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Entwicklung einer sensitiven HPLC-Methode zur Bestimmung der Kontamination von Oberflächen mit Gemcitabin aus Wischproben

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Abstract

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Doctor of Medicine

Development of a Sensitive Wipe-Sampling Procedure coupled to HPLC Analysis for the Determination of Gemcitabine in Surface Contamination

by Leonard ROSENKRANZ

Monitoring of work surfaces in health-care settings still shows detectable levels of antineoplastic drugs. The implementation of recent guidelines for the protection of healthcare workers' safety and health has already resulted in decreased contamination levels of antineoplastic drugs, although compliance with the recommended practices is still inadequate. Hence, very sensitive methods for both environmental sampling and analysis are required to assess the contamination and therefore the risk of exposure of antineoplastic drugs such as gemcitabine in health-care settings. In the present study a high-performance liquid chromatography (HPLC) assay for the sensitive determination of gemcitabine in wipe samples was developed. For sample concentration and especially cleanup and purification a solid-phase extraction (SPE) protocol with appropriate buffer solutions was used. A limit of detection (LOD) of 0.091 µg/ml was obtained. To assess the developed analytical method, surface wipe sampling was performed at eight different work surfaces in health-care settings.

Contents

List of Abbreviations

2dC	2-Deoxycytidine
ADs	Antineoplastic Drugs
DCK	Deoxycytidine Kinase
dFdU	2′,2′-Difluoro-Deoxyuridine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
GC	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
IPR	Ion-Pair Reagent
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MAPK	Mitogen-Activated Protein Kinase
MS	Mass Spectrometry
RP	Reversed-Phase
RR	Ribonucleotide Reductase
SPE	Solid-Phase Extraction
UV	Ultraviolet

Chapter 1

Introduction

1.1 Aim of this Thesis

Cancer is the second leading cause of death worldwide, being responsible for 8.8 million deaths in the year 2015. With the incidence expecting to increase by about 70 % over the next two decades, the importance of the disease as a global health problem will increase even further [1]. As of today, therapeutic strategies can be divided into several core pillars: Surgery, Radiotherapy, Chemotherapy, Targeted Therapy and Immunotherapy. Chemotherapy is the application of one or more cytotoxic drugs to reduce tumor size and induce remission/prolong the life of the patient. A wide variety of pharmacological agents have been used over the years to treat different kinds of cancer, from the earliest non-specific drugs like sulfur mustard to modern targeted therapies via antibodies like Trastuzumab. Since not every type of cancer allows for treatment by targeting specific antigens or metabolites, traditional cytostatic approaches (that target common and unspecific mechanisms of cell division like DNA synthesis) are still the norm today for most cases [2].
Given their toxic nature, many substances widely used for treatment possess a serious hazard potential for the environment and healthcare workers. Individuals at increased risk for drug exposure are nursing staff, pharmacists, physicians, operating room personnel but also shipping and receiving personnel [3]. Workplace exposure to antineoplastic drugs (e.g. through skin contact or alveolar uptake by inhalation) can cause a series of adverse health effects such as infertility, skin rashes, and possibly other malignancies (e.g. leukemia) [4]. There are multiple strategies to protect healthcare workers at risk. First, proper protective equipment during the preparation, administration, and disposal of antineoplastic drugs is crucial. Second, administrative controls and protocols have to be established in order to minimize the potential risk of exposure in healthcare settings [5]. Hence, sensitive and specific analytical methods are required to establish pharmaco-/toxicosurveillance in the work place. Since the risk of unwanted exposure correlates with surface contamination during the preparation and administration of antineoplastic drugs, many analytical methods aim to detect hazardous agents in workplace surfaces [6]. Wipe sampling is a versatile and widely used method to assess surface contamination.
This study focuses on the development of an analytical method for the accurate detection of small amounts of gemcitabine in surface wipe samples. Gemcitabine is a hydrophilic chemotherapeutic agent mainly used for the treatment of solid tumors like breast cancer, ovarian cancer, non-small cell lung cancer, and pancreatic cancer. The developed analytical method combines an isocratic reversed-phase high-performance liquid chromatography (HPLC) with a prior solid-phase extraction for the concentration of the analyte and also reduction of matrix interferences.

1.2 Occupational Hazards of Antineoplastic Drugs

Nowadays, antineoplastic drugs are a cornerstone in the therapy of cancer. In 1943, Alfred Gilman and Louis Goodman performed their therapeutic studies with nitrogen mustard in mice, and laid the foundation for modern chemotherapy in medicine [7]. In the following years, many more substances were discovered and used to treat cancer, beginning with antifolates (methotrexate) in 1948 and 5-fluorouracil in 1957, up to the discovery of cisplatin in 1978 [8–10]. While initially considered as a therapeutic breakthrough, several disadvantages of antineoplastic drugs soon became apparent. Due to their unspecific toxicity, they pose a substantial occupational hazard for healthcare workers [2].
In the 1970s, it was discovered that urine samples of healthcare workers who were involved in the preparation, administration, or disposal of antineoplastic drugs showed higher levels of mutagenic substances, chromosomal aberrations and sister chromatid exchanges compared to nonexposed workers [11–13]. Additionally, acute effects of occupational exposure to antineoplastic drugs have been well described, such as skin rashes, nausea, hair loss, abdominal pain, nasal sores, as well as chronic effects [14]. These effects include the reproductive system, for example spontaneous abortion [15], genotoxic changes [16], and cancers [14, 17]. More recent studies suggest that even staff members without direct patient contact, such as unit clerks, ward aides, dieticians, and shipper/receivers, are at risk of contamination when cytotoxic substances are used within a hospital setting [18]. In summary it has become clear that occupational exposure should be limited to ensure worker's safety in health care.
Following the findings stated above, guidelines for the safe handling of hazardous drugs were established in the US in 1985 by the American Society of Health-System Pharmacists, followed by the Oncology Nursing Society, the National Institutes of Health, and the Occupational Safety and Health Administration. These guidelines were updated and expanded in 1995 [19]. In Germany, workplace safety is covered by a dual system composed of the federal trade supervisory boards (Gewerbeaufsichtsämter) and the employers' liability insurance associations (Berufsgenossenschaften). Established guidelines were updated in 2009 and are in accordance with national and EU law. Since there is no accepted safe level of exposure [20], all official documents stress the significance of workplace monitoring of contaminations with ADs, regardless of the amount of cytotoxic substances being handled.
Different types of exposure have been studied in the past, the most important ones being dermal, inhalative, and oral/gastrointestinal. Since ingestion is relatively unlikely to occur in an occupational setting, analytical methods had to be developed for the measurement of surface and airborne concentration of cytotoxic substances [18]. Surface wipe sampling in combination with different methods of detection (liquid chromatography/gas chromatography and UV/VIS spectrometry/mass spectrometry/tandem mass spectrometry) has been a standard practice to monitor work place contamination with cytotoxic agents for many years [21, 22].

1.3 Gemcitabine

Gemcitabine is a cytostatic drug used for the treatment of a variety of cancer types. Worldwide, it is the third most widely used prescription drug in clinical oncology [23]. In the US, the Federal Food and Drug Administration (FDA) has approved gemcitabine for the first-line treatment of advanced or metastasized non-small cell

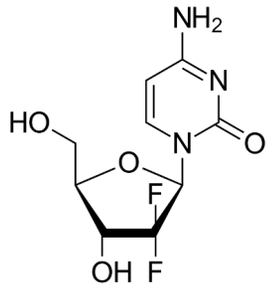


FIGURE 1.1: Chemical structure of gemcitabine

lung cancer and pancreatic cancer in 1996. The approval has been expanded in the years 2004 and 2006 to encompass metastatic breast cancer and advanced ovarian cancer respectively [24]. In Germany, similar approvals exist for gemcitabine.

1.3.1 Pharmacodynamics

The pharmacology of gemcitabine is well understood [23, 25]. Chemically, it belongs to the group of pyrimidine analogues, more specifically, the fluorinated cytidine analogues (Figure 1.1), where both hydrogens at the 2' position of deoxycytidine are replaced by fluorine. Gemcitabine is a weak base with a pK_b of 10.4, and is soluble in water and methanol [26]. From a pharmacodynamic point of view, gemcitabine is as an antimetabolite, which inhibits DNA synthesis through several intracellular targets (Figure 1.2). After administration and cellular uptake via sodium-dependent and -independent nucleoside transporters (due to its hydrophilic nature), the prodrug is phosphorylated by deoxycytidine kinase (DCK) to its mono-, di- and triphosphate forms. The active triphosphate is incorporated into DNA by DNA polymerase alpha and leads to "masked chain termination" [27]. Upon incorporation of further deoxynucleotide triphosphate (dNTP) after gemcitabine triphosphate, common DNA repair mechanisms by 3'5'-exonuclease are inhibited [28]. Therefore, this process is called "masked chain termination" (Figure 1.3). The "masked chain termination" subsequently leads to cessation of DNA synthesis, and subsequently cell death [27].
Another target is ribonucleotide reductase, which catalyses the reaction of cytidine diphosphate to deoxycytidine diphosphate and provides necessary components for DNA synthesis. Activated gemcitabine diphosphate binds to ribonucleotide reductase (RR) and thus inhibits this reaction. Since subsequently less conventional components for DNA are provided, the likelihood of the incorporation of gemcitabine-triphosphate into the DNA is increased. Additionally, deoxycytidine triphosphate acts as an inhibitor of DCK, which leads to a self-potentiation mechanism of gemcitabine, by its inhibition of RR [23, 25, 28, 29].
Another important pathway leading to tumor cell death is caspase signaling. With regard to caspase signaling, gemcitabine functions as a p38 mitogen-activated protein kinase (MAPK) activator. MAPK triggers apoptosis by arbitration of MAPK-activated protein kinase (MK2) [30]. MK2 leads to phosphorylation of heat shock protein Hsp27, which acts as a chaperone during cell growth. Cells with phosphorylated Hsp27 are not able to replicate correctly [31].

1.3.2 Pharmacokinetics

After uptake and distribution, around 90% of intracellular gemcitabine are rapidly metabolized by cytidine deaminase (CDA), which is expressed in high levels in plasma and liver tissue, into the primary metabolite 2',2'-difluoro-deoxyuridine (dFdU). dFdU itself is cytotoxic and acts as a radiosensitizer [32]. It makes DNA more vulnerable to the effects of radiation. This leads to a synergistic effect when combining gemcitabine with radiotherapy. Another less important pathway of intracellular metabolisation is inactivation through dephosphorylation of gemcitabine monophosphate by 5'-nucleotidases. From an extracellular perspective, due to its hydrophilic nature, around 10% of the drug are eliminated by kidney without prior metabolisation. The main metabolite dFdU is also hydrophilic, and thus subject to renal filtration. Both substances are eliminated independently of dose over the linear range, but proportional to creatinine clearance. In doses between 40 mg/m^2 to 3650 mg/m^2, plasma levels of gemcitabine peak after 15 to 30 minutes. Higher doses result in nonlinear pharmacokinetics [33]. Plasma clearance is rapid, with a plasma half-life ($t_{(1/2)}$) of 5 to 20 minutes. After 24 hours, more than 75% of gemcitabine has been metabolized and excreted. In contrast, dFdU is eliminated less rapidly and can be detected in significant concentrations (1 µmol/L) up to one week after administration [34].

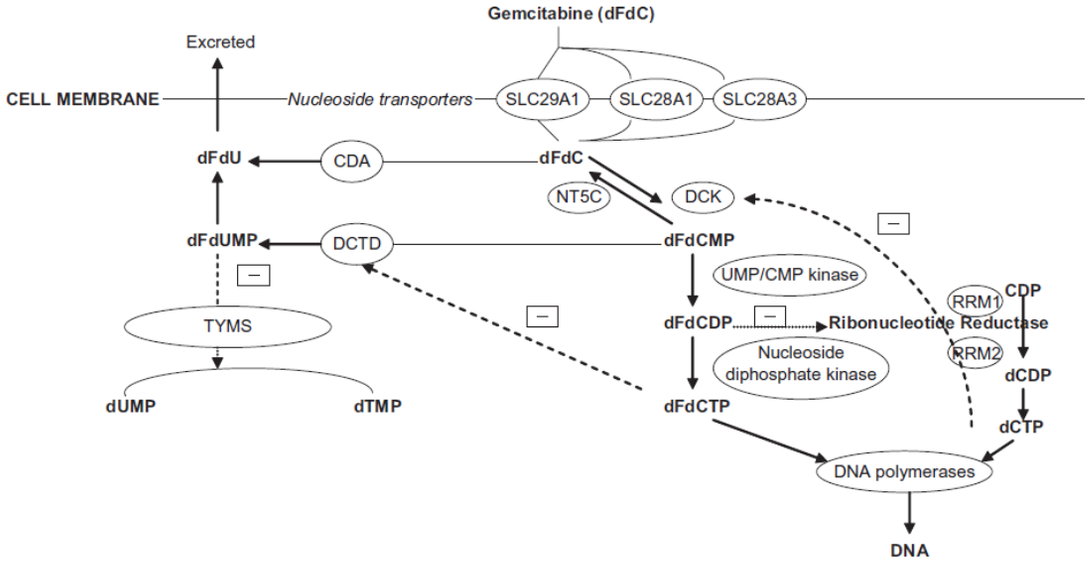


FIGURE 1.2: Pathways of gemcitabine transport, metabolism, and sites of action [25]

1.4 Analytical Detection and Quantification Methods for Gemcitabine

A variety of analytical methods for the detection and quantification of gemcitabine have been described in literature. In order to determine very low concentrations of gemcitabine sensitive analytical techniques are required. Commonly used analytical methods are accelerator mass spectrometry (AMS) [35] and liquid chromatography-mass spectrometry (LC–MS/MS). AMS is well known for its high sensitivity and specificity. However, the low availability and use of radiolabeled drugs makes this

