TRACE ANALYSIS OF CYCLOPHOSPHAMIDE AND ITS METABOLITES IN URINE BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

by

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Abstract

Cyclophosphamide (CP) is an antineoplastic drug used to treat a wide variety of cancers and immune disorders. CP is also a highly toxic alkylating agent, classified as an IARC Group 1 carcinogen. Workers in health-care environments are vulnerable to occupational exposure to CP, primarily via inhalation and dermal absorption. CP is a prodrug; both its therapeutic effectiveness and toxicity are activated through metabolism. To date, however, no study measuring occupational exposure to CP has successfully analyzed its metabolites. The main objective of this work was to develop an analytical method for CP, as well as its metabolites 4-ketocyclophosphamide (KCP) and carboxyphosphamide (CBP), in urine samples collected from health-care workers at risk of CP exposure.

A liquid chromatography-tandem mass spectrometry (LC/MS-MS) method was optimized for CP, KCP and CBP on two different instruments. Post-column infusion showed that the matrix effects resulting from synthetic urine could be separated from the analyte peaks by LC. Estimated instrument limits of quantitation for CP, KCP and CBP in neat solvent were respectively 4.2, 8.2 and 57 ng/L. These parameters were sufficient to meet a quantitation target of 50 ng/L CP in urine, but suggested a need to reduce sample volume to reach a 2.5 ng/L target for KCP and CBP.

Solid-phase extraction (SPE) was explored as a means to exchange the sample matrix for clean solvent and reduce sample volume. Previously developed SPE methods for CP were not designed to include the more polar metabolites, and thus required modification. The best retention of metabolites was seen on a C_{18} sorbent with reduced carbon loading. Retention was improved further under acidic loading conditions, but this had to be controlled carefully since CBP can decompose more rapidly at acidic pH. All three analytes were observed to elute with methanol.

Preface

This study was carried out at the University of British Columbia, as a collaboration between the research groups of Dr. Michael Blades from the Department of Chemistry, and Dr. Winnie Chu from the School of Environmental Health (SoEH). The analysis method developed here was to be applied towards the analysis of urine samples collected as part of Chun-Yip Hon's graduate research, under the supervision of Dr. Kay Teschke at SoEH. No actual biological samples were collected or analyzed for the work presented here, as the present study's scope was limited to analytical method development.

I was responsible for the work presented here, including method development, analytical equipment operation, data analysis and manuscript writing. Experiments were carried out using the facilities available at both SoEH and the Department of Chemistry. Funding for the research was provided by Dr. Michael Blades, Dr. Winnie Chu, and the Natural Sciences and Engineering Research Council of Canada (NSERC).

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Abbreviations

ACN	acetonitrile
ADME	absorption, distribution, metabolism and excretion
ALARA	as low as reasonably achievable
ALDH	aldehyde dehydrogenase
AldoP	aldophosphamide (**Figure 1.2**)
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
CBP	carboxyphosphamide (**Figure 1.2**)
CP	cyclophosphamide (**Figure 1.1**)
CYP	cytochrome P450
d_4-CP	deuterated cyclophosphamide
ESI-MS	electrospray ionization-mass spectrometry
GC	gas chromatography
GSH	glutathione
HN2	trichlormethine (**Figure 1.1**)
HN3	methyl-bis(2-chloroethyl)amine (**Figure 1.1**)
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
IF	ifosfamide (**Figure 1.6**)
KCP	4-ketocyclophosphamide (**Figure 1.2**)
KHP	potassium hydrogen phthalate
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
log D	logarithm of octanol-water distribution coefficient
LOQ	limit of quantitation

m/z	mass-to-charge ratio
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry *or* mass spectrometer
MS/MS	tandem mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
NNM	nor-nitrogen mustard (**Figure 1.4**)
NPD	nitrogen-phosphorus detection
Nuc	nucleophile
OEL	occupational exposure limit
OHCP	4-hydroxycyclophosphamide (**Figure 1.2**)
PBS	phosphate buffered saline
PM	phosphoramide mustard (**Figure 1.1**)
QC	quality control
S/N	signal-to-noise ratio
SAX	strong anion exchange
SoEH	School of Environmental Health, University of British Columbia
SPE	solid-phase extraction
TFAA	trifluoroacetic anhydride
XIC	extracted ion chromatogram

