children.

The problems we experienced with the glutathione assay presented another limitation. Loss of valuable data from four out of the six patients enrolled restricted the conclusions that could be drawn. Furthermore, the lack of a "no amifostine" control arm made it difficult to attribute changes solely to amifostine. While control groups are generally not included in Phase I studies, this aspect may be an important consideration for future work. A control group would also facilitate the determination of whether the amifostine dose used in this study was indeed efficacious in preventing chemotherapy-induced toxicity to the same extent as demonstrated at higher doses.

Finally, other reports suggest that the mechanism of amifostine's modulation of glutathione involves increases in intracellular cysteine uptake, which leads to *de novo* GSH synthesis (Issels and Nagele, 1989). If this hypothesis is true, perhaps the 90-minute study

period available to us was not sufficient to notice changes if they existed. Perhaps monitoring cysteine levels both intracellularly and extracellularly would have yielded more meaningful information and future studies could incorporate this aspect of the potential cytoprotective action of amifostine.

10.0 CONCLUSIONS

In the Phase I study presented here, we concluded that the maximum tolerated dose for twice-daily administration of amifostine with ICE chemotherapy in children is 600 mg/m^2/dose (1200 mg/m^2/day). Within the scope of this study, the pharmacokinetics of amifostine and its metabolites were calculated and compared to published adult data. Important observations were noted. The similarity in the systemic exposure to the cytoprotective metabolite, WR1065, in children and adults proposes that amifostine dosed at a rate of 600 mg/m^2 is equipotent in these two populations. However, the maximum tolerated dose in children appears to be lower than that in adults suggesting that children may not be receiving the same therapeutic advantage (although the effectiveness of this dose has not been adequately studied). This study emphasizes the need for separate determinations of the pharmacokinetics of drugs as well as their appropriate doses in children. Children are not "miniature adults" and often require individualized modifications in treatment protocols that differ from simple dose adjustment based on body size.

A second objective was to investigate the hypothesis that amifostine's mechanism of action is related to its modulation of glutathione levels and this study was a suitable starting point to explore this possibility. In the process, we noticed that a previously published method for sample preparation was causing significant alterations in GSH and GSSG concentrations. This prompted us to identify and validate an improved method for sample preparation that does not compromise sample integrity for at least one month during storage at -80°C. While our original hypothesis could not be adequately addressed within the scope of this clinical study, we now have a reliable tool to investigate the notion of amifostine's modulation of glutathione in future studies. In fact, the applicability of this improved method for sample preparation is clearly demonstrated by its use in several studies currently underway in our laboratory. Examples include *in vitro* investigation of whey protein concentrate's modulation of glutathione in

lymphocytes as well as in microglia. In addition, two clinical studies in progress are investigating the role of oxidative stress in the development and severity of ifosfamide-induced nephrotoxicity and evaluating the effects of chronic acetaminophen administration on glutathione levels in children.

While this improvement in the sample processing method allowed to evaluate amifostine's effect on glutathione in only two patients, emphasizing the need for further work, we can cautiously conclude that amifostine appears to at least prevent a decrease in GSH levels that would be expected upon treatment with ifosfamide.

11.0 REFERENCES

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APPENDIX 1 Clinical Trials in Oncology

One approach to treating cancer may involve research as new therapies may provide hope for some patients. The goals and objectives of each of the phases following preclincal testing are summarized in the table below (Baruchel and Rowell 1998; Finklestein 1997; Hughes 2000). Clinical trials are the essential link between laboratory bench and the bedside. It is because of these clinical trials that such gratifying progress has been made in the treatment of childhood cancer.

Phase I	Phase II	Phase III
• Determination of safe, tolerable and appropriate doses, usually the maximum tolerated dose (MTD) • Characterization of the nature and frequency of toxicity • Identification of dose limiting toxicities (DLT) which preclude use of higher doses • Identification and definition of guidelines for monitoring toxicity • Development of the supportive care that is required to safely administer the agent • Pharmacokinetic studies are carried out to facilitate the selection of an optimal dosing schedule	• Determination of efficacy of the drug against specific malignancies • Drug may be given for longer periods of time • The new treatment may be compared against placebo or standard therapy • Generally requires a larger group of patients	• Often called the "definitive study" • Effectiveness of the drug is studied in great detail in large numbers of patients • Due to the larger numbers of patients, more detailed information regarding the minor and infrequent adverse effects can be obtained

APPENDIX 2 Ethical Approval from the Research Ethics Board the Hospital for Sick Children

THE HOSPITAL FOR SICK CHILDREN

RESEARCH ETHICS BOARD

APR 21 1997

Approval & Terms of Agreement

APPLICANTS: Drs. S. Baruchel, M. Fouladi, R. Lau & M. Greenberg

PROJECT TITLE: Modulation of Ice Toxicity by the Cytoprotective Agent Amifostine. A Phase I/II Study

FILE NUMBER: 97/023

MEMBERS OF THE BOARD*:

Dr.A. Moore, Chair	Dr. L. Buchanan
Dr. R. Abramovitch	Ms. L. Macleod
Rev. Dr. T. Trothen	Mrs. B. Benoliel
Dr. P. Joshi	Ms. M. Rowell
Dr. J. Rutka	Ms. C. Cirilli
Dr. B. McCrindle	Dr. S. Baruchel
Dr. M. Crawford	Dr. P. Dick
Dr. A. Feigenbaum	Ms. S. Doyle

*Meeting may not have been attended by all members.