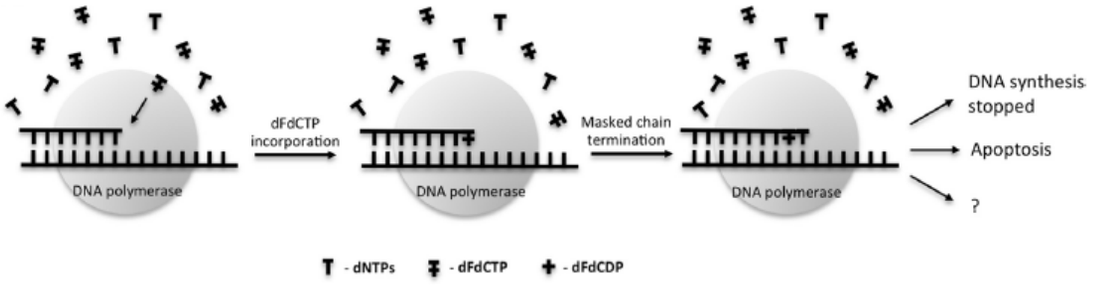


FIGURE 1.3: Masked chain termination by gemcitabine triphosphate [28], deoxynucleotide triphosphate is designated as dNTP, gemcitabine triphosphate as dFdCTP, and gemcitabine diphosphate as dFdCDP

technique expensive. LC-MS/MS is a suitable method with limits of detection in the picogram per milliliter range without the application of radioactive labeling [36]. The methods of choice for the quantification of environmental wipe samples are LC-MS/MS and HPLC methods. An overview of the applied LC/MS-MS methods and HPLC methods for the detection and quantification of gemcitabine in surface wipe samples in literature is shown in Table 1.1. Van Nuland et al. recently developed and validated an ultra sensitive method for the simultaneous quantification of gemcitabine and its metabolite 2′,2′-difluoro-deoxyuridine in human plasma by LC–MS/MS [36]. The samples were prepared with solid phase extraction. The validated assay ranges from 2.5–500 pg/ml for gemcitabine and 250–50,000 pg/ml for dFdU. A simple wipe sampling procedure coupled to LC-MS/MS analysis was developed for simultaneous determination of ten cytotoxic drugs including gemcitabine [21]. Another analytical approach for the quantification of gemcitabine is GC-MS/MS [37]. However, HPLC and MS methods are rather time consuming and expensive. In contrast, UV spectrophotometric methods are a time saving and rapid alternative. Menon et al. developed a simple, rapid, and cost effective UV spectrophotometric method for the determination of gemcitabine HCl in bulk drug and pharmaceutical formulations. Gemcitabine reacts with gold nanoparticles and changes their original red colour to dark blue as a result of aggregation. The initial absorbtion maximum is shifted from 522 nm to 688 nm. A limit of detection of 0.44 µg/ml was found [38]. However, due to the lack in sensitivity, the UV spectrophotometric method may not be appropriate for the detection of gemcitabine in wipe samples. Additionally, for the analysis of complex matrices a prior extraction process has to be implemented. Besides UV spectrophotometry, fluorescence spectroscopy has been successfully applied to quantify gemcitabine with a LOD of 3 to 100 mmol/l. The method is based on the fluorescence quenching of functionalized gold doped quantum dots by gemcitabine via photoinduced charge transfer [39]. Further rapid and versatile analytical methods for the quantification of gemcitabine are immunoassays. For this purpose, a monoclonal antibody was functionalized to polystyrene nanoparticles to develop a homogenous agglutination inhibition assay. Compared to LC-MS/MS methods, immunoassays typically lack sensitivity [40].

TABLE 1.1: Overview of analytical methods for the detection/quantification of gemcitabine in surface wipe samples

Analytical Method	Matrix	Limit of Detection	Limit of Quantification
LC-MS/MS, [21]	wipe sample	n.a.	0.1 ng/cm^2
LC-MS/MS, [41]	wipe sample	0.125 ng/cm^2	0.25 ng/cm^2
LC-MS/MS, [42]	wipe sample	0.10 ng/ml	0.2 ng/ml
LC-MS/MS, [43]	wipe sample	0.0020 ng/ml	0.0068 ng/ml
LC-MS/MS, [44]	wipe sample	n.a.	0.5 ng/ml
HPLC/UV, [45]	plasma/urine	n.a.	50 ng/ml / 20 µg/ml
LC-MS/MS, [46]	urine	0.05 µg/l	0.2 µg/l
HPLC-DAD, [47]	plasma	0.10 µg/l	0.20 µg/l
HPLC/UV, [48]	plasma	n.a.	20 µg/ml

1.5 High Performance Liquid Chromatography

Chromatography describes an analytical technique, which is applied for the separation, detection, and quantification of specific substances. Hereby, the components are distributed between two phases: stationary phase and mobile phase [49]. High performance liquid chromatography (HPLC), which is a special form of Liquid Chromatography (LC), is the most widely used form of chromatography for soluble analytes. Whereas LC is a general term and describes any chromatographic procedure in which the mobile phase is a liquid (other examples being open column and thin layer chromatography), HPLC enhances the performance (hence "High Performance") of LC principles, by optimizing key factors of the method. In conventional open column LC, the solvent containing the analytes is applied onto a column consisting of large particles (~150 - 250 µm) and gravity is used to guide the mobile phase through the stationary phase. After passage of the mobile phase, the solvent is analyzed and separated analytes can be detected after certain intervals of time. Separation occurs as consequence of the distribution of the analytes between the mobile and stationary phases (and their different affinities for both of them) [50]. Figure 1.4 displays an overview of the technique. There is a huge variety of chromatographic principles, such as adsorption, partition, ion exchange, and size exchange.

However, basic LC has several disadvantages: (1) low resolution due to relatively large particle size; (2) slow procedure and operator dependent results; (3) high costs, one column is used for one analysis. HPLC overcomes these problems by introducing a reusable column, containing a stationary phase packed with small particles (~2 - 50 µm), and using pumps to apply high pressure within the chromatographic circuit, to improve resolution and speed up the process [51].
Figure 1.5 gives a schematic overview of a HPLC workstation. A HPLC pump transports the mobile phase forward through the injection loop. There, the sample containing the analytes enters the mobile phase and the mixture is pumped through the HPLC column (usually within a column heater) containing the stationary phase. Separation results during transport by the mobile phase as the analyte interacts with the stationary phase in a different way than the other components of the matrix through varying principles, depending on the applied column and composition of

the mobile phase. After separation, the mobile phase containing the distinct analytes enters a detector. Commonly used detectors are UV detectors or mass spectrometers.

1.5.1 Separation

HPLC utilizes different principles to separate an analyte from its matrix. All principles are based on the interaction between the analyte, and the mobile/stationary phase. Table 1.2 provides an overview of the most commonly applied separation techniques by HPLC and their associated retention mechanisms. By deliberate selection of a specific stationary phase and a corresponding mobile phase, different retention mechanisms can be used to separate analytes. Furthermore, different principles can be combined, as was the case during the experiments performed for this thesis (combination of Ion Exchange and Ion Pair chromatography).

TABLE 1.2: Overview of chromatographic principles [51]

Chromatographic principle	Mechanism of retention
Adsorption	Surface adsorption on basis of polarity.
Ion Exchange chromatography	Charge interaction between solute ions and counter ionophores on packing.
Ion Pair	Ions in solution are paired or neutralised and separated as an ion pair on a reversed-phase column.
Size exclusion	Filtering effect on the basis of hydrodynamic volume.
Chiral	Diastereoisomeric interactions between solute enantiomers and chiral sites within the packing.
Affinity	Bio-specific binding of solute to immobilised ligand.

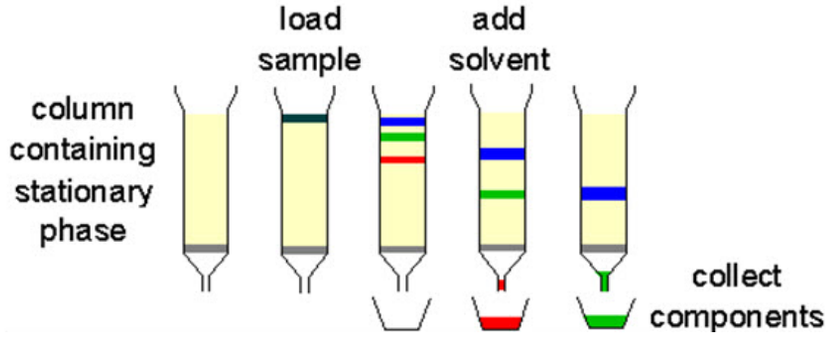


FIGURE 1.4: Principle of Liquid Chromatography [B.M.Tissue1996]

1.5.2 Mobile and Stationary Phase

The mobile phase in HPLC consists of one or more liquid chemicals, used to transport and interact with the analyte of a given assay. For Reversed-phase HPLC, most mobile phases have a hydrophilic base (adjusted to a specific pH by buffers), combined with varying percentages of more hydrophobic components such as methanol

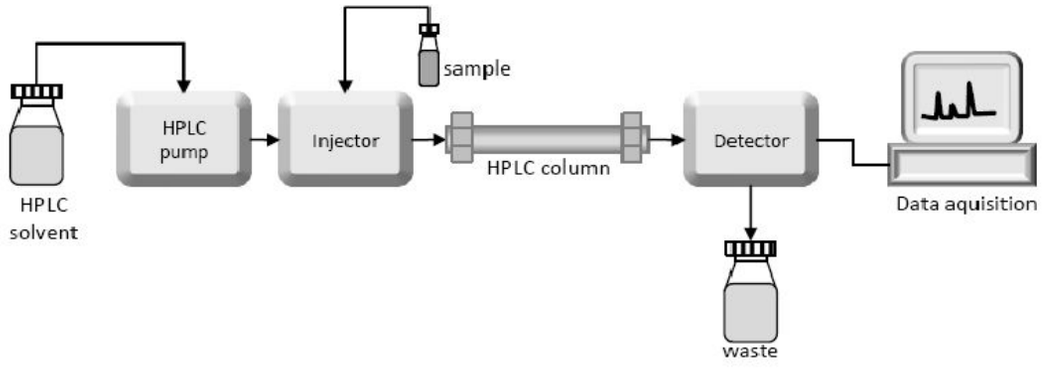


FIGURE 1.5: Overview of HPLC Instrumentation [laboratory-info.com/hplc]

or acetonitrile [52]. Another widely used possibility is the continuous adjustment of thecomposition of the mobile phase by applying gradient pump systems. In the case of ion pair chromatography, which is used during this thesis, the mobile phase was altered by the addition of an ion pair reagent. This reagent subsequently binds to the charged analyte, and forms an uncharged complex which can in turn interact with the stationary phase [53].

HPLC stationary phases consist of columns with varying length and diameter and packings therein. Typical columns are stainless steel lined with glass to withstand high pressure and to prevent metal catalysis of solvent-solute reactions within. Packings come in different forms. Generally, the particle size of the spherical supports used to pack a column has a crucial impact on the performance of the HPLC column. The smaller the particle size, the better the separation of the analyte due to the higher surface area and therefore the higher number of theoretical plates in a given column. Hence, the particle size is usually kept ≤ 5 µm [54, 55]. The choice of packing material results in the mode of separation within the column (see above). The majority of widely used packings consist of microporous silica based particles, which can be chemically modified. Three types of derivatisation are common:

Hydrocarbon Groups Long ($C_{18}H_{37}$) or short (C_2, C_8, etc.) chain hydrocarbons result in a hydrophobic surface area and are used in Reversed-Phase HPLC.

Polar Groups Amino, ether, diol and cyanopropyl groups present a polar surface area, used for Normal Phase HPLC.

Ion Exchange Groups Quarternary ammonium, sulphonic acid, etc., used for Ion-Exchange Chromatography.

A special form of derivatisation is the use of chiral compounds, in chiral chromatography. By utilizing substances like cyclodextrines or chiral polysaccharides with R- or S-configuration, a racemic analyte can be separated into its diastereoisomers. Separation occurs because diastereoisomeric forms of the analyte interact differently with the chiral compounds of the stationary phase[51].

1.5.3 Detectors

After separation, the desired analyte is detected by a suitable device. The list below provides a short summary of the most common detectors in HPLC [50, 56].

Ultraviolet/Vis/Photo Diode Array Light absorbing properties of electrons within a molecule at particular wavelengths are used. Light of a specific wavelength passes through a glass tube containing the analyte. Electrons in that molecule absorb the light and thus change its intensity. The flexibility of UV detectors can be enhanced by using a photo diode array, and scanning several wavelengths simultaneously. Figure 1.6 illustrates an UV/Vis detector coupled with a photodiode array.

Fluorescence Specific analytes possess the ability to emit fluorescent light after absorbing radiation at a longer wavelength. If an analyte is not capable of fluorescence, it may be chemically modified by coupling to a fluorescing compound. Generally, fluorescent detectors are more selective and sensitive than UV detectors, as they don't measure a small difference in intensity of absorbed light, but rather an intensity compared to a baseline of zero.

Electrochemical Analytes usually contain a number of functional groups. Many of them can be oxidised or reduced by applying an electric potential greater than the analytes half-wave potential. An electrochemical detector uses a fixed potential and monitors the changes in its current. When an analyte passes through and is oxidised/reduced, a change in this current occurs and detection is possible.

Refractive Index Solutes change the refractive index of a mobile phase. By comparing two cells, one purely filled with mobile phase and the other one containing mobile phase and the analyte, detection can be achieved. The main advantage of a refractive index detector is its broad spectrum: all substances can be detected, regardless of their chemical composition. However, its sensitivity is inferior to that of an UV detector.

Evaporative Light Scattering The mobile phase containing the analyte is nebulized and evaporated into fine particles. A laser beam is subsequentially used and the scattered radiation after hitting the particles is measured. Depending on concentration, every non-volatile analyte can be detected as it scatters the laser radiation.

Mass Spectrometry Mass spectrometry measures the mass-to-charge ratio of an ionised analyte. After separation by HPLC, the analyte enters an ion source and is ionized by a variety of methods (electron ionization, chemical ionization, photoionisation, spray ionisation, etc.). The charged particles are subsequently detected by utilizing their electro-magnetic properties (ion trapping, time of flight, quadrupole filters, etc.).

1.5.4 Internal Standard

Internal standardisation is a technique used in chromatography to improve accuracy of an analysis. A constant amount of internal standard is added to each analyzed sample, and the ratio of analyte to internal standard is calculated. A calibration

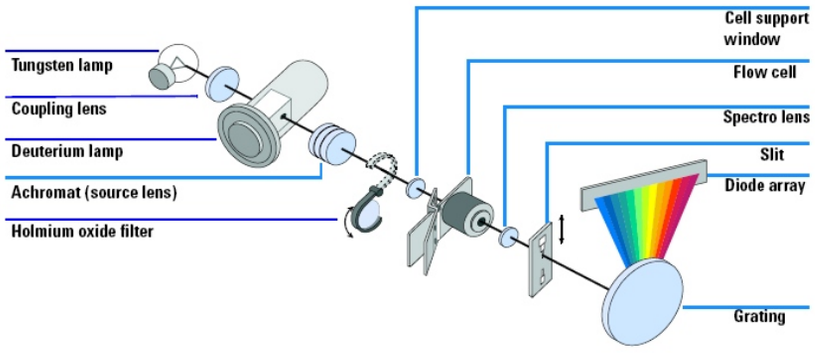


FIGURE 1.6: Principle of Diode Array Detector (©Agilent Technologies): Visible light emitted by the tungsten lamp and UV light emitted by the deuterium lamp pass the flow cell and is subsequently detected by the diode array.

curve, containing known amounts of analyte with fixed amounts of internal standard per calibration sample can be obtained. Subsequently, the initial amount of analyte in the sample of interest can be calculated. Especially in methods utilizing several steps of sample preparation, which may result in a certain loss of analyte, an internal standard can be useful for correction. However, the internal standard has to meet following requirements [57]:

Similar Structure The structure of an internal standard has to be as similar as possible to the analyte of interest, ensuring similar retention times, extraction characteristics, detectability, etc.