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I must thank Derek Smith and Dr. Robin Stoodley, who have patiently entertained my endless questions about thesis writing, and provided help with editing and proofreading. I also offer my gratitude to Chun-Yip Hon, who helped to review the occupational health component of this report; and to the many members of the Chu group who have opened my mind to possibilities that lie beyond my field of study. Special thanks go out to Dr. Nikolay Stoynov of the Centre for High-Throughput Biology for providing access to data analysis software on short notice.

To all the friends I have made at UBC and throughout Vancouver, and to my friends who are geographically distant but always close to my heart, I couldn't have done it without you.

Last but not least, I give thanks to my family for their unwavering confidence and for the many ways in which they have supported me.

1. Introduction

In this section, a brief overview is provided of antineoplastic drugs, with a focus on the history and development of cyclophosphamide (CP). The metabolic fate of CP is presented, and the risks of occupational exposure to CP are discussed. Literature methods for analysis of CP and metabolites are reviewed, and background information is presented for the analytical methods utilized in this study.

1.1. Antineoplastic drugs

According to the British Columbia Cancer Agency's statistics, there were 492 new cancer diagnoses per 100,000 residents in 2007, and 202 cancer deaths per 100,000 residents. Cancer mortality rates decreased from 1980 to 2005 in all adult age groups except for women over 65, but adult incidence rates have not seen any comparable decrease [1]. With a growing population, an escalating number of patients are entering the health care system for cancer treatments such as radiation therapy and chemotherapy.

In its broadest sense, *chemotherapy* refers to the use of chemicals to treat any illness, but it most commonly refers to cancer treatments. *Cytotoxic agents* have a toxic effect on cells, regardless of whether any beneficial applications exist. A subset of these are *antineoplastic agents*, used to prevent or inhibit the maturation and proliferation of cancerous cells in abnormal tissue (neoplasm), typically by targeting the DNA [2].

Antineoplastic drugs can be classified based on their mode of action. The categories include alkylating agents, antimetabolites, mitotic spindle inhibitors, hormone-related drugs, antibiotics, and miscellaneous drugs with rarer mechanisms. *Alkylating agents* have electrophilic functionalities which form covalent bonds with endogenous nucleophilic functional groups such as those found on proteins, RNA and DNA [2]. Some of these compounds have two or more alkylating functionalities, which can create crosslinks between biological molecules.

Some of the earliest known antineoplastics were the alkylating agents tris-(2-chloroethyl)amine ("HN2", trichlormethine) and methyl-bis(2-chloroethyl)amine ("HN3", mechlorethamine), nitrogen mustards used during World War I (**Figure 1.1**). Subsequent research suggested these mustards had cytotoxic properties useful in cancer treatment [3, 4]. Of course, an ideal treatment should aim to minimize harm to the patient while maximizing therapeutic effectiveness; on the contrary, HN2 and HN3 were designed to be powerfully toxic chemical warfare agents.

One way to reduce drug toxicity is to design a *prodrug*, a less toxic precursor molecule which becomes pharmacologically active after metabolic transformations. A 1948 study suggested high levels of phosphamidase enzyme activity in malignant cells [5]. This led to a hypothesis that a phosphorylated nitrogen mustards could undergo enzymatic hydrolysis of the P—N bond in cancerous cells, liberating the nitrogen mustard as an active metabolite [6]. Phosphoramide mustard (PM, **Figure 1.1**) was thus synthesized in 1954 [7]; during the same decade, a number of additional phosporylated mustards were synthesized at the Asta-Werke Company in Germany. Among these, in 1958 cyclophosphamide (CP, **Figure 1.1**) was reported to have the highest therapeutic index – a measure of the drug's effectiveness relative to its toxicity [8]. Later studies revealed PM to be cytotoxic *in vitro*, but ineffective and highly toxic in clinical trials [9, 10]. In 1959, CP was approved by the United States Food and Drug Administration [11].