I agree to carry out the proposed research involving human subjects in accordance with the protocol approved by the Research Ethics Board using the approved consent form/s. I shall notify the department/division chief and the Research Ethics Board prior to implementing any modifications in the protocol and of any adverse or unexpected events as soon as possible.

SIGNATURE (INVESTIGATOR) _____ DATE April 9, 1997

I agree to monitor the protocol on an ongoing basis, and to notify the Research Ethics Board as appropriate.

SIGNATURE (DEPARTMENT/DIVISIONHEAD) _____ DATE April 10 97

The Research Ethics Board of the Hospital for Sick Children has reviewed and approved the above-named project.

Chair, Research Ethics Board _____ DATE April 16 97

DATE OF APPROVAL APR 16 1997

EXPIRY DATE APR - - 1998

APPENDIX 3 Research Information and Consent Forms

RESEARCH CONSENT FORM
(16 Years Old and Over)

MODULATION OF ICE TOXICITY BY THE CYTOPROTECTIVE AGENT AMIFOSTINE. A PHASE I/II STUDY

Investigators(s): All can be reached by calling 813-7742

Dr. M. Greenberg
Dr. S. Weitzman
Dr. H.S.L. Chan
Dr. E. Boyle
Dr. R. Grant
Dr. S. Baruchel
Dr. D. Malkin
Dr. M. Leaker

Phase I Trials in Pediatric Oncology

The goal of a Phase I study is to find a safe dose of a new drug that can be used in the future treatment of cancer. Phase I studies are performed by giving different dosages of the new drug to different patients and by doing blood tests to measure levels of the drug and check for side effects.

Introduction:

You have been told that you have a recurrent solid tumor . Recurrent solid tumors are difficult to treat as they do not respond well to most forms of chemotherapy, and are therefore often progressive.

In order to treat your tumor, it will be appropriate to deliver a high dose of chemotherapy. This chemotherapy may control your tumor, but may cause damage to different organs such as the bone marrow, the kidney, the heart, the gut, and the central nervous system.

We are asking your permission to enroll you in a research study that will test a new drug that is expected to protect the body from the toxicity of chemotherapy. This will allow the chemotherapy to be delivered at the right dose and with the right schedule which will better control the progression of your tumor.

The name of this new drug is **AMIFOSTINE**. This is a new drug being used in children. We first need to define the optimum dose and the monitoring care to be given during the administration of the drug. This drug has been used in hundreds of adults with cancer and has been proven to be safe and to protect normal tissues against some of the severe side effects of chemotherapy (ie. damage to the bone marrow, gut, kidney, and heart). Amifostine does not protect the tumor.

If you participate in this study, you may be asked to participate in one of the two different arms of the study.

The first step of this clinical research is to find out what is the best dose of Amifostine that will not produce unacceptable side effects. You will receive a dose of Amifostine established by your doctor, based on current knowledge about the side effects at the time you are registered on the study. At that stage of the protocol, the dosages of chemotherapy (Ifosfamide, Carboplatin, VP-16) will be similar to the standard treatment (i.e. treatment that you would have received if you were not part of this study).

Once the optimum dose of Amifostine is defined, we will start the second stage of the research. (If you are part of the first stage of this research, you will not be asked to be part of the second stage). During the second stage of this research, the drug carboplatin will be administered at a higher dose (than the standard treatment). It will be given with the optimum dose of Amifostine as defined by the results of the first stage of this clinical research. We will evaluate if the addition of Amifostine will allow us to deliver this higher dose of Carboplatin. The two other chemotherapy drugs, Ifosfamide and VP-16, will be administered at the standard dosage.

Your doctor will inform you of the dose of Carboplatin that you will receive and the potential side effects that could be experienced. It is possible that you will be the first one to receive this higher dose of carboplatin and in this situation, it may not be possible for your doctor to give you any information on children treatedat this higher dose level.

Very strict guidelines are in place in order to detect and reduce excessive toxicity of the Amifostine and chemotherapy regimen.

Purpose of Research:

1) To see if the drug Amifostine is well tolerated and can reduce the side effects of chemotherapy when administered prior to and following certain chemotherapy drugs.

2) To define a new dose for Carboplatin when given with Amifostine.

3) To see if a higher dose Carboplatin, given with Ifosfamide and VP-16, produces a better killing of tumor cells.

4) To determine the side effects of Amifostine and the higher dose of Carboplatin when given with the standard doses of Ifosfamide and VP-16 in patients with recurrent cancer.

Description of Research Procedure:

Before starting the study, blook work, urine tests, a chest X-Ray and a hearing test will be done.
You will be admitted to hospital to receive a chemotherapy regimen that includes 3 drugs at a standard dosage. The drugs are **Carboplatin, Ifosfamide** and **VP-16**.

On day 1 and 2 of the protocol you will receive both Ifosfamide and VP 16 and on day 3 Carboplatin will be given. All drugs will be given by IV infusion (directly into a vein or central line).

Amifostine will be given by IV infusion 15 minutes before and 2 hours after both Ifosfamide and Carboplatin are given.

Other children before you have been treated with Amifostine at a certain dose level. We will give the first three patients an established dose of Amifostine. If the first three patients do not experience any major side effects, then the next three patients will receive a 20% higher dose of Amifostine. This will continue until the optimum dose is reached. When severe side effects occur which are hard to control then we know that the optimum dose (maximum tolerated dose) of Amifostine is the one that was given before these severe side effects occured.