Well resolved For HPLC the internal standard has to exhibit a distinct chromatographic signal without interference from the analyte.

Not present in sample An internal standard may never be inherently present in the sample of interest.

Detectable The internal standard has to be detectable and quantifiable, but not necessarily at the same detection parameters.

Stability The internal standard has to be stable during sample preparation and chromatographic analysis.

1.5.5 Optimization of Separation and Detection

To optimize separation and detection, several parameters of a HPLC method can be modified [58]:

Mobile Phase Composition and therefore affinity of the analyte towards the mobile phase can be modified and optimized. Additionally, gradient systems, which contain several different mobile phases at varying concentrations throughout an analytic run, can be applied.

Stationary Phase Different stationary phases and various principles of separation (normal-phase, reversed-phase, ion exchange, adsorption, etc.) are available. In addition to that, particle size and surface area also affect separation.

Detector Method of detection can be optimized, choosing from a wide array of available detectors (ultraviolet photometry, mass spectrometry, refractive index monitoring, photo diode arrays, optical rotation, etc...).

Flow Rate has a crucial effect on separation by influencing the time of analyte interaction between the stationary and mobile phase. Flow rate also plays an important role in potential upscaling of the method for obtaining a high throughput method.

Temperature directly affects mass transfer and viscosity of the analyte and mobile phase. Higher temperatures may improve peak resolution, but also may result in a decrease in specificity.

1.6 Solid Phase Extraction

1.6.1 Overview and Principle

Solid Phase Extraction is a form of Liquid Chromatography (LC), which is typically used for the extraction of analytes from complex matrices. This technique enables the isolation (extraction), clean up, as well as the concentration of the analytes prior to their identification or quantification [59]. In principle, a typical SPE protocol involves four steps: 1. Conditioning the packing, 2. Applying (loading) the sample, 3. Washing the packing (removal of interferences), 4. Eluting and recovering the analyte [60]. In the following section these steps are described in detail. Figure 1.7 provides an overview of the technique.

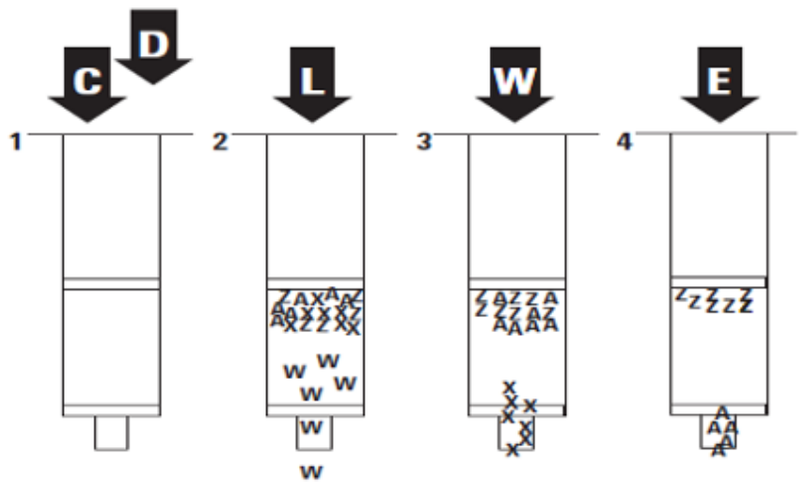


FIGURE 1.7: Working steps in Solid Phase Extraction Chromatography; **Sample Components:** A = Analyte(s) of interest, W = Weakly retained, undesired matrix component(s), X = Intermediate undesired matrix component(s), Z = Strongly retained undesired matrix compontent(s); **Solvents:** C = Conditioning solvent, D = Equilibration solvent, L = Loading solvent, W = Washing solvent, E = Eluting solvent [61]

1. **Conditioning the packing**: First, the column package is conditioned by passaging solvent through the SPE column in order to remove any present impurities (e.g. from manufacturing). Second, the conditioning of the column

allows the sorbent to be solvated, which may decrease the sample retention. Solvation is especially important for reversed-phase silica-based packings.

2. **Applying (loading) the sample**: Next, the analyte is dissolved in a weak solvent, designated as "L", and carefully applied ("loaded) on the SPE column. Usually, a column material with a high affinity for the analyte is used, to quantitatively bind the substance to the stationary phase. By using the weak solvent the applied analyte is strongly retained. As a weak solvent water or a buffer is used for reversed phase SPE (RP-SPE). In contrast, for ion exchange SPE a solvent with very low ionic strength should be used. The flow-rate of the sample loading can easily be adjusted by varying the vacuum.

3. **Washing (Rinsing)**: In the washing step, the cartridge is carefully washed with a solvent of intermediate strength ("W") in order to remove interfering substances. An extensive washing step should be avoided, since this may result in a partial elution of the analyte and thus a decreased analyte recovery. Usually, the rinsing step should be stopped before the analyte starts to leave the cartridge to avoid any loss of the analyte. Typical washing solutions are water or buffer. The addition of a small amount of organic solvents contributes to the removal of hydrophobic substances.

4. **Eluting**: Finally, for the quantitative elution of the desired analyte an elution solution with a higher affinity to the stationary phaseis used, which elutes the analyte [62]. During the elution step the analyte is eluted and collected. Hereby, the analyte should be collected in a solvent volume which is as small as possible to allow a sensitive detection of the analyte. This can be achieved by using a strong elution solvent (E). However, an intermediate strength elution solvent can also be used.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 List of Materials

- High Performance Liquid Chromatography (Shimadzu, Duisburg, Germany)
  - Double LC-20 AD pumps, gradient unit
  - UV-VIS SPD 20 AV detector
  - CTO-10 AC column oven
  - Class-VP 7 HPLC software suite
- Mixed-Mode Cation-Exchange SPE, MCX 3cc, 60mg (Waters, Eschborn, Germany)
- Thermo Accucore XL C18 HPLC Column, 150mm/ID4.6mm/Particle Size 4 µm (ThermoFisher Scientific, Schwerte, Germany)
- LiChrospher RP-18 HPLC Column, 25cm/ID4.6mm/Particle Size 5 µm (Merck, Darmstadt, Germany)
- Supelcosil LC-18 HPLC Column, 15cm/ID4.6mm/Particle Size 3 µm (Merck, Darmstadt, Germany)
- Vapotherm Evaporator (Barkey, Leopoldshöhe, Germany)
- Ika HS260 basic Shaker (Ika, Staufen, Germany)
- Hettich Universal 32 Centrifuge (Hettrichlab, Tuttlingen, Germany)
- Analytical Balance (Sartorius Lab Instruments, Göttingen, Germany)
- 50 mL Round-Neck Bottles (Omnilab, Munich, Germany)
- Gemcitabine Hydrochloride $\geq$ 98% (Merck, Darmstadt, Germany)
- 2'-Deoxycytidine $\geq$ 99% (Merck, Darmstadt, Germany)
- Adenosine $\geq$ 99% (Merck, Darmstadt, Germany)
- Hydrochloric Acid 37% (Merck, Darmstadt, Germany)
- Sodium Hydroxide $\geq$ 97% (Merck, Darmstadt, Germany)
- Sodium Dihydrogen Phosphate (Merck, Darmstadt, Germany)
- Methanol anhydrous, 99.8% (Merck, Darmstadt, Germany)

- Acetonitrile anhydrous, 99.8% (Merck, Darmstadt, Germany)
- Fluka Phosphoric Acid for HPLC, 85-90% (ThermoFisher Scientific, Schwerte, Germany)
- Millipore Ultrapure Lab Water (in-lab purification system)

List of buffers:
Acidified Water: 1 mL 4% Phosphoric acid (v/v), deionized water ad 100 mL SPE Washing buffer (50 mM NaH_2PO_4, pH = 2.9): 3.5 g NaH_2PO_4, 200 µl H_3PO_4 85%, deionized water ad 500 ml
SPE elution buffer: 2 mL NH_4OH, MeOH ad 100 mL

2.2 Methods

2.2.1 Preparation of Standard Solutions

Stock solutions of gemcitabine and the internal standard 2'-deoxycytidine were prepared in methanol at a concentration of 10 µg/ml. The stock solutions were stored at 4 - 8 °C up to 1 week. Working solutions were created by diluting the stock solution to obtain final concentrations of 0.01 to 1.0 µg/ml for the analyte.

2.2.2 Surface Wipe Sample Preparation

For sampling, a new set of clean, disposable, powder-free gloves was used to avoid any contamination. First, three cellulose filters (grade 391, Sartorius) were moistened with 6 drops Millipore water each. An area of 20 x 20 cm^2 was wiped, according to a previously mentioned wiping scheme (Figure 2.1) [22]. Different areas (table, scale, floor, sink, hood etc.) were tested, each in triplicates. Real samples were taken at an oncological outpatient clinic in Munich. The filters where then placed in a glass vial and frozen at $-20\,°C$ for long-term storage. Before testing, the filters were thawed and 20 ml of acidified water, containing 4 % of phosphoric acid, was added . The filters were then shaken at 180 rpm on a horizontal shaker for 20 min. 15 ml of this solution was concentrated by solid phase extraction as described below. Blank samples without wiping were prepared analogously.

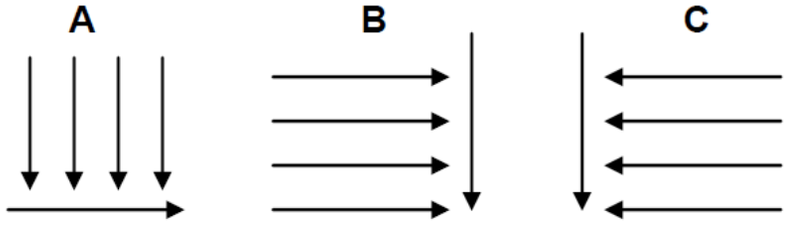


FIGURE 2.1: Wipe Scheme of analyzed surface

2.2.3 Solid Phase Extraction (SPE) in Wipe Samples

Our SPE-Method used cation exchange columns, which were primed with 2 mL MeOH followed by 2 ml NaH_2PO_4 buffer solution (pH = 2.9). After priming, the

working solutions of gemcitabine and the internal standard were pipetted onto the column. A mild vacuum was applied until the columns ran dry. Then, washing steps were performed, starting with 2 mL washing buffer, followed by 2 mL MeOH. It has to be noted that columns were allowed to run dry only after the application of buffer. After each washing step, samples were collected and analyzed analogously by our HPLC method. Finally, analyte and internal standard were eluted with a solution of 1 ml of 2% NH_4OH in MeOH.
Subsequently, samples were concentrated on a tempered water bath at 50 °C N_2 atmosphere. 100 µl 4% H_3PO_4 was subsequently added. Samples were analyzed by RP-HPLC.

2.2.4 Final Standard Operating Procedure

The filter was wetted with 20 mL acidified water and shaken for 20 minutes on a laboratory shaker. Subsequently, an aliquot of 15 ml was used for each experiment. For the following solid phase extraction, up to five SPE columns were processed simultaneously using a 5 - port vacuum manifold. The columns (MCX 3cc, 60mg Waters) were pretreated with both 2 ml MeOH and 2 ml SPE washing buffer (50 mM NaH_2PO_4, pH = 2.9) to ensure the desired acidic pH value for the sample. 25 µl of the internal standard (10 µg/ml in water) was added to the column. Consequently, the sample (15 mL) was processed through the SPE column until running dry. The SPE columns were thoroughly washed with 2 mL SPE washing buffer and 2 mL methanol. Then, the sample was eluted by adding 1 mL of 2 % ammonium hydroxide in methanol. The sample was concentrated at 50 °C under nitrogen flow. After the concentration step, the dried compounds were dissolved in 100 µl of phosphoric acid and gently mixed for several minutes. The sample was consequently analyzed with RP-HPLC. For quantification of gemcitabine a standard curve of gemcitabine solutions ranging from 0 - 2.5 µg/ml was performed. HPLC method parameters are listed in Table 2.1 below.

TABLE 2.1: Parameters of RP HPLC-Method

Column	Thermo Accucore XL C18 (150 mm / ID 4.6 mm / Particle Size 4 µm)
Solvent	CH_3CN:MeOH:NaH_2PO_4buffer (pH=3), 5:5:90, + octanesulfonate sodium 11 mM
Detection wavelength	275 nm
Injection volume	25 µl
Temperature	35 °C
Flow	1.2 ml/min

Chapter 3

Results

3.1 Preliminary Testing of a RP-HPLC Method for the Detection of Gemcitabine

To analyze the basic chromatographic properties of gemcitabine various solutions with concentrations of gemcitabine ranging from 0.5 to 10 µg/ml were directly injected into the HPLC system and measured by RP-HLPC. The initial parameters of the described method were adapted from Rahul et al. [26] with minor deviations and are summarized in Table (Table 3.1). Using these conditions, initial HPLC runs were performed and improved systematically.

TABLE 3.1: Parameters of preliminary HPLC-Method

Column	LiChrospher RP-18 HPLC Column, 25cm/ID4.6mm/Particle Size 5 µm
Solvent	CH_3CN:H_2O, 10:90
Detection wavelength	275 nm
Injection volume	25 µl
Temperature	35 °C
Flow	1.2 ml/min

TABLE 3.2: Preliminary analysis of different gemcitabine concentrations (n=3)

Concentration (µg/ml)	**Mean peak area (gemcitabine)**	**Standard deviation (relative in %)**
0.5	28,014	676 (2.4%)
1	56,565	517 (0.9%)
2	100,042	1,498 (1.5%)
5	240,696	17,223 (7.1%)
10	449,819	10,959 (2.4%)

As shown in Figure 3.1, a sharp gemcitabine peak was observed at a retention time of 3 minutes. Hence, the compound eluted quickly showing Gaussian peak shape with good peak symmetry (asymmetry factor= 1.02). It was possible to detect 12.5 ng gemcitabine per injection with an average peak area of approximately 28,000. Measurements were performed in triplicates.