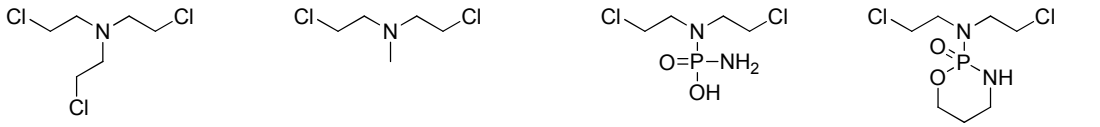

Figure 1.1: Structures of alkylating agents trichlormethine (HN2), mechlorethamine (HN3), phosphoramide mustard (PM) and cyclophosphamide (CP). Each contains the characteristic bis(chloroethyl)amine (nitrogen mustard) functional group.

1.2. Cyclophosphamide

A recent review paper names cyclophosphamide as one of the most effective anti-cancer drugs ever synthesized [11]. CP is sold under the brand names Cytoxan®, Neosar® and Procytox®, available both as tablets and for injection. It is used both alone and in combination with other antineoplastics for breast cancer, leukemias, lung cancer, lymphomas, multiple myeloma, ovarian cancer, and other cancers. CP also has non-cancer uses for severe active rheumatoid arthritis, as an immunosuppressant, and in conditioning regimens for bone marrow transplant patients [12].

1.2.1. Metabolic fate

In studies of drug metabolism, the acronym ADME is used to indicate four stages of disposition of a compound: Absorption, Distribution, Metabolism and Excretion. The parent compound is absorbed into the bloodstream by intravenous injection; or if administered orally, it also has the opportunity to undergo first-pass metabolism before entering the bloodstream. In the distribution stage, the drug leaves the bloodstream and can enter tissue cells to interact with receptors and other macromolecules. At any of these sites, the drug can be metabolized by interacting with enzymes [13].

Phase I metabolism consists of simple chemical transformations which serve to detoxify the drug or promote further metabolism. These include oxidation, reduction and hydrolysis, which can each introduce or expose reactive functionalities such as hydroxyl, thiol, amine and carboxylate groups. In phase II metabolism, these functionalities are conjugated with endogenous molecules to create products such as glucuronides, sulfates, amides, esters and glutathione adducts. Some detoxication reactions such as acylation and methylation result in greater lipophilicity; however, most metabolic transformations serve to increase a molecule's hydrophilicity so that it can be excreted more easily [13].

Cyclophosphamide is a prodrug which activates through metabolism, and has a terminal half-life of 2-12 h [14]. An outline of its phase I metabolism is given in **Figure 1.2**. CP

has a minor inactivation pathway in which a cytochrome P450 enzyme (CYP) releases inactive dechloroethyl-CP and the neurotoxic agent chloroacetaldehyde by oxidative dealkylation of CP [15]. The competing activation pathway is a CYP oxidation of CP to form 4-hydroxycyclophosphamide (OHCP), mainly in the liver. There is also a recognized autoinduction effect: CP administration leads to an increase in CYP enzymes, which in turn accelerates metabolism of CP [16].

The metabolite OHCP is in equilibrium with its open-chain tautomer aldophosphamide (AldoP). Both of these have inactivation pathways: OHCP can oxidize further via CYP to 4-ketocyclophosphamide (KCP), and AldoP can oxidize via aldehyde dehydrogenase (ALDH) to carboxyphosphamide (CBP). These enzymatic mechanisms compete with the spontaneous β-elimination from AldoP to form the alkylating agent phosphoramide mustard (PM), and the highly reactive byproduct acrolein [17]. The OHCP/AldoP tautomeric pair is often referred to as OHCP for simplicity.

As noted above, PM alone was found ineffective as a cancer drug in clinical trials; ultimately this is because PM is mostly ionized at physiological pH and unable to enter cells. Conversely OHCP enters cells readily, and thus acts as a transport molecule to form intracellular and even intratumoural PM [17]. Cells with high levels of ALDH, such as bone marrow cells, are less vulnerable since the enzyme can deactivate OHCP, preventing the formation of an active alkylating agent [6]. There is also evidence that CBP is produced locally within the kidney, and excreted in the urine without returning to circulation [18]. About 20% of CP excretes unchanged [19].