Because Ifosfamide can cause bladder irritation, a drug called Mesna will be given by IV infusion over 3 hours after each dose of Ifosfamide and then over 15 minutes at 3, 6 and 9 hours after the last dose of Ifosfamide is given. Mesna helps protect the bladder from irritation.

It is possible that we may also have to use a drug called G-CSF which will be given by injection under the skin at home. This drug helps the white blood count to recover rapidly and should help to prevent infections. It will be given every day until the blood counts increase.

A scan will be done 3 weeks after treatment is given. If the results show stable disease or improvement a second course of treatment will be given.

Three weeks after the second course of chemotherapy, all the scans (CT, MRI and Bone scans) will be repeated.

To study the way the body handles Amifostine, special blood studies will need to be done called pharmacokinetics. After the first dose of Amifostine is given 11 blood samples will be drawn over 1 1/2 hours. Less than 1/2 teaspoon is drawn each time which is less than 5% of your total blood volume and is a safe amount even for very small children. These samples cannot be drawn from the central line. It will be necessary to put a small plastic tube called a heplock into a vein in your arm which will be used to draw all of the samples from.

Once the maximum tolerated dose of Amifostine has been established, you may be asked to participate in the second phase of the study which will try to find a higher, yet safe, dose of Carboplatin that can be given in association with a standard dose of Ifosfamide and VP-16 and with Amifostine.

<u>Description of Standard Treatment</u>:

At the time of diagnosis of tumor recurrence, you may be offered various types of treatment. The combination of ifosfamide , carboplatin and VP-16 is a combination of drugs frequently used in cases similar to yours, and is recognized as a standard treatment in our institution.

Potential Harms:

Side effects of Amifostine are transient and will stop a few minutes after the infusion of the drug. They are: nausea, vomiting, sneezing, warm flushed feeling, mild somnolence (drowsiness), metallic taste during the infusion, occasional allergic reaction and transient lowering of blood calcium. Blood work will be done every 12 hours to measure the calcium level as well as other blood levels. The central line can be used for this. All of your urine will be collected tor testing during your hospital stay.
The most frequent side effect is a drop of the blood pressure. For that reason your blood pressure will be taken every 5 minutes during the infusion of amifostine (15 minutes) and for 15 minutes after that. Amifostine will be stopped if your blood pressure drops below a certain level. Side effects have always been reversible, and no major or irreversible side effects have been reported in adults receiving Amifostine. Temperature and other vital signs will also be monitored.

Medications will be given in order to decrease the amount of nausea and vomiting that you may experience during the administration of the chemotherapy and Amifostine.

Ifosfamide , Carboplatin and VP-16 may cause severe lowering of blood counts. This may lead to infection or bleeding. Both of these can be treated and we may use G-CSF with the intention of speeding up the recovery of the white blood cell count and aid recovery from infection. Ifosfamide may cause kidney damage that may result in the need for you to take mineral supplements by mouth.

Side effects of **Ifosfamide** may include: low blood counts, decreased resistance to infection, bleeding, anemia, bladder irritation, blood in the urine, kidney damage, brain toxicity, nerve damage, lung damage, heart damage, drowsiness, seizures, nausea, vomiting, liver damage and hair loss. **Rare** instances of secondary cancer, leukemia, and pre-leukemia have been reported. **Very rarely** death may occur due to one or more complications.

Side effects of **VP-16** may include: low blood counts, with a period of increased risk of infection, bleeding and anemia, nausea and vomiting, hair loss, diarrhea, nerve disorders, mouth sores, serious allergic reactions with chills, fever, low blood pressure and difficulty breathing, rapid heart beat, swelling or itching of the face, hands, and feet, a risk of developing secondary leukemia, or pre- leukemia.

Side effects of **Carboplatin** may include: low blood counts, with a brief period of increased risk of infection, bleeding, bruising and anemia, fatigue, severe nausea and vomiting, serious allergic reaction, with low blood pressure and breathing difficulty, severe damage to the kidneys, hearing loss, ringing in the ears, spasm of the muscles or seizures due to imbalance of blood chemicals , nerve damage, and damage to peripheral nerves that transmit sensations and control body movement. **Rarely** damage to the liver or eyes, secondary malignancies, leukemia and pre-leukemia have been reported. Side effects from certain antibiotics can be made worse when given at the same time as Carboplatin.

Common side effects of **G-CSF** may include: bone pain, chills, fever, headache, generalized ill feeling, nausea, low blood pressure and shortness of breath. Mild toxicity to the liver and

spleen have been noted. Bruising may occur at the site of injection. Pre-existing skin rashes or inflammation of blood vessels may recur while using G-CSF.

You will be given medication to help prevent and control the side effects mentioned. In previous studies and in extensive experience at HSC, the side effects of this drug were found to be tolerable and usually disappeared after the treatment was stopped.

Side effects of **Mesna** may include: bad taste (common), abdominal pain, nausea and vomiting, diarrhea, headache, limb and joint pain, drowsiness, rash, and **rarely** low blood pressure.

Potential Benefits:

It is not known whether you will have any benefit from this research study. However, it is hoped that the treatment offered will be effective in controlling your tumor permanently, or at least temporarily. The knowledge obtained from this study will possibly be of help to others in the future.

Should new scientific evidence indicate that this treatment is no longer in your best interest, this treatment will be stopped and further alternatives will be discussed with you.

Alternatives:

If you choose not to participate in this study, you may receive the treatment currently used by HSC. If you choose not receive any further therapy, the tumor will probably continue to progress, during which time you will continue to receive the best available supportive care.