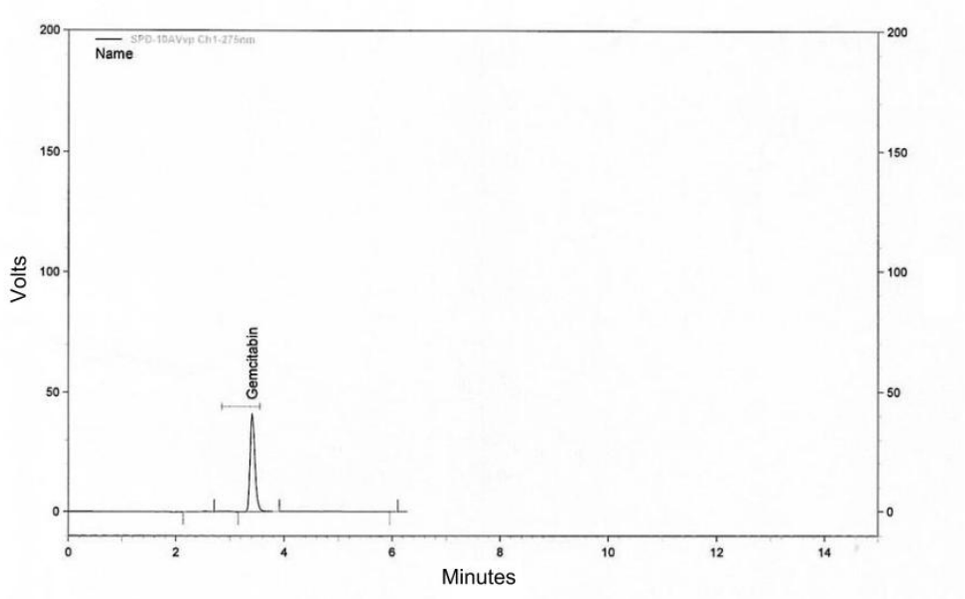


FIGURE 3.1: Exemplary chromatogram of gemcitabine (5 µg/ml)

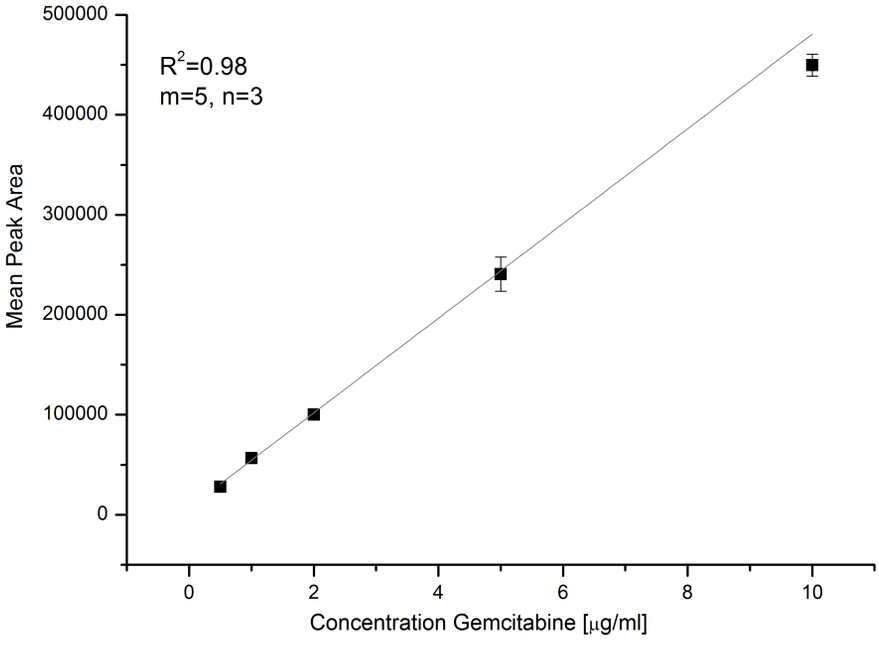


FIGURE 3.2: Standard curve of gemcitabine stock solutions by RP-HPLC. Standard deviations (n=3) are displayed as error bars.

Additionally, Figure 3.2 displays a standard curve obtained by measuring different concentrations of gemcitabine.

3.2 Development of a RP-HPLC Method for the Detection of Gemcitabine

Next, an ion pairing reagent (IPR) was added to optimize chromatographic behavior of the analyte by increasing its retention time. Furthermore, different internal standards were tested to improve the precision of the method. All experiments were initially performed out of stock solutions in absence of filter matrix interference, or wipe sample impurities to optimize the method under the best possible conditions.

3.2.1 Internal Standards

Simultaneously to the early IPR experiments, the addition of internal standards was analyzed in order to improve the precision of the analytical method. Therefore, the chemically similar internal standards adenosine and 2dC (as described by [63]) at a concentration of 2 µg/ml were added. The concentration of gemcitabine was set to 2 µg/ml, while the rest of the parameters of the method were kept constant, as displayed above. For comparison, the influence of both the analyte and the internal standards on mean peak area and retention time was evaluated. As shown in Table 3.3, the internal standard 2dC was eluted at 4.8 minutes, whereas adenosine was eluted at 3.7 minutes. The retention time of gemcitabine was at 8.1 minutes. Due to possible early eluting pollutants or matrix components in the real sample, the internal standard 2dC with the higher retention time of 4.8 minutes was chosen for our method.

TABLE 3.3: Chromatographic Parameters of Internal Standards and Gemcitabine, c=2 µg/ml, n=3

Parameter	Gemcitabine	2′-Deoxycytidine	Adenosine
Mean Peak Area	100,042 $\pm$ 1.5%	322,282 $\pm$ 2.2%	145,548 $\pm$ 2.3%
Mean Retention Time	8.1 $\pm$ 1.1 min	4.8 $\pm$ 0.4 min	3.7 $\pm$ 0.6 min

3.2.2 Addition of Ion Pair Reagent (IPR)

An IPR was used to increase the retention of gemcitabine and allow its detection in a real sample also containing interfering contaminants. The chosen IPR was sodium 1-octanesulfonate. To evaluate the effect of the added IPR, the influence on retention time of gemcitabine was analyzed. Consequently, the IPR sodium 1-octanesulfonate was added to the mobile phase at pH 3. Parameters of the RP HPLC method are shown in Table 3.4. In addition to the IPR, the column type was changed to the shorter column Thermo Accucore XL C18 in order to decrease the HPLC analysis time. The mobile phase was changed to a triphasic mixture, as previously described by [63].

3.2.3 Influence of IPR Concentration

Next, the influence of the IPR sodium 1-octanesulfonate on the RP-HPLC method was evaluated. For this study, different concentrations of octanesulfonate sodium (1 mM, 3 mM, 11 mM) were added to the mobile phase at pH 3 and analyzed with various concentrations of gemcitabine (0.1, 0.5, 1, 10 µg/ml) and the internal standard

TABLE 3.4: Parameters of RP HPLC-Method

Column	Thermo Accucore XL C18 (150 mm / ID 4.6 mm / Particle Size 4 µm)
Solvent	CH_3CN:MeOH:NaH_2PO_4buffer (pH=3), 5:5:90, + octanesulfonate sodium 3 mM
Detection wavelength	275 nm
Injection volume	25 µl
Temperature	35 °C
Flow	1.2 ml/min

2dC (10 µg/ml). Therefore, all other parameters of the RP HPLC method were kept constant (Table 3.4).

TABLE 3.5: Influence of different IPR concentrations on retention of gemcitabine and 2dC. Averaged retention times (n=3) are displayed.

Concentration of IPR (mol/L)	**Retention time of 2dC (min)**	**Retention time of gemcitabine (min)**
1	7.5 ± 0.1	11.3 ± 0.1
3	11.7 ± 0.2	16.4 ± 0.1
11	18.5 ± 0.5	27.7 ± 1.3

By adding 1 mM, 3 mM, and 11 mM of IPR, retention times for 2'-deoxycytidine of 7.5 minutes, 11.7 minutes and 18.5 minutes were obtained (Table 3.5). Additionally, higher concentrations of the IPR of 1 mM, 3 mM, and 11 mM analogously increased the retention time of gemcitabine from 11.3 minutes to 16.4 minutes and 27.7 minutes, respectively. However, peak symmetry deteriorated with decreasing IPR concentrations with peaks showing an increment in tailing. For following experiments, the highest concentration of 11 mM sodium octanesulfonate was applied for our method. To improve analysis speed with the higher retention times, a shorter chromatographic column (Thermo Accucore XL C18, 150mm / ID4.6mm / particle size 4 µm) was used, which resulted in retention times of 9 min for gemcitabine and 6 min for 2dC.

3.2.4 Influence of Absorption Wavelength

In this study, the influence of the absorption wavelength on the developed RP-HPLC method for gemcitabine was evaluated. Samples of the analyte and the internal standard 2dC with a concentration of 10 µg/ml were analyzed at two different absorption wavelengths (267 nm; 275 nm) by RP-HPLC. As shown in (Table 3.6), a detection wavelength of 267 nm shows a mean peak area (gemcitabine) of approximately 395, whereas the absorption wavelenghts of 275 nm resulted in a slightly higher main peak area of 443. The same effect can be seen for the internal standard 2dC. 275 nm was used for detection wavelength throughout the following studies.

TABLE 3.6: Influence of Absorption Wavelength on mean peak area

Wavelength (nm)	**Mean peak area gemcitabine (10 µg/ml)**	**Mean peak area 2'dC (10 µg/ml)**
267	395,380	358,757
275	443,039	478,209

3.2.5 Summary

The influence of several parameters on the retention times and mean peak areas of gemcitabine such as absorption wavelengths, type and concentration of IPR, and selection of internal standards were investigated during the stepwise method optimization. A limit of detection of 0.091 µg/ml was obtained.
A summary of all optimized parameters is listed in Table 3.7.

TABLE 3.7: Summary: Optimized parameters for the dection of gemcitabine by RP-HPLC

Parameter	
Internal standard	2'-deoxycytidine
Ion pairing reagent	sodium 1-octanesulfonate, c = 11 mM
Detection wavelength	275 nm
HPLC column	(Thermo Accucore XL C18, 150mm / ID4.6mm / particle size 4 µm
Mobile phase	CH_3CN:MeOH:NaH_2PO_4 buffer (pH=3), 5:5:90
Injection volume	25 µl
Column temperature	35 °C
Flow	1.2 ml/min

3.3 Analysis of Gemcitabine in Wipe Samples

3.3.1 Wipe Sample Preparation

As described in chapter 2, sample acquisition was performed by collecting wipe samples. Therefore, a specific wipe scheme (Figure 2.1) was applied. For the experiments, the desired surface area was selected, and wiped with three folded filter papers previously wetted with six drops of ultrapure water. After sample acquisition, the filters were stored in a 50 mL round neck bottle and frozen at $-20\,^{\circ}C$ until analysis. Due to the high water solubility of gemcitabine, the extraction solvent was deionized water. Before analysis, samples where thawed at room temperature. Subsequently, 20 mL of acidified water were added in order to extract gemcitabine from the filter matrix (see chapter 2). For method optimization, different filter materials and pH values of the extraction solution were considered and analyzed.

3.3.2 Influence of Filter Matrix on Interference

For sampling, the surface of interest has to be wiped with a wet filter paper to absorb any environmental gemcitabine. The solvent is subsequently added to the filter to quantitatively extract the gemcitabine from the filter. Not only gemcitabine, but also components of the filter matrix itself may be co-extracted by this procedure. In order to assess the extraction of inherent components of the filter matrix, different filter papers were prepared according to the established procedure for the analyte, without the addition of gemcitabine or the internal standard. First, filter discs grade 391 from Sartorius were tested.

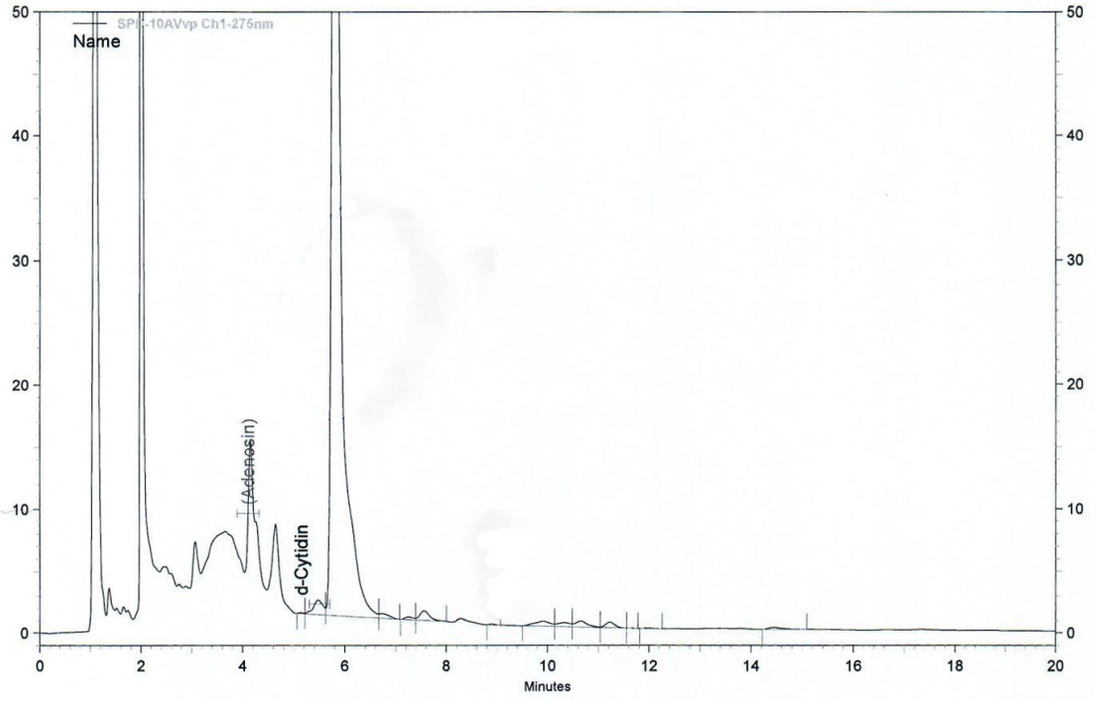


FIGURE 3.3: Chromatogram of an extract from Sartorius filter paper, prepared with the established procedure without analyte or internal standard

The retention time of gemcitabine with the established method was between 8 to 9 minutes. A prominent signal was present at 6 minutes, which is in direct vicinity to the retention time of the internal standard 2dC. Consequently, various filter materials were assessed to examine the interference between filter material and analyte as well as internal standard. Second, Whatman grade 42 ashless filter paper for instrumental analysis was evaluated.