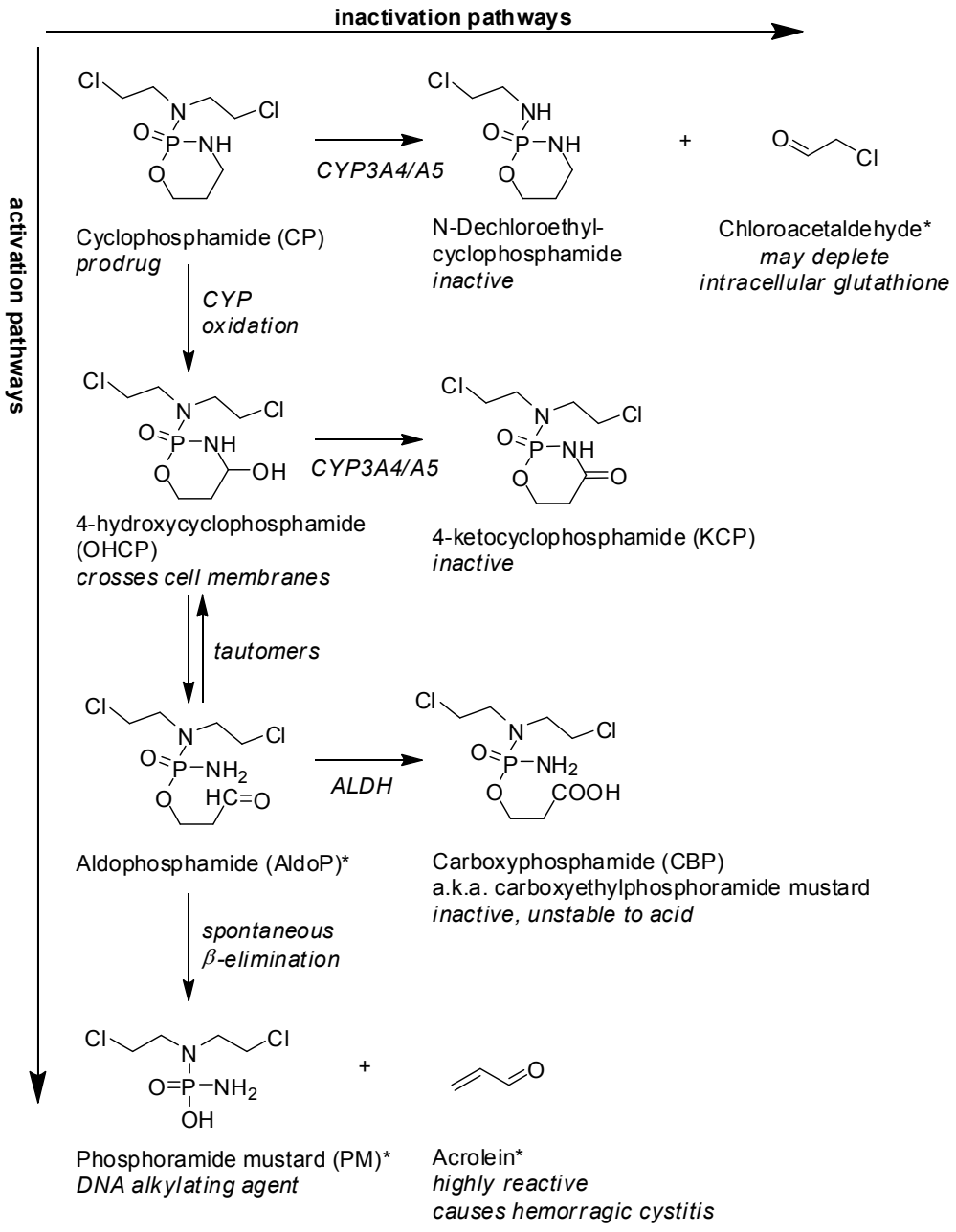

Figure 1.2: Phase I metabolism of cyclophosphamide. Adapted from [17] with additional information from [11, 15, 20].
** asterisk denotes electrophiles which may conjugate with glutathione.*