Disclosure of Records:

A record of your progress while on this study will be kept in a confidential form at HSC. No identifying information will be released or published. Pathological materials, including slides, may be sent to a central office or laboratory for review or for further diagnostic studies. Tvv che research consent form will be inserted into your health record.

Participation:

Participation in research is voluntary. If you choose not to participate, you and your family will continue to have access to quality care at HSC. If you choose to participate, you can withdraw from this study at any time. Again, you and your family will continue to have access to quality care at HSC.

Consent:

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for myself and for other members of family. As well, the potential harms and discomforts have been explained to me and I also understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that records relating to my care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I, _____, have read the above and hereby give consent to participate. I understand that I shall receive a copy after signing this form.

_____	___	_____	_____	_____
Patient's Name	Age	Patient's Signature	Phone No.	Date

_____	_____	_____	_____
Witness Name	Witness Signature	Phone No.	Date

_____	_____	_____	_____
HSC Physician Name	Physician Signature	Phone No.	Date

RESEARCH CONSENT FORM
(Under 16 years old)

MODULATION OF ICE TOXICITY BY THE CYTOPROTECTIVE AGENT AMIFOSTINE. A PHASE I/II STUDY

Investigators(s): All can be reached by calling 813-7742

Dr. M. Greenberg
Dr. S. Weitzman
Dr. H.S.L. Chan
Dr. E. Boyle
Dr. R. Grant
Dr. S. Baruchel
Dr. D. Malkin
Dr. M. Leaker

Phase I Trials in Pediatric Oncology

The goal of a Phase I study is to find a safe dose of a new drug that can be used in the future treatment of cancer. Phase I studies are performed by giving different dosages of the new drug to different patients and by doing blood tests to measure levels of the drug and check for side effects.

Introduction:

You have been told that your child has a recurrent solid tumor . Recurrent solid tumors are difficult to treat as they do not respond well to most forms of chemotherapy, and are therefore often progressive.

In order to treat your child's tumor, it will be appropriate to deliver a high dose of chemotherapy. This chemotherapy may control your child's tumor, but may cause damage to different organs such as the bone marrow, the kidney, the heart, the gut, and the central nervous system.

We are asking your permission to enroll your child in a research study that will test a new drug that is expected to protect the body from the toxicity of chemotherapy. This will allow the chemotherapy to be delivered at the right dose and with the right schedule which will better control the progression of your child's tumor.

The name of this new drug is **AMIFOSTINE**. This is a new drug being used in children. We need first to define the optimum dose and the monitoring care to be given during the administration of the drug. This drug has been used in hundreds of adults with cancer and has been proven to be safe and to protect normal tissues against some of the severe side effects of chemotherapy (ie. damage to the bone marrow, gut, kidney, and heart). Amifostine does not protect the tumor.

If your child participates in this study, he/she may be asked to participate in one of the two different arms of the study.

The first step of this clinical research is to find out what is the best dose of Amifostine that will

not produce unacceptable side effects. Your child will receive a dose of Amifostine established by your doctor, based on current knowledge about the side effects at the time your child is registered on the study. At that stage of the protocol, the dosages of chemotherapy (Ifosfamide, Carboplatin, VP 16) will be similar to the standard treatment (i.e. treatment that your child would have received if he/she was not part of this study).

Once the optimum dose of Amifostine is defined, we will start the second stage of the research. (If your child is part of the first stage of this research, he/she will not be asked to be part of the second stage). During the second stage of this research, the drug Carboplatin will be administered at a higher dose (than the standard treatment). It will be given with the optimum dose of Amifostine as defined by the results of the first stage of this clinical research. We will evaluate if the addition of Amifostine will allow us to deliver this higher dose of Carboplatin. The two other drugs, Ifosfamide and VP 16, will be administered at the standard dosage.

Your doctor will inform you of the dose of Carboplatin that your child will receive and the potential side effects that could be experienced. It is possible that your child could be the first one to receive this higher dose of carboplatin and in this situation, it may not be possible for your doctor to give you any information on children treated at this higher dose level.

Very strict guidelines are in place in order to detect and reduce excessive toxicity of the Amifostine and chemotherapy regimen.

Purpose of Research:

1) To see if the drug Amifostine is well tolerated and can reduce the side effects of chemotherapy when administered prior to and following certain chemotherapy drugs.

2) To define a new dose for Carboplatin when given with Amifostine.

3) To see if a higher dose of Carboplatin, given with Ifosfamide and VP-16, produce a better killing of tumor cells.

4) To determine the side effects of Amifostine and the higher dose of Carboplatin when given with standard doses of Ifosfamide and VP-16 in patients with recurrent cancer.

Description of Research Procedure:

Before starting the study, blood work, urine tests, heart tests, a chest X-Ray and a hearing test will be done.

Your child will be admitted to hospital to receive a chemotherapy regimen that includes 3 drugs at a standard dosage. The drugs are **Carboplatin, Ifosfamide** and **VP-16.**
On day 1 and 2 of the protocol your child will receive both Ifosfamide and VP 16, and on day 3 Carboplatin will be given. All drugs will be given by IV infusion (directly into a vein or central line).

Amifostine will be given by IV infusion 15 minutes before and 2 hours after both Ifosfamide and Carboplatin.

Other children before your child have been treated with Amifostine at a certain dose level. We will give the first three patients an established dose of Amifostine. If the first three patients do not experience any major side effects, then the next three patients will receive a 20% higher dose of Amifostine. This will continue until the optimum dose is reached. When severe side effects occur that are hard to control then we know that the optimum dose (maximum tolerated dose) of Amifostine is the one that was given before these severe side effects occured.