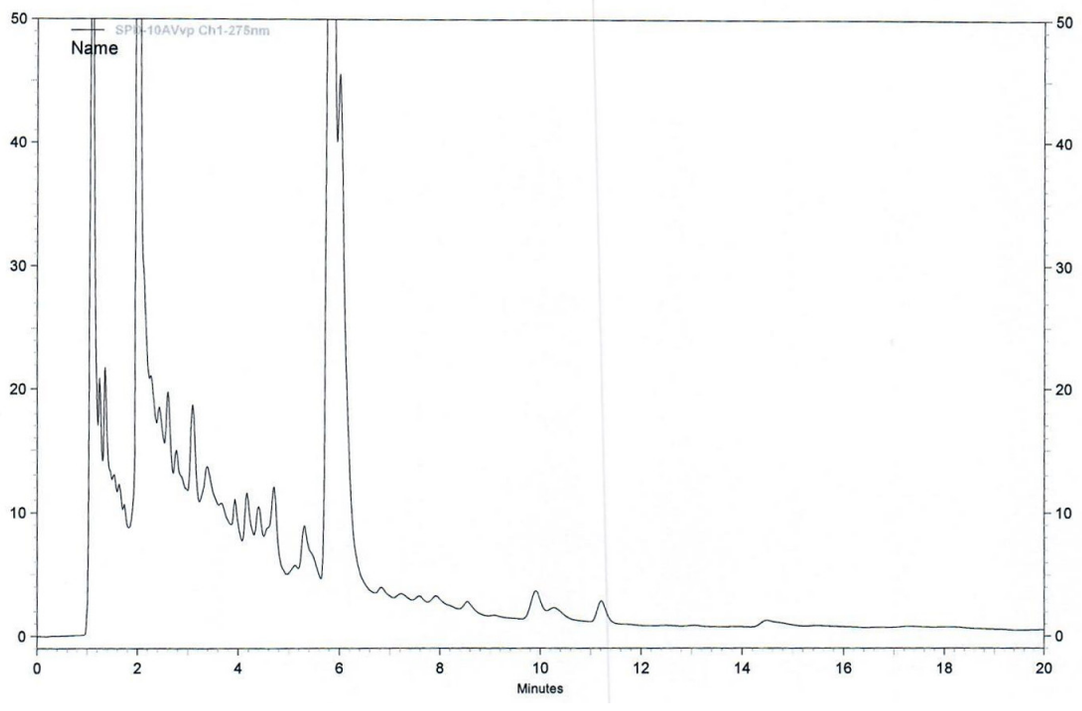


FIGURE 3.4: Chromatogram of an extract from Whatman filter paper, prepared with the established procedure without analyte or internal standard

As shown in Figure 3.4, the chromatogram clearly displays a prominent signal at 6 minutes. At a retention time of 6 minutes, the internal standard was detected. Additionally, the background noise was increased compared to the Sartorius filter material. Thus, the Whatman filter paper was not appropriate for our developed method. Lastly, a third filter paper was examined to reduce the high interference signal at 6 minutes. The third examined filter paper was a Schleicher& Schuell 589 round filter disk. Similar to the Whatman filter, a high background noise was prominent. Additionally, the strong signal at 6 minutes was similarly present. Sartorius filter papers were used for further studies.

3.3.3 Influence of pH Value of Filter Extraction Solution

Quantitative extraction of gemcitabine from the filter is a crucial part of sample preparation. Three different aqueous solutions, acidic water at pH 3, distilled water at pH 6, and alkaline water at pH 9 were prepared. The pH of the solutions was adjusted by the addition of either H_3PO_4 4% (v/v) or NH_3 2% (v/v). Then, standard sample preparation was executed and three different samples, containing 40 ng gemcitabine were analyzed. In order to compare the aqueous solutions, the influence on the mean peak area of gemcitabine, which represents the amount of gemcitabine in

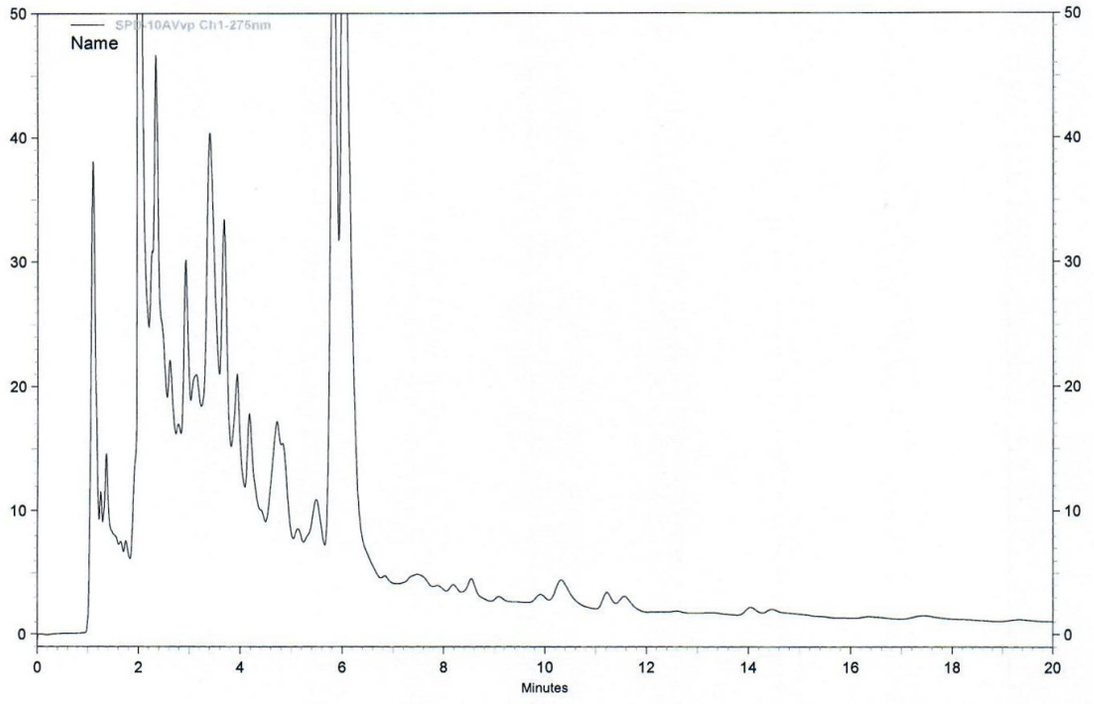


FIGURE 3.5: Chromatogram of an extract from Schleicher& Schuell filter paper, prepared with the established procedure without analyte or internal standard

the sample, was analyzed. As shown in Figure 3.6, all three aqueous extraction solutions showed a similar peak area, the mean peak area being 279,347 with a RSD of 0.99%. For further experiments, we chose an aqueous acidic extraction solution with a pH of 3.

3.3.4 Extraction of Gemcitabine in Wipe Samples by Solid Phase Extraction (SPE)

Prior to each RP-HPLC analysis, a SPE process was performed for extraction of gemcitabine. For our experiments, working solutions of gemcitabine (0.1 µg/ml) and internal standard (2 µg/ml) were prepared and directly measured by our HPLC method. To evaluate the recovery of gemcitabine after SPE, solutions of the analyte and internal standard at the same concentration with a prior SPE extraction process were analogously analyzed. Additionally, all washing eluates were measured to evaluate potential losses of analyte or internal standard during the washing steps. The recovery of gemcitabine after SPE was calculated according to Equation 3.1.

$$\frac{\text{Main peak area of gemcitabine after SPE extraction}}{\text{Mean peak area of gemcitabine}} = \text{Recovery (\%)} \qquad (3.1)$$

No visible peaks of gemcitabine or internal standard could be detected in any of our washing solutions (blank spectrum not shown). The achieved recovery of gemcitabine after SPE was 98.32 % (Table 3.8).

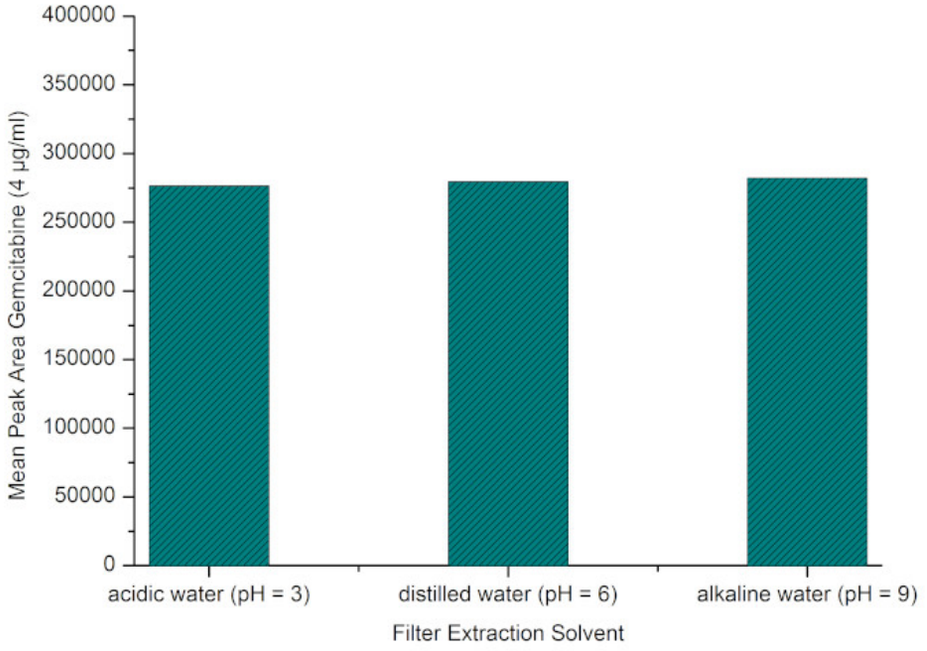


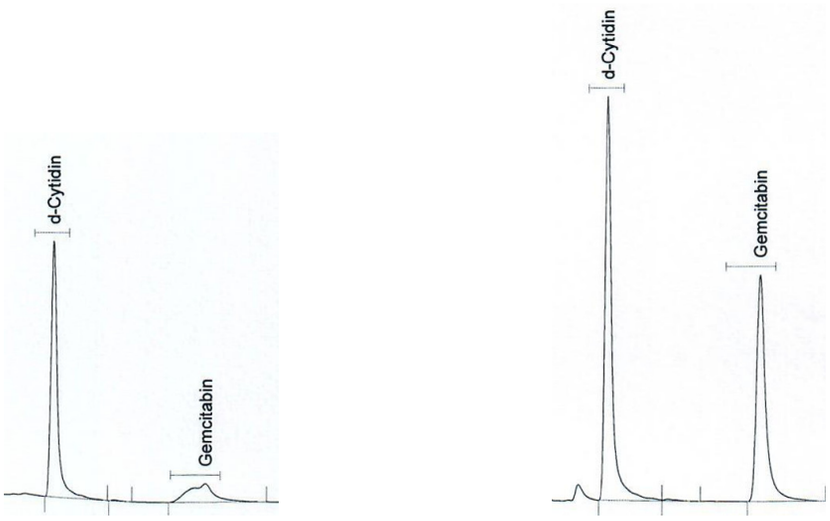
FIGURE 3.6: Influence of various aqueous extraction solvents on gemcitabine mean peak area (4 µg/ml)

TABLE 3.8: Recovery Rate: Stock Solution vs SPE Eluate ($c_{gemcitabine}$=0.1 µg/ml, n=3)

Mean Area Stock	**Mean Area SPE Eluate**	**Recovery Rate in %**
12,498.7 ± 10.68 %	12,288.7 ± 17.63 %	98.32

3.3.5 Influence of pH Value of SPE Eluate

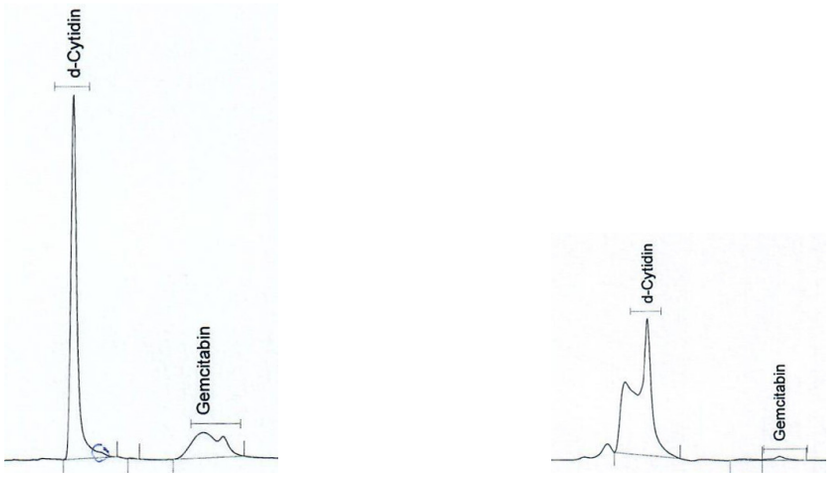
After optimization of the SPE, the influence of the pH value on the chromatographic parameters of the wipe sample was investigated. As previously described, before injection in the HPLC system, all samples were reconstituted in mobile phase, and different concentrations of H_3PO_4 were added to obtain various pH values in the solutions. To compare the influence of the pH value of the sample (SPE eluate) on the RP-HPLC method for gemcitabine, both peak area and peak symmetry were compared at various pH values of the mobile phase.
Figure 3.7 and Figure 3.8 show sample chromatograms at different pH values ranging from pH 1 to pH 7. At pH 1, gemcitabine showed a deformed signal at 8.4 min with a mean peak area of 56,856. 2dC appeared at 5.3 min with a mean peak area of 194,415. Peak symmetry of gemcitabine was 0.78 and for 2dC 1.2.
At pH 3, gemcitabine showed a distinct signal at 8.4 min with a mean peak area of 247,162. 2dC appeared at 5.3 min with a mean peak area of 326,817. Peak symmetry of gemcitabine was 1.2 and for 2dC 1.3.
At pH 5, gemcitabine showed a deformed signal at 8.4 min with a mean peak area of 56,856. 2dC remained a distinct signal at 5.3 min with a mean peak area of 194,415.



pH = 1 pH = 3

FIGURE 3.7: Chromatograms of gemcitabine and 2′-dC at different pH values

Peak symmetry of gemcitabine was 0.8 and for 2dC 1.3.
Finally at pH 7, gemcitabine signal lost most of its intensity at a retention time of 8.3 min with a mean peak area of 8,004. The 2dC signal appeared heavily deformed at 5.3 min with a mean peak area of 363,473. Peak symmetry of gemcitabine was 1.1 and for 2dC 0.68. Consequently, for further method development, SPE samples were adjusted to a pH value of 3 prior to HPLC analysis.



pH = 5 pH = 7

FIGURE 3.8: Chromatograms of gemcitabine and 2′-dC at different pH values

3.3.6 Summary

In summary, the RP-HPLC method for the quantitative detection of gemcitabine in wipe samples was successfully developed. The standard protocol for the wipe sample preparation as well as a SPE process prior to each RP-HPLC measurement was established in order to extract the analyte as well as to concentrate the gemcitabine sample to increase the assay sensitivity (volumetric concentration factor: 200). With regard to possible contaminations during sampling on the surface and the filter matrix, the influence of several parameters such as filter material, pH value of filter extraction solution, and pH value of SPE eluate was investigated during the stepwise method optimization.
A summary of all optimized parameters is listed in Table 3.7.