Nitrogen mustards such as PM are powerful alkylating agents due to an intramolecular cyclization reaction, in which the nitrogen atom displaces chlorine to form a 3-membered aziridinium cation (**Figure 1.3**). Endogenous nucleophiles such as guanine from DNA can easily attack the strained ring and form strong covalent bonds. With a second chloroethyl group available, a DNA-DNA crosslink can be formed. A similar mechanism can also produce crosslinks with RNA and proteins [11]. In the case of PM, the nitrogen is a weaker nucleophile than an amine due to the electron-withdrawing P=O group; but the deprotonation of phosphate compensates for this decrease and stabilizes the energy barrier to chloride loss. The parent drug CP, a cyclic ester, has no phosphate proton to remove; therefore cyclization of CP is much less favourable without further metabolism [21].

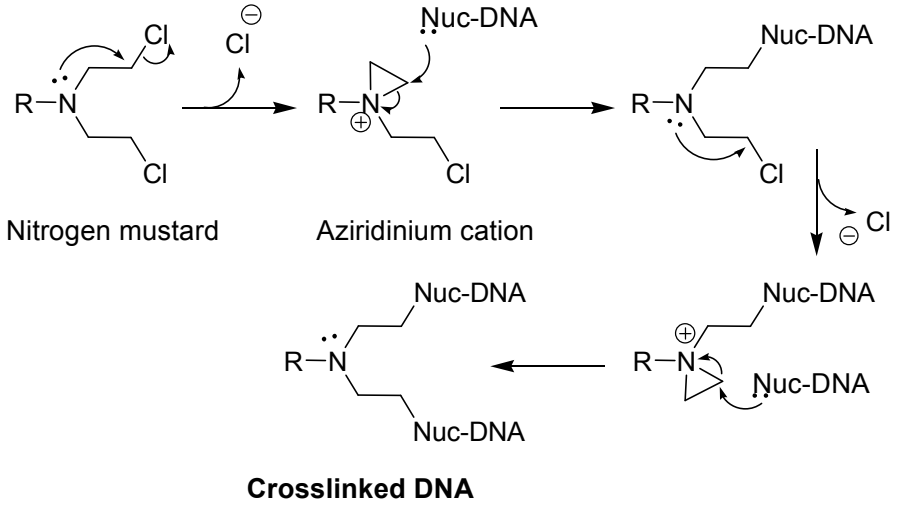

Figure 1.3: Mechanism of DNA alkylation by a nitrogen mustard. Adapted from [11]. Nuc-DNA refers to a nucleophilic functional group (e.g. the N-7 position of guanine) in a DNA molecule.

In addition to PM, other electrophilic metabolites of CP include the aldehydes AldoP, chloroacetaldehyde and acrolein, all of which can bind to endogenous nucleophiles. A significant detoxication mechanism for electrophiles is conjugation with glutathione (GSH), a tripeptide with a nucleophilic thiol group. This phase II metabolic transformation can be spontaneous or mediated by glutathione S-transferase enzymes.

Chloroacetaldehyde and acrolein may thus promote CP cytotoxicity by depleting cellular GSH levels, leaving less GSH available to detoxify the active metabolites [17].

1.2.2. Toxicity and workplace hazards

Although cyclophosphamide has some selectivity for cancer cells, it can damage healthy cells as well. Adverse effects include hemorrhagic cystitis of the bladder, bone marrow suppression, cardiac toxicity, infertility in both men and women, liver and kidney toxicity, teratogenicity and carcinogenesis [2, 11]. CP is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). Occupational exposure to antineoplastic drugs such as CP can also result in adverse health effects, and there is presently no recognized safe exposure limit. Such exposures are likely to occur within health-care facilities. Workers can be exposed when preparing and administering the drug, cleaning up accidental spills, handling patients or disposing of waste. The primary routes of exposure are inhalation and dermal absorption [2].

A 2005 retrospective cohort study surveyed hospitals and health care centres in British Columbia to determine whether nurses have elevated risk of cancer incidence or mortality with respect to antineoplastic drugs, ionizing radiation and anaesthetic gases [2]. Among the hospital pharmacies surveyed, 92% reported use of cyclophosphamide. Some nurses indicated that until the early 1980s, nurses would mix and administer cytotoxic agents without specific training nor personal protective equipment.