Because Ifosfamide can cause bladder irritation, a drug called Mesna will be given by IV infusion over 3 hours after each dose of Ifosfamide and then over 15 minutes at 3, 6 and 9 hours after the last dose of Ifosfamide is given. Mesna helps protect the bladder from irritation.

It is possible that we may also have to use a drug called G-CSF which will be given by injection under the skin at home. This drug helps the white blood count to recover rapidly and should help to prevent infections. It will be given every day until the blood counts increase.

A scan will be done 3 weeks after treatment is given. If the results show stable disease or improvement a second course of treatment will be given.

Three weeks after the second course of chemotherapy, all the scans (CT, MRI and Bone scans) will be repeated.

To study the way the body handles Amifostine, special blood studies will need to be done called pharmacokinetics. After the first dose of Amifostine is given 11 blood samples will be drawn over 1 1/2 hours. Less than 1/2 teaspoon is drawn each time which is less than 5% of your childs total blood volume and is a safe amount even for very small children. These samples cannot be drawn from the central line. It will be necessary to put a small plastic IV tube called a heplock into a vein in your childs arm which will be used to draw all of the samples from.

Once the maximum tolerated dose of Amifostine has been established, your child may be asked to participate in the second phase of the study which will try to find a higher, yet safe, dose of Carboplatin that can be given in association with a standard dose of Ifosfamide and VP-16 and with Amifostine.

Description of Standard Treatment:

At the time of diagnosis of tumor recurrence, you may be offered various types of treatment. The combination of Ifosfamide , Carboplatin and VP-16 is a combination of drugs frequently used in cases similar to your child's case, and is recognized as a standard treatment in our institution.

Potential Harms:

Side effects of Amifostine are transient and will stop a few minutes after the infusion of the drug. They are: nausea, vomiting, sneezing, warm flushed feeling, mild somnolence (drowsiness), metallic taste during the infusion, occasional allergic reaction and transient lowering of blood calcium. Blood work will be done every 12 hours to measure the calcium level as well as other levels. The central line can be used for this. All of your child's urine will be collected during the hospital stay.
The most frequent side effect is a drop of the blood pressure. For that reason your child's blood pressure will be taken every 5 minutes during the infusion of Amifostine (15 minutes) and for 15 minutes after that. Amifostine will be stopped if your child's blood pressure drops below a certain level. Side effects have always been reversible, and no major or irreversible side effects have been reported in adults receiving Amifostine. Temperature and other vital signs will also be monitored.

Medications will be given in order to decrease the amount of nausea and vomiting that your child may experience during the administration of the chemotherapy and Amifostine.

Ifosfamide , Carboplatin and VP-16 may cause severe lowering of blood counts. This may lead to infection or bleeding. Both of these can be treated and we may use G-CSF with the intention of speeding up the recovery of the white blood cell count and aid recovery from infection. Ifosfamide may cause kidney damage that may result in the need for your child to take mineral supplements by mouth.

Side effects of **Ifosfamide** may include: low blood counts, decreased resistance to infection, bleeding, anemia, bladder irritation, blood in the urine, kidney damage, brain toxicity, nerve damage, lung damage, heart damage, drowsiness, seizures, nausea, vomiting, liver damage and hair loss. **Rare** instances of secondary cancer, leukemia, and pre-leukemia have been reported. **Very rarely** death may occur due to one or more complications.

Side effects of **VP-16** may include: low blood counts, with a period of increased risk of infection, bleeding and anemia, nausea and vomiting, hair loss, diarrhea, nerve disorders, mouth sores, serious allergic reactions with chills, fever, low blood pressure and difficulty breathing, rapid heart beat, swelling or itching of the face, hands, and feet, a risk of developing secondary leukemia, or pre- leukemia.

Side effects of **Carboplatin** may include: low blood counts, with a brief period of increased risk of infection, bleeding, bruising and anemia, fatigue, severe nausea and vomiting, serious allergic reaction, with low blood pressure and breathing difficulty, severe damage to the kidneys, hearing loss, ringing in the ears, spasm of the muscles or seizures due to imbalance of blood chemicals , nerve damage, and damage to peripheral nerves that transmit sensations

and control body movement. **Rarely** damage to the liver or eyes, secondary malignancies, leukemia and pre-leukemia have been reported. Side effects from certain antibiotics can be made worse when given at the same time as Carboplatin.

Common side effects of G-CSF may include: bone pain, chills, fever, headache, generalized ill feeling, nausea, low blood pressure and shortness of breath. Mild toxicity to the liver and spleen have been noted. Bruising may occur at the site of injection. Pre-existing skin rashes or inflammation of blood vessels may recur while using G-CSF.
Your child will be given medication to help prevent and control the side effects mentioned. In previous studies and in extensive experience at HSC, the side effects of this drug were found to be tolerable, and usually disappeared after the treatment was stopped.

Side effects of **Mesna** may include: bad taste (common), abdominal pain, nausea and vomiting, diarrhea, headache, limb and joint pain, drowsiness, rash, and **rarely** low blood pressure.

Potential Benefits:

It is not known whether your child will have any benefit from this research study. However, it is hoped that the treatment offered will be effective in controlling your child's tumor permanently, or at least temporarily. The knowledge obtained from this study will possibly be of help to others in the future.

Should new scientific evidence indicate that this treatment is no longer in your child's best interest, this treatment will be stopped and further alternatives will be discussed with you.