3.4 Outpatient Clinic Surface Measurements

To verify our developed analytical method in a real working environment, wipe samples were taken at a clinic for oncology and measured according to the developed SOP.

3.4.1 Sample Acquisition and Results

Wipe sample acquisition was performed on site as described in Chapter 2. Hereby, three filters per site were used and stored until further use. After SPE, the samples were analyzed by our developed RP-HPLC method. A standard curve of gemcitabine solutions ranging from 0 - 2.5 µg/ml was performed in order to quantify the amount of gemcitabine in an outpatient oncolocy clinic (Figure 3.9). A correlation coefficient of 0.99 was obtained.

An exemplary chromatogram overlay of real wipe samples containing gemcitabine at different sample acquisition sites in an outpatient oncology clinic is shown in Figure 3.10. A clear and distinct gemcitabine peak at the retention time of around 8.2 minutes was visible. The overview of sample acquisition sites and results are shown in Table 3.9 .
As shown in Table 3.9, samples *"Bathroom Floor" (2)*, *"Patient Recreation Area" (3)* and *"Cytostatic Isolator" (6)* contained quantifiable traces of gemcitabine with a maximum of 3.98 µg/ml of the anti-neoplastic drug.
Traces of gemcitabine could also be detected in the patient recreation area (c = 0.2935 µg/ml) and the patient bathroom floor (c = 0.0622 µg/ml). In work benches, printer, floors and the reception desk of the outpatient clinics, the analyte concentration was below the LOD and therefore was designated as not detected.

TABLE 3.9: Sample Acquisition Sites and Concentrations in a Outpatient Oncology Clinic

Site No	**Site**	$\mathbf{c_{gemcitabine}}$ **(µg/ml)**	$\mathbf{m_{gemcitabine}}$ **(ng/cm²)**
1	Reception desk	n.d.	–
2	Patient bathroom floor	0.0622	0.0207
3	Patient recreation area	0.2935	0.0978
4	Work bench 1	n.d.	–
5	Work bench 2	n.d.	–
6	Cytostatic isolator	3.9797	1.3266
7	Printer	n.d.	–
8	Floor	n.d.	–

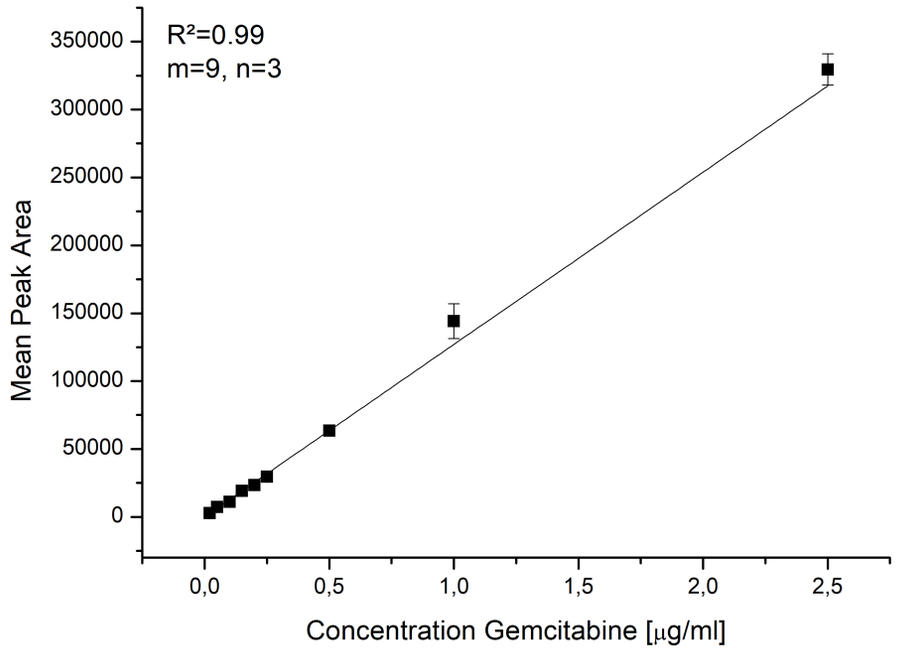


FIGURE 3.9: Standard curve of gemcitabine calibration solutions by RP-HPLC for outpatient samples

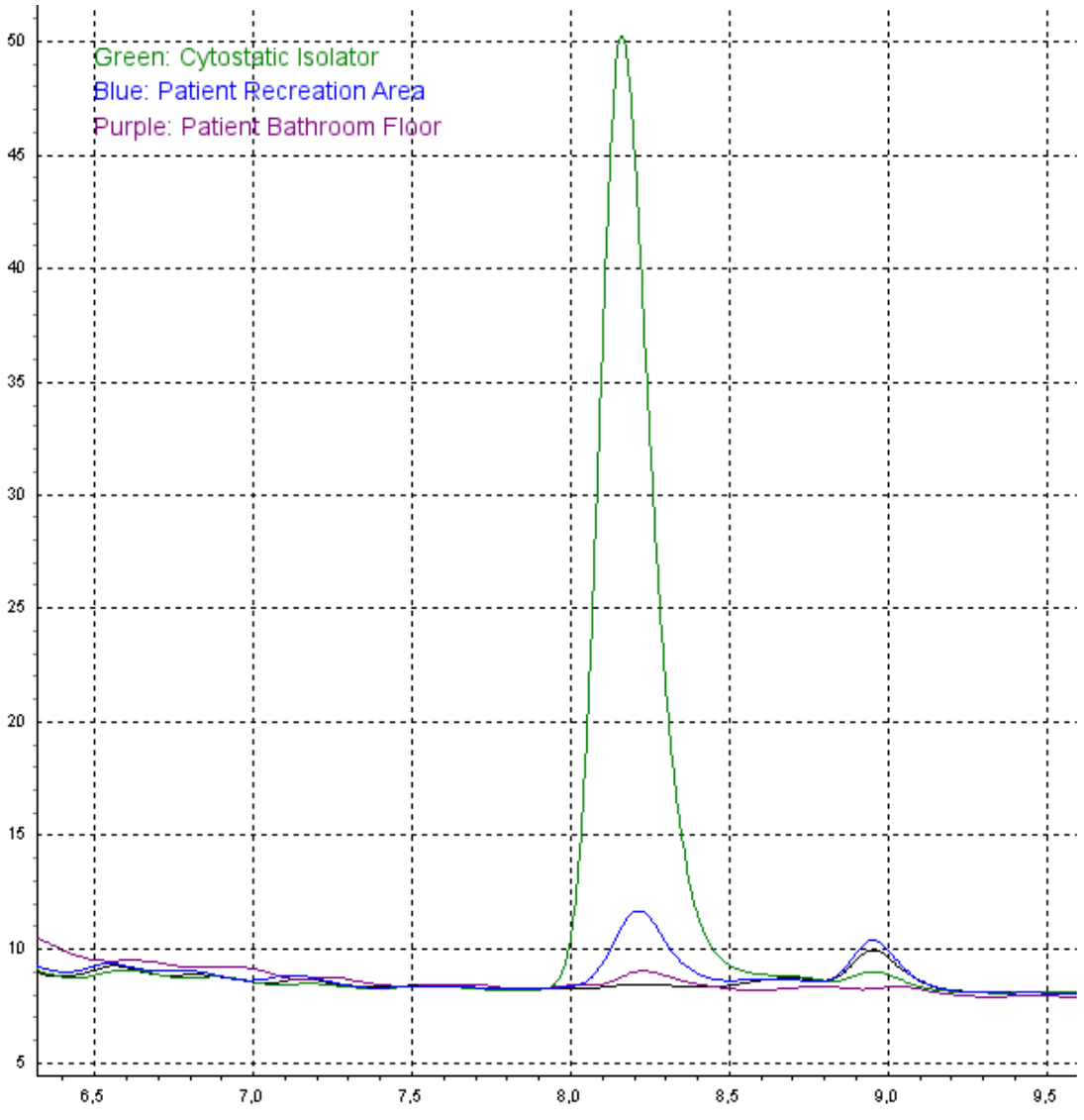


FIGURE 3.10: Chromatogram overlay of wipe samples at different sample sample acquisition sites in an outpatient oncology clinic

Chapter 4

Discussion

4.1 Preliminary Testing of a RP-HPLC Method for the Detection of Gemcitabine

The first aim of this thesis was to develop a RP-HPLC method for the quantitative analysis of gemcitabine. Preliminary tests adapting HPLC parameters with minor deviations from a previously published method [26] were conducted. Since parameters such as temperature, column length and material, pH value of solvent, and detection wavelength have a great influence on method sensitivity and specifity, HPLC runs were performed and improved systematically. Our results showed that gemcitabine can be both rapidly and quantitively detected by RP-HLPC in the range from 0.5 µg/ml to 10µg/ml. However, the low retention time of gemcitabine at only three minutes was a major challenge. Since the higher aim of this thesis was to detect and quantify the analyte gemcitabine in real wipe samples, early eluting hydrophilic impurities, either from filter matrix or surface contaminants, could easily interfere with the desired analyte peak. Hence, a longer retention time (> 10 min) is essential to analyze gemcitabine without any matrix interference.

4.2 Development of a RP-HPLC Method for the Detection of Gemcitabine

4.2.1 Internal Standards

In order to improve the precision and accuracy of our analytical method an internal standard was applied. 2'-deoxycytidine and adenosine were selected, since both compounds are chemically similar compounds to gemcitabine and are therefore expected to exhibit similar chromatographic characteristics. As shown in the figures below (Figure 4.1) and (Figure 4.2), all substances share the chemical backbone of a (deoxy)ribonucleoside, modified by a di-fluoro group in the case of gemcitabine. Whereas the analyte and 2'-deoxycytidine are both conjugated to the base cytosine, adenosine is conjugated to the base adenine.
Our results showed, that the internal standard adenosine was eluted at 3.7 min and 2'dC at 4.8 min compared to gemcitabine at 8.1 minutes. The peaks were perfectly separated and showed no overlapping effects.
Due to early eluting pollutants or matrix components in real wipe samples, the internal standard 2dC with the higher retention time of 4.8 minutes was chosen for our

2′-deoxycytidine Adenosine

FIGURE 4.1: Chemical Structure of Internal Standards

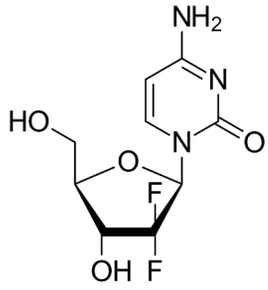


FIGURE 4.2: Chemical Structure of Gemcitabine

method. Experiments indicated that chromatographic signals (peak area and retention time) stayed consistent during the time of analysis. Hence, it can be concluded that internal standards were stable under storage conditions.

In summary, the RP-HPLC method for the quantitative detection of gemcitabine as pure substance was successfully optimized. The aims were to achieve an increased retention time to avoid interference with early eluting pollutants and to achieve appropriate mean peak areas, which allows a quantitative detection of gemcitabine.

4.2.2 Addition of Ion Pair Reagent (IPR)

After the preliminary chromatographic experiments, a further optimization of the analytical method was performed. Since the retention time of native gemcitabine was too short for adequate detection of gemcitabine in a real wipe sample also containing early eluting contaminants, an ion-pairing reagent was applied [63–65]. The aim was to increase the retention of gemcitabine for effectively separating the matrix components and wipe sample impurities from the analyte peak. Additionally, the sensitivity of detection can be increased by the application of an ion pairing reagent. Since sodium octanesulfonate was successfully used by Khatri et. al for the detection of gemcitabine in human plasma by RP-HPLC [63], sodium octanesulfonate was also chosen as IPR in this study. The interaction of the polar compound gemcitabine with the non-polar surface of the stationary phase appeared to be too low and gemcitabine consequently eluted out immediately without much retention at 3 minutes. Gemcitabine is a weak base with a pK_b of 10.4, which is predominantly protonated at pH 3, and is thus an excellent target for ion pairing at pH 3. The anion of the ion pairing reagent (octanesulfonate sodium), octanesulfonate, binds to the protonated positively charged analyte and forms a non-ionic compound via ionic interactions and thus increases the hydrophobicity of gemcitabine. As a result, the affinity of the gemcitabine-octanesulfonate ion-pair to the stationary phase of the column is increased and, as a consequence, the analyte interacts stronger with the stationary phase and its retention time increases.
Depending on the concentration of the IPR, the retention of the analyte can be increased [65]. Both peak symmetry and signal intensity may significantly differ depending on IPR concentration.

4.2.3 Influence of IPR Concentration

As shown by our study, increasing the concentration of the IPR octanesulfonate sodium from 1 mM to 11 mM in the mobile phase clearly led to an increase of the retention times of both the internal standard and the analyte gemcitabine. The retention time of 2'-dC could be increased up to 18.5 minutes at an IPR concentration of 11 mM in the mobile phase.
The reason for the increased retention time can be attributed to the increased hydrophobicity of analyte-IPR or internal standard-IPR. Hydrophobic substances show a higher affinity to the stationary RP-Phase and the retention times increase [65].
Since higher concentrations of octanesulfonate sodium resulted in an improved peak symmetry and a longer retention time of both internal standard and gemcitabine, a concentration of 11 mM of the IPR octanesulfonate sodium was used for our further experiments. By applying this concentration, retention times of 27 minutes for gemcitabine and 18 minutes for 2'dC were achieved.
With the increased retention time a better separation of gemcitabine and internal standard from filter matrix components and wipe sample impurities could be obtained. Nevertheless, due to long retention times, the chromatographic column was eventually switched to a shorter type, a Thermo Accucore XL C18 (150mm / ID4.6mm / particle size 4 µm) in order to guarantee a rapid analytical detection method for gemcitabine. By applying the shorter HPLC-column retention times of 9 min for gemcitabine and 6 min for 2'dC were obtained, which proved to be satisfactory for further experiments.

4.2.4 Influence of Absorption Wavelength

Absorption wavelength may have a significant influence on sensitivity of detection of analytes during HPLC chromatography. Therefore, two different absorption wavelengths (267 nm and 275 nm) were tested for our RP-HPLC method. In literature, 267 nm is commonly used in methods for detection of gemcitabine [63, 64]. Our study showed, that the detection wavelength of 275 nm was more beneficial since higher mean peak areas could be obtained. Thus, 275 nm was used as detection wavelength for our method.