Numerous studies have revealed CP contamination in hospital environments – including drug administration and preparation areas – that could lead to occupational exposure. CP has been detected in air samples in preparation areas [22-24], on surfaces such as floors, door handles, intravenous infusion bags, countertops, chairs and tables [22, 25, 26], and on the exterior of sealed drug packaging [27, 28]. In response to these findings, further

research has investigated preventative measures, including better personal protective equipment [29], closed-system devices [26] and cleaning solvents [30].

Biological monitoring methods for occupational exposure to CP have typically involved analysis of urine samples. In a 1984 Canadian study, CP was detected in urine of two nurses at a cancer ward [31]; subsequently in a 1986 Dutch study, CP was detected in urine of 5 out of 20 hospital workers who had handled the drug [32]. Similar findings have been reported since, using even more sensitive detection methods [29, 33, 34]. Increased genetic damage was found in nurses who handled CP and other antineoplastics compared to a control group [35]. Following the adoption of protective measures throughout a health-care facility, several studies have found that CP levels in urine and environmental samples are reduced but not completely eliminated [29, 36, 37].

Like many other pharmaceuticals, CP is excreted into waste streams. A recent German study demonstrated CP contamination in surface water and landfill effluent [19]. The authors estimate a low risk of adverse effects from the use of untreated surface water as drinking water, while also acknowledging the current lack of methodology and knowledge for risk assessment of carcinogenic drugs in the aquatic environment. The same study cites a German Federal Environmental Agency (*Umweltbundesamt*) report recommending a limit of 10 ng/L in drinking water for highly active genotoxic compounds over a 70-year lifetime.

1.3. Analysis of cyclophosphamide and its metabolites

1.3.1. Physical and chemical properties

Cyclophosphamide monohydrate is a white solid at room temperature, soluble in both water and methanol. Based on its conjugate acid pK_a of 2.84 [38], CP is not expected to be ionized at pH 7. CP has an octanol-water distribution coefficient (log D) of 0.23 [38], which suggests that it will partition easily into both organic and aqueous phases.

The metabolites 4-ketocyclophosphamide (KCP) and carboxyphosphamide (CBP) are also white solids at room temperature, soluble in methanol and slightly soluble in water. KCP has an acidic amide proton with a calculated pK_a of 7.18 [39], and CBP is a carboxylic acid with a calculated pK_a of 4.29 [39].

1.3.2. Stability

The parent drug CP is stable in aqueous solutions under certain temperature and pH conditions. After 7 days at 20°C, CP is >95% intact from pH 3.4-8.6; after 2 days at pH 1.2 and 20°C, only 37% remains. At biological conditions of pH 7.4 and 37°C, CP has a degradation half-life of 6.6 days [40]. In practical terms, CP can withstand changes in pH and temperature during laboratory handling, but sample storage conditions must be more precisely controlled. In urine, some spontaneous decomposition of CP may be already expected at the time of sample collection.

In neutral or slightly acidic solutions, CP degrades via an intramolecular alkylation of the ring nitrogen, displacing chloride from one chloroethyl group and leading to a bicyclic intermediate (**Figure 1.4**). The P—N bridge bond hydrolyzes immediately, yielding a nine-membered oxadiazaphosphacyclononane heterocycle, as identified by ^{31}P NMR [41]. Under strongly acidic conditions, CP degrades instead by hydrolysis of both P—N bonds, leading to nor-nitrogen mustard (NNM) and 3-aminopropylphosphoric acid [42].

At pH 7.8-7.9, KCP is reported to degrade in urine [41], but it is otherwise stable in the pH range 3.0-7.5 [43]. No further details on KCP's degradation pathways have been reported. Conversely, the stability of CBP in human urine has been thoroughly studied, as it is a major metabolite of CP. At 25°C, CBP degrades at pH 7 by less than 10% in 24 h; but at pH 5.5 it degrades by 50%, and it has a stability maximum at pH 8. At -80°C over 6 months, CBP degrades by 30% regardless of pH [44]. Degradation products include NNM, 3-(phosphonooxy)propanamide, 3-(phosphonooxy)propanoic acid and phosphoramidic acid (**Figure 1.5**) [41].