Alternatives:

If you choose, on behalf of your child, not to participate in this study, your child may receive the treatment currently used at HSC. If you choose, on behalf of your child, not to receive any further therapy, the tumor will probably continue to progress during which time your child will continue to receive the best available supportive care.

Disclosure of Records:

A record of your child's progress while on this study will be kept in a confidential form at HSC. No identifying information will be released or published. Pathological materials, including slides, may be sent to a central office or laboratory for review or for further diagnostic studies. The research consent form will be inserted into your child's health record.

Participation:

Participation in research is voluntary. If you choose on behalf of your child not to participate, you and your family will continue to have access to quality care at HSC. If you choose on behalf of your child to participate, you can withdraw from this study at any time. Again, your child and your family will continue to have access to quality care at HSC.

Consent:

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at The Hospital for Sick Children for my child and for other members of my family. As well, the potential harms and discomforts have been explained to me and I also understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that records relating to my child and his/her care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I have read the above and hereby give consent for my child _____ to participate. I understand that I shall receive a copy after signing this form.

Parent/Legal Guardian Name	Patient/Legal Guardian's Signature	Phone No.	Date
Witness Name	Witness Signature	Phone No.	Date
HSC Physician Name	Physician Signature	Phone No.	Date

INFORMATION FORM

TITLE OF STUDY:
MODULATION OF ICE TOXICITY BY AMIFOSTINE A CYTOPROTECTIVE AGENT. A PHASE I/II STUDY

INVESTIGATORS: All can be reached by calling 813-7742

Dr. M. Greenberg	Dr. R. Lau	Dr. E Boyle
Dr. S. Weitzman	Dr. R. Grant	Dr. D. Malkin
Dr. H.S.L. Chan	Dr. S. Baruchel	Dr. M. Leaker

Why are we doing this study?
You know that you have a tumor that has grown back. We are trying to find out if using a new medicine called Amifostine will protect you against the side effects of the chemotherapy we want to give you so that our chances of curing the tumor that you have are better. The names of the chemotherapy medicines are Ifosfamide, VP-16 and Carboplatin. Sometimes these medicines can make you sick in other ways and we need to find out if that will happen when we give them to you.
Amifostine has been given to many adult patients but not very often to children. Being part of this research you will help us find the best dose of this medicine for children.
If the side effects of the medicine are too bad we will stop the treatment.

What will happen during the study?
You will be given the medicine Amifostine into your IV for 3 days. You will get it before and after the chemotherapy drugs Ifosphamide and Carboplatin are given. You might get quite sick and we will give you other medicines to help you get better fast. Three weeks after you get the amifostine and chemotherapy for the first time, we will do a scan of your tumor to see how the chemo is working. Then we will decide whether you will get more treatment or not. The very first time you get Amifostine we need to do 11 special blood tests so we know how the Amifostine is working in your body. We will put an IV needle (called a heplock) into one of your veins and we will take all of the blood tests from that needle.

Are there good things and bad things about this study?
This chemo will hopefully make your tumor go away. The medicines can sometimes do other things like make you sick in your stomach, or make you feel tired, or give you a fever. You and your family will be asked to tell us if these things, or other bad things happen to you. We can give you other medicine to help you feel better.

Who will know about what I did in the study?
If you are part of this study, your name, address, information about how you feel, and information about your tumor will be shared with other doctors in Canada and the United States to see if the medicines are working. No one will write or talk about your illness using your name or other identifying information.

Can I decide if I want to be in the study?
If you do not want to be a part of this study that is O.K. No one will be upset or disappointed. If you say yes now but change your mind later, you can say no to your doctor or nurse and that will be O.K. Your mother or father is also reading some information about this study. They will talk to you about it. Ask them questions if you do not understand what you have read or been told. They will help you to understand. Please ask the nurse or doctor any questions that you may have. They will also help you to understand.

Assent

I was present when _____ read this form and gave his/her verbal assent.

_____
Name of person who obtained assent

_____
Signature

APPENDIX 4 Hydrodynamic Voltammograms

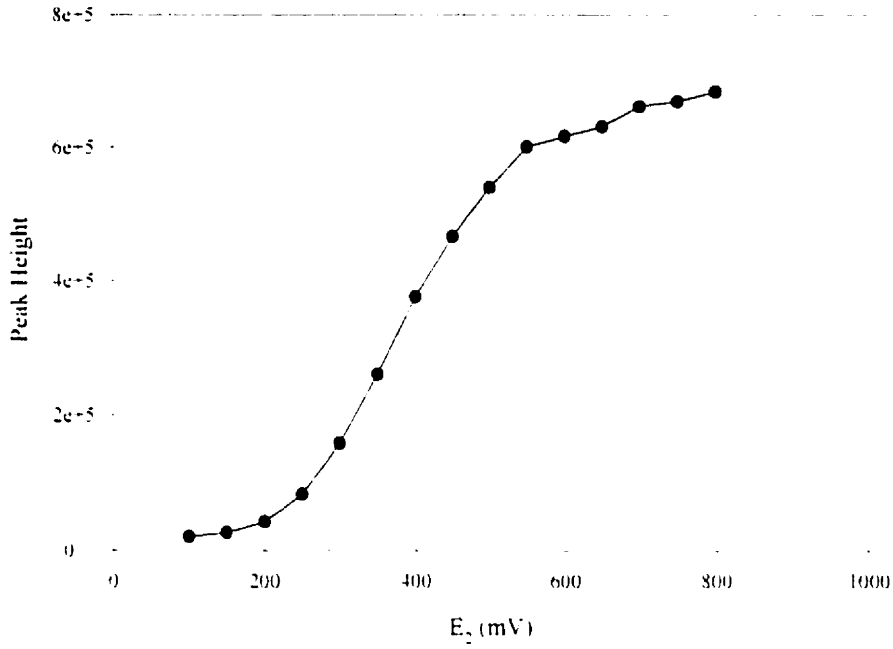
In order to use an electrochemical detector successfully for HPLC, one must know the appropriate potential to effect the desired electrochemical reaction (*i.e.*, oxidation or reduction of the compound of interest). This potential can be determined experimentally by generating a hydrodynamic voltammogram (HDV) curve, also known as the current/voltage (C/V) curve. It is important to always obtain a HDV curve for each compound of interest when the system is initially set up and each time a major component (*e.g.*, the cell) is changed.