4.3 Analysis of Gemcitabine in Wipe Samples

The higher aim of this thesis was the quantitative detection of gemcitabine in real wipe samples. On the basis of our previous results of the detection of pure gemcitabine, the method in real wipe samples was stepwise established and optimized.
After the chromatographic conditions of our method were successfully tested, further experiments on preanalytics were performed. Preanalytics consists of two phases, sample acquisition via wipe-sampling and extraction. As extraction method a SPE approach was chosen. Our goal was to quantitatively collect gemcitabine on surfaces and to further extract the analyte from possible contaminants prior to HPLC analysis.

4.3.1 Influence of Filter Matrix on Interference

An important aspect for the method development is the selection of the optimal filter material. The appropriate filter material should not interfere with the analyte or the internal standard and be chemically inert to solvents used in our study. Therefore, several filter materials, which all consist of alpha-cellulose (Sartorius filter paper, Whatman filter paper, and Schleicher& Schuell filter paper) were tested.
In comparison, all three filter matrices showed a prominent peak at retention time of around 6 minutes. Therefore, they may interfere with the internal standard, which typically appeared at this retention time. Since all three filters are composed of high levels of alpha-cellulose it can be assumed, that the interfering signal may possibly be alpha-cellulose (or fragments thereof), which is dissolved during sample preparation.
However, only the Whatman and the Schleicher& Schuell filters additionally showed a relatively high background noise, which would later complicate quantitative detection of low amounts of gemcitabine. Therefore, we decided to use the Sartorius filter papers for further studies.

4.3.2 Influence of pH Value of Filter Extraction Solution

The next crucial step was the extraction of gemcitabine from the filter material of the wipe sample. In order to achieve a quantitative extraction of gemcitabine from

the filter material, the pH value of the filter extraction solution is essential. Gemcitabine is a weak base with a pK_b of 10.4, the corresponding acid having a pK_a of 3.6. It typically shows a poor stability in moderate alkaline and extreme acidic environments [26]. Therefore, three different aqueous solutions, acidic water at pH 3, deionized water at pH 6 and alkaline water at pH 9, were evaluated. Since the mean peak area represents the amount of the analyte in the sample, the mean peak areas of gemcitabine were analyzed in order to choose the optimal pH value of the extraction solution. Our study clearly demonstrated that different pH values of the filter extraction solution have no impact on the mean peak area of analyzed gemcitabine and therefore no effect on filter extraction process of gemcitabine within the range of pH 3-9. The slight deviations between the solutions can be attributed to measurement artefacts. We decided to use acidified water with a pH value of 3 for further extraction steps, since the following solid phase extraction requires an acidic solution for loading the analyte onto the column.

4.3.3 Extraction of Gemcitabine in Wipe Samples by Solid Phase Extraction (SPE)

To isolate gemcitabine from possible contaminants in the wipe sample a SPE was performed prior to each chromatographic analysis. Additionally, the SPE, which enables a concentration of the sample, may significantly increase the sensitivity of the HPLC-method. SPE makes use of the affinity between the chemical surface of a packed column and an analyte to retain the latter. Thus, several washing steps can be performed, to purify the bound analyte of any impurities and subsequently concentrate it. The bond between the analyte and the surface of the column is subsequently reversed (in this instance via a pH change of the solvent), and the analyte can be eluted freely.
To test the efficiency of the SPE, recovery of gemcitabine before and after SPE was determined. The absence of gemcitabine was additionally confirmed in the washing solutions, which verifies that no analyte losses occur during washing.
A total recovery of gemcitabine after SPE of 98.32 % was achieved. Thus, the SPE is perfectly suitable as purification method for our analyte, since no analyte loss occurred during the purification and isolation process via SPE.

4.3.4 Influence of pH Value of SPE Eluate

Next, the influence of the pH value of the sample on chromatographic parameters after SPE was investigated. Since gemcitabine and 2dC both have primary amino groups Figure 4.2, which can be protonated, an acidic solvent transforms both substances into their ionized form. Positively charged molecules show a higher affinity to the negatively charged ion-pairing reagent sodium octanesulfonate, and the corresponding ion pair has increased lipophilicity and affinity to the stationary phase. A comparison of the peak symmetry and peak area of gemcitabine and internal standard at different pH values was evaluated. Our results clearly demonstrate that different pH values had a crucial impact on both peak symmetry and mean peak area. We assumed that incomplete ionization can have a substantial impact on peak

symmetry and area. A high pH value of pH 5 and 7 leads to poor ionization of gemcitabine and 2dC, thereby reducing mean peak area and worsening peak symmetry. The 2dC peak appears to be deformed, possibly by the hydrophile gemcitabine, which elutes at a lower retention time and overlaps with the area of the internal standard.
In an extremely acidic environment at pH 1, gemcitabine is not stable and thus detection is poor [26]. The optimal pH value was discovered to be at a pH value of 3. At pH 3, both gemcitabine and 2dC showed a distinct signal with a peak symmetry between 0.9 and 1.1. However, a minor tailing effect is visible. Consequently, for further method development, the pH of the SPE eluate was adjusted to 3 before injection onto the column.

4.4 Outpatient Clinic Surface Measurements

After finalisation of our HPLC Method and Standard Operation Procedure, real wipe samples were taken at an outpatient clinic for oncology. Standard cytostatic therapy regimes for cancer include a variety of chemotherapeutic drugs, i.e. taxanes, platin derivates, alkylating agents. In this study, it should be verified whether the developed method for gemcitabine is applicable in an environment full of antineoplastic chemicals that could act as potential contaminants. To test our developed method for the detection of gemcitabine in wipe samples under real work conditions, outpatient clinic surface measurements were performed at various sampling sites. Compared to the samples analyzed under laboratory conditions, the gemcitabine peak in real life samples showed no interference with other cytostatic drugs or surface contaminants. The peak for gemcitabine was always symmetrical and distinct, with no other substances influenced detection or sensitivity. Considering that the prospect of the presence of other cytostatic drug in an outpatient setting is high, it can be assumed that our method and preanalytics facilitate a selective detection of gemcitabine under real world conditions. Thus, our developed method is applicable at real working conditions and we could successfully detect gemcitabine in outpatient clinics.

As expected, the highest concentration of the analyte was detected directly at the preparation site in the isolator. Besides that, traces of gemcitabine could also be detected in the patient recreation area and the patient bathroom floor. Gemcitabine is a hydrophile antineoplastic drug that is almost quantitatively eliminated by the kidneys either as unmetabolized substance (~10 %) or as uracil metabolite 2',2'-difluoro-deoxyuridine (dFdU) [23]. Therefore, a possible contamination in the patient restrooms may be likely. Our finding confirms these results. Additionally, the patient recreation area was also tested positive for gemcitabine, which could be an indication for unsafe practices during application of chemotherapy by staff as well as contamination of surfaces by skin contact to furniture. Similar findings have been published before in the study of Kopp et. al. [30] No traces of gemcitabine were found in work benches, printer, floors, and the reception desk of the outpatient clinics. Either these location sites are not contaminated with Gemcitabine or the developed detection method in wipe samples with an LOD of 0.091 µg/ml is not sensitive enough to detect traces of gemcitabine. To determine traces of gemcitabine or to exclude any contamination with gemcitabine on those sites, a mass spectrometry-based method with higher sensitivity could be applied.

Work place contamination with cytostatic drugs is a well known problem in clinics

and private practices. However, analytical studies about workplace contaminations in outclinic patient settings is rather scarce. A summary of the recent studies about workplace contamination with gemcitabine in outpatient clinic settings using wipe samples is listed in Table 4.1. Kopp et al., applied a LC-MS/MS method for the detection of several antineoplastic drugs including gemcitabine on workplaces using wipe samples [30]. They demonstrated that around 60 % of forty assessed institutions were tested positive for the contamination of one or more wipe samples, containing one or more of the following agents (5-fluorouracil, platinum, gemcitabine, ifosfamide, methotrexate, docetaxel, paclitaxel). For gemcitabine, in 64 % of the samples contamination was detected, with a median of 1.2 pg/cm^2. Since our method is based on UV detection and Kopp et al. used a MS/MS based detection method, their LOD is lower compared to our developed method. Hence our method is only suitable for higher concentrations of gemcitabine. The specific distribution of gemcitabine contaminated samples also varied from our findings. Whereas our outpatient clinic samples tested positive in the preparation area for antineoplastic agents (cytostatic isolator) and showed a high sample contamination of 1326 pg/cm^2, the most contaminated sampling site in other clinics was the floor, with a 75th percentile of 88.6 pg/cm^2. Whereas in our real samples traces of gemcitabine were detectably in the patient restrooms, Kopp et al. could not detect any contamination of gemcitabine in the patient restrooms.
In the ESOP study, wipe samples for the contamination of cytotoxic drugs in 15 European hospitals were tested and measured by LC/MS/MS. Measurable amounts of at least one agent were detected on sampled surfaces in each hospital. The highest level of drug contamination was detected on isolators and floors (in pharmacies and wards) as well as the armrests of patient's chairs. These findings are in accordance with our results [66].

TABLE 4.1: Overview of gemcitabine concentrations at different workplaces

Detection system, Literature	**Surface contamination [ng/cm^2]: median / maximum**	**Sampling sites**
GC/MS, [30]	0.0012 / 750	Outpatient oncology health care settings (n=28)
LC/MS/MS, [67]	5 / 600	Outpatient infusion center
LC/MS/MS,[66]	0.003 / 171	Hospitals (n=15)
LC/HPLC, [68]	- / 37	Pharmacy cytotoxic compounding section, oncology wards.
HPLC-MS/MS, [69]	- / 1,888	Pharmacies (n=130)

Chapter 5

Summary and Outlook

Antineoplastic drugs such as gemcitabine play a crucial role in cancer therapy and are widely used in clinics and outpatient clinical settings.

Since a vast majority of antineoplastic drugs are classified as carcinogenic, mutagenic and teratogenic for humans, a minimization of contamination with antineoplastic drugs such as gemcitabine is essential to reduce health risks for employees in clinics and outclinic patient settings. The aim of this study was to develop a sensitive HPLC method for the detection of low levels of gemcitabine in wipe samples. Wipe sampling enables the assessment of exposures caused by surface contamination and can be also used to verify the effectiveness of decontamination procedures. In this thesis, a wipe sampling procedure coupled to RP-HPLC was developed for the determination of gemcitabine. The procedure includes a prior solid phase extraction process for purification and concentration of gemcitabine in the wipe sample. Various chromatographic parameters as well as the purification process were tested and further optimized.

Finally, the developed method was successfully applied for the determination of surface contamination in an outpatient clinical oncology setting.

LC-MS/MS is a highly sensitive method, which enables the detection of low amounts of drug contamination. However, monitoring by HPLC-UV is a cheap, broadly applicable, and robust technique, which might be especially beneficial for third world countries. Future work should be focused on the monitoring of other relevant antineoplastic drugs such as paclitaxel to reduce the potential risk of contamination in work surfaces.

Bibliography

1. Siegel Rebecca L., Miller Kimberly D. & Jemal Ahmedin. Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians* **68,** 7–30 (Jan. 4, 2018).
2. Chabner, B. A. & Roberts, T. G. Chemotherapy and the war on cancer. *Nature Reviews Cancer* **5,** 65–72 (Jan. 1, 2005).
3. Ramphal, R., Bains, T., Goulet, G. & Vaillancourt, R. Occupational exposure to chemotherapy of pharmacy personnel at a single centre. *The Canadian journal of hospital pharmacy* **68,** 104–112. JSTOR: 25964681 (2015).
4. Connor, T. H., Lawson, C. C., Polovich, M. & McDiarmid, M. A. Reproductive health risks associated with occupational exposures to antineoplastic drugs in health care settings: a review of the evidence. *Journal of occupational and environmental medicine* **56,** 901–910. JSTOR: 25153300 (Sept. 2014).
5. Landeck, L., Gonzalez, E. & Koch, O. M. Handling chemotherapy drugs—Do medical gloves really protect? *International Journal of Cancer* **137,** 1800–1805. (2019) (Oct. 15, 2015).
6. Colombo, M., Jeronimo, M., Astrakianakis, G., Apte, C. & Hon, C.-Y. Wipe Sampling Method and Evaluation of Environmental Variables for Assessing Surface Contamination of 10 Antineoplastic Drugs by Liquid Chromatography/Tandem Mass Spectrometry. *Annals of Work Exposures and Health* **61,** 1003–1014. (2019) (Aug. 3, 2017).
7. DeVita, V. T. & Chu, E. A History of Cancer Chemotherapy. *Cancer Research* **68,** 8643 (Nov. 1, 2008).
8. Alderden, R. A., Hall, M. D. & Hambley, T. W. The Discovery and Development of Cisplatin. *Journal of Chemical Education* **83,** 728 (May 1, 2006).
9. Bertino, J. R. in *Methotrexate* (eds Cronstein, B. N. & Bertino, J. R.) 1–7 (Birkhäuser Basel, Basel, 2000).
10. Rich, T. A., Shepard, R. C. & Mosley, S. T. Four Decades of Continuing Innovation With Fluorouracil: Current and Future Approaches to Fluorouracil Chemoradiation Therapy. *Journal of Clinical Oncology* **22,** 2214–2232 (June 1, 2004).
11. Falck, K., Gröhn, P., Sorsa, M., Vainio, H., Heinonen, E. & Holsti, L. Mutagenicity in urine of nurses handling cytostatic drugs. *The Lancet* **313,** 1250–1251 (June 6, 1979).
12. Connor Thomas H. Hazardous Anticancer Drugs in Health Care. *Annals of the New York Academy of Sciences* **1076,** 615–623 (Oct. 5, 2006).
13. S Baker, E. & H Connor, T. *Monitoring occupation exposure to cancer chemotherapy drugs* 2713 pp. (Dec. 1, 1996).
14. Graeve, C. U., McGovern, P. M., Alexander, B., Church, T., Ryan, A. & Polovich, M. Occupational Exposure to Antineoplastic Agents: An Analysis of Health Care Workers and Their Environments. *Workplace Health & Safety* **65,** 9–20 (Oct. 6, 2016).

15. Lawson, C. C., Rocheleau, C. M., Whelan, E. A., Lividoti Hibert, E. N., Grajewski, B., Spiegelman, D. & Rich-Edwards, J. W. Occupational exposures among nurses and risk of spontaneous abortion. *American Journal of Obstetrics & Gynecology* **206,** 327.e1–327.e8.

16. Villarini, M., Dominici, L., Piccinini, R., Fatigoni, C., Ambrogi, M., Curti, G., Morucci, P., Muzi, G., Monarca, S. & Moretti, M. Assessment of primary, oxidative and excision repaired DNA damage in hospital personnel handling antineoplastic drugs. *Mutagenesis* **26,** 359–369 (May 1, 2011).

17. Skov, T., Maarup, B., Olsen, J., Rørth, M., Winthereik, H. & Lynge, E. Leukaemia and Reproductive Outcome among Nurses Handling Antineoplastic Drugs. *British Journal of Industrial Medicine* **49,** 855–861 (1992).