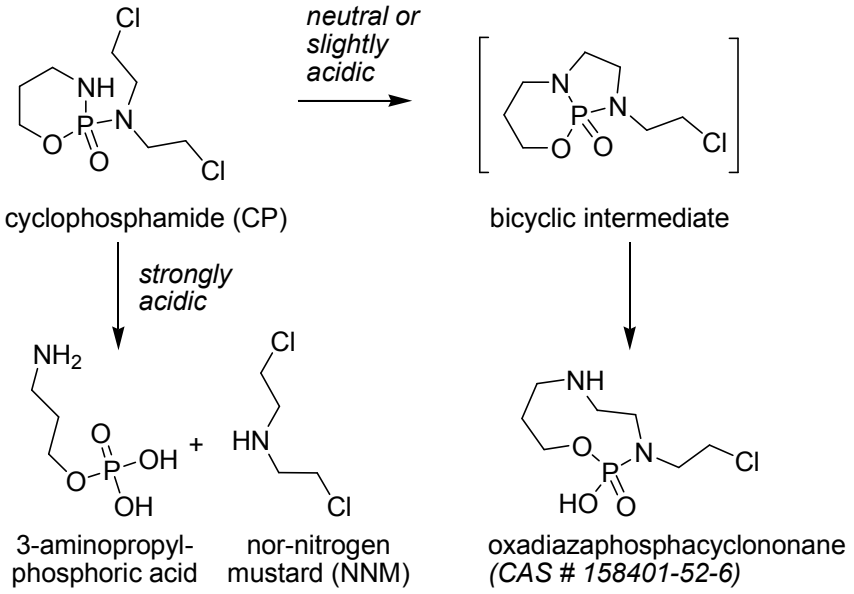

Figure 1.4: Degradation pathways for cyclophopshamide. CP is stable for >7 days at pH 3.4-8.6, 20°C; however $t_{1/2}$ = 6.6 days at physiological conditions of pH 7.4, 37°C. Adapted from [40-42].

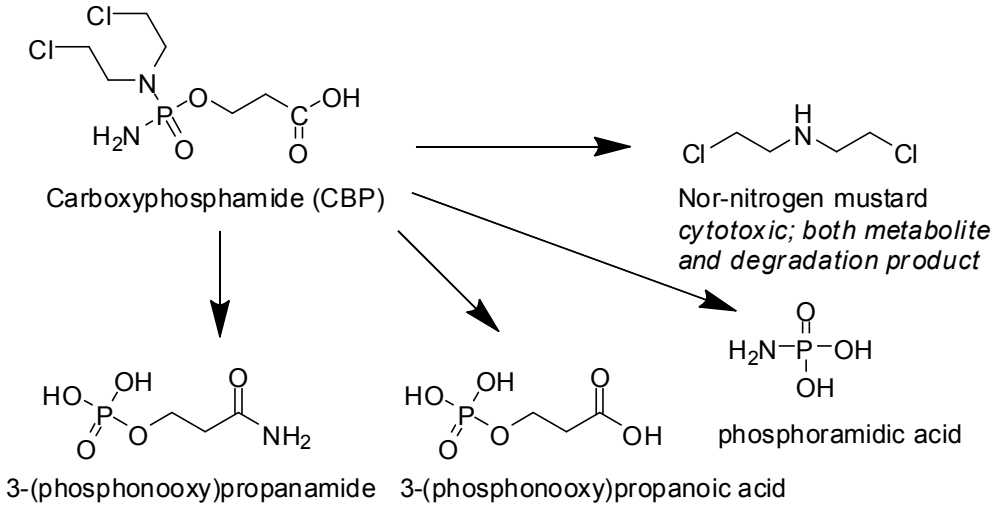

Figure 1.5: Degradation of carboxyphosphamide under acidic conditions. Adapted from [41, 44].