An HDV curve is generated by initially setting the potential on both channels (i.e., E1 and E2) to a value where it is known that the electrochemical activity of the compound of interest is low (*i.e.*, no response is seen). A sample of known concentration of the species of interest is then injected and the chromatogram is recorded. The potential on E2 is increased by 50 mV and once the baseline is stable, the same sample (of fixed concentration) is injected again. This process is repeated until no further increases in the peak height are observed. The current generated (peak height) is plotted against the applied potential. Visual inspection of the curve indicates where the oxidative (using positive potentials) or reductive (using negative potentials) response is the greatest (*i.e.*, once the curve plateaus). The potential selected for future use in analyzing this particular compound should be approximately 50 mV higher than the potential at which the peak height reached a plateau (ESA Inc. 1993).

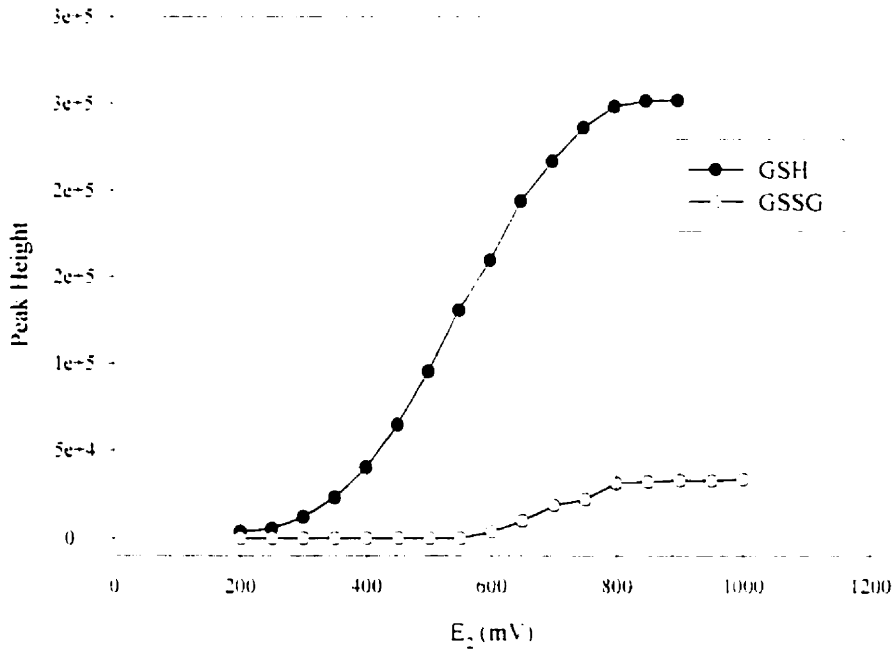
The following two figures are representative HDV curves for WR1065 and glutathione respectively. New HDV curves were generated each time the analytical cell was replaced to determine the optimum electrochemical settings for the compounds of interest. In general, the optimum settings for WR1065 analysis were approximately 650, 200 and 600 mV for the guard cell, E1 and E2 respectively and for glutathione they were 850, 400, 800 mV. In some cases we

had to compromise some of the glutathione signal in order to lower the electrochemical settings, as higher voltages shorten the life of the analytical cell.

A typical WR1065 voltamogram. 30 µl of a 100 µM standard solution were analyzed at a gain range of 10 µA.



A typical GSH Voltamogram. 10 µl of a standard solution containing 100 µM GSH and GSSG was analyzed with a gain range of 100 µA for GSH and 1 µA for GSSG.



APPENDIX 5 Plasma concentration-time data for patients enrolled in the Phase I clinical trial of amifostine (receiving a dose of 600 mg/m^2).

Patient 1

Time (min.)	Amifostine Concentration (μM)	WR1065 Concentration (μM)	Disulfide Concentration (μM)
pre infusion	0.0	0.0	0.0
3	22.3	31.2	96.3
5	11.2	26.9	78.6
15	5.5	18.8	36.0
25	3.4	13.1	28.4
32	2.9	11.1	25.6
45	1.8	4.4	22.6
60	1.0	1.8	18.9
88	0.4	0.5	14.2

Patient 2

Time (min.)	Amifostine Concentration (μM)	WR1065 Concentration (μM)	Disulfide Concentration (μM)
pre infusion	0.0	0.0	0.0
3	7.5	51.6	58.2
5	11.7	42.3	52.4
10	7.9	31.6	47.6
15	1.2	43.3	17.9
20	3.6	44.9	12.2
33	0.5	22.9	11.0
43	2.9	14.2	10.5
60		11.1	8.6
90	2.3	7.2	6.7