18. Hon, C.-Y., Teschke, K., A Demers, P. & Venners, S. *Antineoplastic Drug Contamination on the Hands of Employees Working Throughout the Hospital Medication System* (Mar. 18, 2014).

19. Connor Thomas H. & McDiarmid Melissa A. Preventing Occupational Exposures to Antineoplastic Drugs in Health Care Settings. *CA: A Cancer Journal for Clinicians* **56,** 354–365 (Dec. 31, 2008).

20. Turci, R., Sottani, C., Spagnoli, G. & Minoia, C. Biological and environmental monitoring of hospital personnel exposed to antineoplastic agents: a review of analytical methods. *Journal of Chromatography B* **789,** 169–209 (June 15, 2003).

21. Nussbaumer, S., Geiser, L., Sadeghipour, F.-s., Hochstrasser, D., Bonnabry, P., Veuthey, J.-L. & Fleury-Souverain, S.-d. Wipe sampling procedure coupled to LC–MS/MS analysis for the simultaneous determination of 10 cytotoxic drugs on different surfaces. *Analytical and Bioanalytical Chemistry* **402,** 2499–2509 (Mar. 1, 2012).

22. Schmaus, G., Schierl, R. & Funck, S. Monitoring surface contamination by antineoplastic drugs using gas chromatography-mass spectrometry and voltammetry. *American Journal of Health-System Pharmacy* **59,** 956–961. (2019) (May 15, 2002).

23. Ciccolini, J., Serdjebi, C., Peters, G. J. & Giovannetti, E. Pharmacokinetics and pharmacogenetics of Gemcitabine as a mainstay in adult and pediatric oncology: an EORTC-PAMM perspective. *Cancer Chemotherapy and Pharmacology* **78,** 1–12. JSTOR: {PMC}4921117 (2016).

24. Toschi, L., Finocchiaro, G., Bartolini, S., Gioia, V. & Cappuzzo, F. Role of gemcitabine in cancer therapy. *Future Oncology* **1,** 7–17. (2019) (Feb. 1, 2005).

25. Wong, A., Soo, R. A., Yong, W.-P. & Innocenti, F. Clinical pharmacology and pharmacogenetics of gemcitabine. *Drug Metabolism Reviews* **41,** 77–88 (May 1, 2009).

26. Rahul, S., Ashok, S., Rajashri, N. & Naeem, S. Stability-Indicating HPLC Determination of Gemcitabine in Pharmaceutical Formulations. *International Journal of Analytical Chemistry* **2015,** 12 (2015).

27. Pourquier, P., Gioffre, C., Kohlhagen, G., Urasaki, Y., Goldwasser, F., Hertel, L. W., Yu, S., Pon, R. T., Gmeiner, W. H. & Pommier, Y. Gemcitabine (2,2-Difluoro-2-Deoxycytidine), an Antimetabolite That Poisons Topoisomerase I. *Clinical Cancer Research* **8,** 2499 (Aug. 1, 2002).

28. De Sousa Cavalcante, L. & Monteiro, G. Gemcitabine: Metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. *European Journal of Pharmacology* **741,** 8–16 (Oct. 15, 2014).

29. Mini, E., Nobili, S., Caciagli, B., Landini, I. & Mazzei, T. Cellular pharmacology of gemcitabine. *Annals of Oncology* **17,** v7–v12 (suppl_5 May 1, 2006).

30. Kopp, B., Schierl, R. & Nowak, D. Evaluation of working practices and surface contamination with antineoplastic drugs in outpatient oncology health care settings. *International Archives of Occupational and Environmental Health* **86,** 47–55 (Jan. 1, 2013).

31. Nakashima, M., Adachi, S., Yasuda, I., Yamauchi, T., Kawaguchi, J., Itani, M., Yoshioka, T., Matsushima-Nishiwaki, R., Hirose, Y., Kozawa, O. & Moriwaki, H. Phosphorylation status of heat shock protein 27 plays a key role in gemcitabine-induced apoptosis of pancreatic cancer cells. *Cancer Letters* **313,** 218–225 (Dec. 27, 2011).

32. Hodge, L. S., Taub, M. E. & Tracy, T. S. The Deaminated Metabolite of Gemcitabine, 2,2-Difluorodeoxyuridine, Modulates the Rate of Gemcitabine Transport and Intracellular Phosphorylation via Deoxycytidine Kinase. *Drug Metabolism and Disposition* **39,** 2013 (Nov. 1, 2011).

33. Peters, G., Clavel, M., Noordhuis, P., Geyssen, G., Laan, A., Guastalla, J., Edzes, H. & Vermorken, J. Clinical Phase I and Pharmacology Study of Gemcitabine (2′, 2′-Difluorodeoxycytidine) Administered in a Two-Weekly Schedule. *Journal of Chemotherapy* **19,** 212–221 (Apr. 1, 2007).

34. De Lange, S. M., van der Born, K., Kroep, J. R., Jensen, H. A., Pfeiffer, P., Cleverly, A., van Groeningen, C. J. & Peters, G. J. No evidence of gemcitabine accumulation during weekly administration. *European Journal of Clinical Pharmacology* **61,** 843–849 (Dec. 1, 2005).

35. Scharadin, T. M., Zhang, H., Zimmermann, M., Wang, S., Malfatti, M. A., Cimino, G. D., Turteltaub, K., de Vere White, R., Pan, C.-x. & Henderson, P. T. Diagnostic Microdosing Approach to Study Gemcitabine Resistance. *Chemical Research in Toxicology* **29,** 1843–1848 (Nov. 21, 2016).

36. Van Nuland, M., Hillebrand, M., Rosing, H., Burgers, J., Schellens, J. & Beijnen, J. Ultra-sensitive LC–MS/MS method for the quantification of gemcitabine and its metabolite 2,2-difluorodeoxyuridine in human plasma for a microdose clinical trial. *Journal of Pharmaceutical and Biomedical Analysis* **151,** 25–31 (Mar. 20, 2018).

37. Böhlandt, A. & Schierl, R. Benefits of Wipe Sampling: Evaluation of Long-Term 5-Fluorouracil and Platinum Monitoring Data. *Pharmaceutical Technology in Hospital Pharmacy* **1,** 139–150. (2019) (2016).

38. Menon, S. K., Mistry, B. R., Joshi, K. V., Sutariya, P. G. & Patel, R. V. Analytical detection and method development of anticancer drug Gemcitabine HCl using gold nanoparticles. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **94,** 235–242 (Aug. 1, 2012).

39. Najafi, S., Amani, S. & Shahlaei, M. Rapid determination of the anti-cancer agent Gemcitabine in biological samples by fluorescence sensor based on Au-doped CdTe. *Journal of Molecular Liquids* **266,** 514–521 (Sept. 15, 2018).

40. Kozo, D., Ross, M. W., Jarrah, J., Barrett, M., Harney, R. L., Courtney, J. B., Baburina, I., Holleran, J. L., Beumer, J. H., Peters, G. J., Honeywell, R. J. & Salamone, S. J. Rapid Homogeneous Immunoassay to Quantify Gemcitabine in Plasma for Therapeutic Drug Monitoring. *Therapeutic Drug Monitoring* **39** (2017).

41. Sottani, C., Turci, R., Schierl, R., Gaggeri, R., Barbieri, A., Violante, F. & Minoia, C. Simultaneous determination of gemcitabine, taxol, cyclophosphamide and ifosfamide in wipe samples by high-performance liquid chromatography/tandem mass spectrometry: Protocol of validation and uncertainty of measurement. **21,** 1289–1296 (Apr. 15, 2007).

42. Tuerk, J., Kiffmeyer, T. K., Hadtstein, C., Heinemann, A., Hahn, M., Stuetzer, H., Kuss, H.-M. & Eickmann, U. Development and validation of an LC–MS/MS procedure for environmental monitoring of eight cytostatic drugs in pharmacies. *International Journal of Environmental Analytical Chemistry* **91,** 1178–1190 (Oct. 1, 2011).

43. Hetzel, T., vom Eyser, C., Tuerk, J., Teutenberg, T. & Schmidt, T. C. Microliquid chromatography mass spectrometry for the analysis of antineoplastic drugs from wipe samples. *Analytical and Bioanalytical Chemistry* **408,** 8221–8229 (Nov. 1, 2016).

44. Fleury-Souverain, S., Mattiuzzo, M., Mehl, F., Nussbaumer, S., Bouchoud, L., Falaschi, L., Gex-Fabry, M., Rudaz, S., Sadeghipour, F. & Bonnabry, P. Evaluation of chemical contamination of surfaces during the preparation of chemotherapies in 24 hospital pharmacies. *European Journal of Hospital Pharmacy* **22,** 333 (Nov. 1, 2015).

45. Freeman, K. B., Anliker, S., Hamilton, M., Osborne, D., Dhahir, P. H., Nelson, R. & Allerheiligen, S. R. Validated assays for the determination of gemcitabine in human plasma and urine using high-performance liquid chromatography with ultraviolet detection. *Journal of Chromatography B: Biomedical Sciences and Applications* **665,** 171–181 (Mar. 10, 1995).

46. Sottani Cristina, Zucchetti Massimo, Zaffaroni Marco, Bettinelli Maurizio & Minoia Claudio. Validated procedure for simultaneous trace level determination of the anti-cancer agent gemcitabine and its metabolite in human urine by high-performance liquid chromatography with tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **18,** 1017–1023 (Apr. 8, 2004).

47. Lin, N.-m., Zeng, S., Ma, S.-l., Fan, Y., Zhong, H.-j. & Fang, L. *Determination of gemcitabine and its metabolite in human plasma using high-pressure liquid chromatography coupled with a diode array detector* 1584 pp. (Dec. 1, 2004).

48. Lanz Christian, Früh Martin, Thormann Wolfgang, Cerny Thomas & Lauterburg Bernhard H. Rapid determination of gemcitabine in plasma and serum using reversed-phase HPLC. *Journal of Separation Science* **30,** 1811–1820 (July 23, 2007).

49. Ettre, L. S. Nomenclature for chromatography (IUPAC Recommendations 1993). *Pure and Applied Chemistry* **65,** 819–872 (1993).

50. Snyder, L. R. Peer Reviewed: HPLC: Past and Present. *Analytical Chemistry* **72,** 412 A–420 A (June 1, 2000).

51. Braithwaite, A. & Smith, J. *Chromatographic Methods* 5th ed. 580 pp. ISBN: 978-0-7514-0158-5 (Springer Netherlands, 1999).

52. McCalley David V. Selection of suitable stationary phases and optimum conditions for their application in the separation of basic compounds by reversed-phase HPLC. *Journal of Separation Science* **26,** 187–200 (Mar. 10, 2003).

53. Meyer, V. in *Practical High-Performance Liquid Chromatography* 4th Edition, 195–201 (Mar. 8, 2006).

54. Neue, U. A Review of: HPLC Columns, Theory, Technology, and Practice. *Journal of Liquid Chromatography & Related Technologies* **21,** 910–912 (Mar. 1, 1998).

55. Neue, U. D., Alden, B. A., Grover, E. R., Grumbach, E. S., Iraneta, P. C. & Méndez, A. in *Separation Science and Technology* (eds Ahuja, S. & Rasmussen, H.) 45–83 (Academic Press, Jan. 1, 2007). ISBN: 1877-1718.

56. Swartz, M. HPLC DETECTORS: A BRIEF REVIEW. *Journal of Liquid Chromatography & Related Technologies* **33,** 1130–1150 (July 13, 2010).

57. Wang, M., Wang, C. & Han, X. Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry-What, how and why? *Mass spectrometry reviews* **36,** 693–714. JSTOR: 26773411 (Nov. 2017).

58. Dorsey, J. G., Cooper, W. T., Siles, B. A., Foley, J. P. & Barth, H. G. Liquid Chromatography: Theory and Methodology. *Analytical Chemistry* **70,** 591–644 (June 1, 1998).

59. Poole, C. in *Encyclopedia of Separation Science* (ed Wilson, I. D.) 1405–1416 (Academic Press, Oxford, Jan. 1, 2000). ISBN: 978-0-12-226770-3.

60. Wells, D. A. *Chapter 12 Solid-phase extraction: Strategies for method development and optimization* (ed Wells, D. A.) Jan. 1, 2003.

61. Majors, R. E. *Sample Preparation Fundamentals for Chromatography* https://www.agilent.com/cs/library/primers/Public/5991-3326EN_SPHB.pdf (2014).

62. Nickerson, B. & Colón, I. in *Sample Preparation of Pharmaceutical Dosage Forms: Challenges and Strategies for Sample Preparation and Extraction* (ed Nickerson, B.) 63–92 (Springer US, Boston, MA, 2011). ISBN: 978-1-4419-9631-2.

63. Khatri, A., Fisher, J. E. & Kirstein, M. N. Enhanced Sensitivity Method for Measuring Gemcitabine in Human Plasma. *Chromatographia* **72,** 1005–1008 (Nov. 1, 2010).

64. Kirstein, M. N., Hassan, I., Guire, D. E., Weller, D. R., Dagit, J. W., Fisher, J. E. & Remmel, R. P. High-performance liquid chromatographic method for the determination of gemcitabine and 2,2-difluorodeoxyuridine in plasma and tissue culture media. *Journal of Chromatography B* **835,** 136–142 (May 1, 2006).

65. Cecchi, T. Ion Pairing Chromatography. *Critical Reviews in Analytical Chemistry* **38,** 161–213 (Aug. 1, 2008).

66. Korczowska, E., Jankowiak-Gracz, H., Tuerk, J., Hetzel, T. & Meier, K. PP-001Contamination with cytotoxic drugs in the workplace – ESOP pilot study. *European Journal of Hospital Pharmacy* **23,** A195 (Suppl 1 Mar. 1, 2016).

67. West, C. & Beaucham. *Evaluation of Chemotherapy Drug Exposure in an Outpatient Infusion Center* https://www.cdc.gov/niosh/hhe/reports/pdfs/2013-0019-3205.pdf.

68. Lee Su-Gil, Tkaczuk Michael, Jankewicz Ganyk & Ambados Fotios. Surface Contamination from Cytotoxic Chemotherapy Following Preparation and Administration. *Journal of Pharmacy Practice and Research* **37,** 271–276. (2018) (Apr. 13, 2015).

69. Kiffmeyer, T. K., Tuerk, J., Hahn, M., Stuetzer, H., Hadtstein, C., Heinemann, A. & Eickmann, U. Application and Assessment of a Regular Environmental Monitoring of the Antineoplastic Drug Contamination Level in Pharmacies - The MEWIP Project. *The Annals of Occupational Hygiene* **57,** 444–455 (May 1, 2013).

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