Patient 3

Time (min.)	Amifostine Concentration (μM)	WR1065 Concentration (μM)	Disulfide Concentration (μM)
pre infusion	0.0	0.0	0.0
1	15.2	16.4	60.6
3	11.0	16.6	59.9
8	9.0	28.9	48.2
10	5.7	25.4	51.9
18	4.5	19.9	48.9
20	0.4	15.9	37.3
30	0.1	12.7	30.5
40	0.6	6.3	28.2
60		2.6	23.0
90		0.6	19.8

Patient 4

Time (min.)	Amifostine Concentration (μM)	WR1065 Concentration (μM)	Disulfide Concentration (μM)
pre infusion	0.0	0.0	0.0
2	40.3	32.5	43.0
5	16.0	27.3	25.1
10	4.1	35.5	2.1
15	3.0	23.0	1.9
22	1.5	16.3	2.0
30	0.8	12.7	2.1
40	1.2	7.7	4.3
60	0.2	2.8	4.7
92	0.1	0.8	2.7

Patient 5

Time (min.)	Amifostine Concentration (μM)	WR1065 Concentration (μM)	Disulfide Concentration (μM)
pre infusion	0.0	0.0	0.0
1	78.0	19.9	81.6
5	32.2	15.5	87.0
10	6.3	10.7	80.7
15	4.3	21.2	50.2
20	2.7	14.6	35.3
30	1.3	8.7	25.8
42	1.4	4.8	23.1
60	1.1	2.1	20.1
90		1.0	16.2

Patient 6

Time (min.)	Amifostine Concentration (μM)	WR1065 Concentration (μM)	Disulfide Concentration (μM)
pre infusion	0.0	0.0	0.0
2	7.6	14.6	61.6
5	3.1	10.6	53.3
10	1.4	16.1	29.8
15	0.6	9.8	18.4
20	0.1	6.7	17.2
30		2.9	12.9
41		1.4	10.2
61		0.1	7.3
90			4.9

APPENDIX 6

Whole blood glutathione concentrations of patients treated with amifostine. Samples were collected just before and for 90 minutes following the amifostine infusion (total glutathione is expressed in GSH equivalents *i.e.*, GSH + 2 GSSG).

Patient 1 (2.5% PCA was used: samples were stored for 4 months prior to analysis)

Time (min.)	GSH (μM)	GSSG (μM)	Total Glutathione (μM)
preinfusion	N/A	N/A	N/A
3	63.17	214.58	492.33
5	79.15	170.03	419.21
15	107.39	172.71	452.80
25	120.36	211.19	542.75
32	109.00	214.42	537.84
45	37.25	246.30	529.86
60	135.56	240.05	615.65
88	154.01	250.18	654.38

N/A = sample not obtained

Patient 2 (2.5% PCA was used: samples were stored for 2 weeks prior to analysis)

Time (min.)	GSH (μM)	GSSG (μM)	Total Glutathione (μM)
preinfusion	284.72	307.15	899.02
3	88.61	243.30	575.22
5	130.08	233.60	597.28
10	81.84	268.98	619.79
15	152.19	211.38	574.96
20	263.85	206.10	676.05
33	240.62	224.3	689.22
43	233.82	250.89	735.60
60	198.32	308.63	815.59
90	265.25	279.24	823.73

Patient 3 (2.5% PCA was used: samples were stored for 3 months prior to analysis)

Time (min.)	GSH (μM)	GSSG (μM)	Total Glutathione (μM)
preinfusion	N/D	214.22	
1	N/D	220.25	
3	N/D	203.22	
8	47.20	224.75	496.70
10	42.63	105.55	253.73
18	52.16	142.60	337.36
20	46.33	75.22	196.77
30	37.42	162.58	362.58
40	N/D	190.18	
60	N/D	142.41	
90	126.66	196.27	519.20

N/D = below limit of detection

Patient 4 (2.5% PCA was used: samples were stored for 1 day prior to analysis)

Time (min.)	GSH (μM)	GSSG (μM)	Total Glutathione (μM)
preinfusion	521.45	31.80	585.05
2	512.61	36.32	585.25
5	400.62	40.98	482.58
10	424.66	20.52	465.70
15	450.62	36.86	524.34
22	431.81	34.06	499.93
30	475.30	56.30	587.90
40	380.89	68.96	518.81
60	438.57	60.62	559.81
92	432.94	61.21	555.36

Patient 5 (15% PCA was used: samples were stored for 1 week prior to analysis)

Time (min.)	GSH (μM)	GSSG (μM)	Total Glutathione (μM)
preinfusion	674.58	121.01	916.60
1	575.40	38.48	652.36
5	611.10	45.82	702.74
10	618.06	64.49	747.04
15	698.07	54.67	807.41
20	691.15	50.69	792.53
30	825.08	77.99	981.06
42	754.52	84.63	923.78
60	720.82	75.05	870.92
90	708.18	69.58	847.34

Patient 6 (15% PCA was used: samples were stored for 1 week prior to analysis)

Time (min.)	GSH (μM)	GSSG (μM)	Total Glutathione (μM)
preinfusion	660.67	74.05	808.77
2	725.34	56.03	837.40
5	741.32	72.80	886.92
10	907.51	73.71	1054.93
15	899.42	83.47	1066.36
20	715.16	61.27	837.70
30	702.71	70.20	843.11
41	678.22	86.68	851.58
61	773.28	98.47	970.22
90	672.60	85.37	843.34