1.3.3. Concentrations of CP and metabolites in urine

The literature methods for CP analysis fall broadly into two categories, clinical and trace concentrations. Clinical studies operate at the urine and plasma concentrations seen in patients who receive the drug; for example, a 1.0-2.6 g dose of CP leads to 24-hour urine concentrations of 108-175 mg/L [45]. Typically the patient is receiving multiple medications as part of a chemotherapy regimen, administered over the course of a few months. Following remission, the patient is no longer exposed to antineoplastics. Analytical methods designed for clinical concentrations, including pharmacokinetic studies, often do not need to achieve trace detection limits.

Trace studies include the analysis of CP in blood and urine of exposed workers, surface and aerosol samples, and the natural environment. Occupationally exposed workers show trace amounts of the drug ranging from 20 ng/L to 10 μg/L of CP in urine [34, 46], as many as six orders of magnitude below clinical levels. It is estimated that chronic exposure with a mean daily excretion of 180 ng CP would result in up to 400 cancer cases per million during a 40-year working period [47, 48]. With no occupational exposure limit (OEL) established for any antineoplastics to date, the only reduction target available is the ALARA principle (as low as reasonably achievable) [36]. Therefore any improvement in a method's detection limit can ultimately lead to valuable information about risk.

Efforts to reduce exposure to antineoplastics have in some cases been so successful that most or all subjects of a multi-year study have drug levels below the detection limit by the final year [36, 37]. But are these workers safe, or are they now subject to an untraceable risk? While this reduction is a positive development, it does present challenges from a monitoring standpoint.

1.3.4. Review of analytical methods

A variety of analytical methods have been used to characterize and quantify CP and its metabolites. In some studies where 24-hour urine samples were collected, results are reported exclusively as mass per sample, since this is a more useful exposure metric; however analytical method development studies tend to report in units of mass or moles per volume. A normal 24-hour urine volume in humans is 800-2000 mL [49]. Thus to facilitate analytical comparison between the two formats, results from the literature are converted here to mass units per litre where possible, and as mass per sample where volume information was not reported.

The first gas chromatography-mass spectrometry (GC-MS) method to identify CP, PM and NNM unambiguously was reported in 1978 [50]; prior to that time, quantitative clinical studies of CP and metabolites typically used radiolabeled drugs, chemical assays for alkylating activity and mutagenicity, or biomarkers for the same. The method required liquid-liquid extraction (LLE) of plasma or urine aliquots, followed by derivatization with trifluoroacetic anhydride (TFAA) and diazomethane prior to GC-MS with isobutene as a chemical ionization (CI) reagent. A lower limit of sensitivity of 3 μg/L was reported for CP in plasma and urine.

Bahr *et al*. reported the use of high-performance liquid chromatography (HPLC), combined offline with field desorption mass spectrometry (FD-MS) in 1981 to detect CP, KCP and CBP in the urine of a multiple sclerosis patient. Sample preparation used liquid-liquid extraction, and notably did not require derivatization [51, 52].

In 1984, the first biological monitoring study of occupational CP exposure which detected CP directly was reported by Hirst *et al*. Gas chromatography with nitrogen-phosphorous detection (GC-NPD) was used after liquid-liquid extraction of the entire 24-hour urine volume with TFAA derivatization. Selected samples were also confirmed by GC-MS. Subjects included five volunteer cancer patients who received topical CP

doses to assess the dermal exposure route, and two exposed nurses. The limit of detection (LOD) for CP was 250-300 ng per 24-hour urine sample (volume units not reported) [31].

Burton *et al*. reported in 1988 a CP analysis of 250 µL plasma aliquots by HPLC with sample cleanup by solid-phase extraction (SPE). LOD for CP was 1 mg/L, sufficient for pharmacokinetic studies, although the authors noted they could reach better sensitivities with greater sample volume [53].

A 1988 method by Hadidi *et al*. combined thin-layer chromatography (TLC) with a colorimetric assay for alkylating activity, followed by photography and densitometry [54]. This was reported as the first method to combine detection of CP, KCP, CBP, PM and NNM without radiolabeling; however it was later reported unreliable for PM and NNM [55]. LOD was 1 mg/L for CP and 0.5 mg/L for both KCP and CBP.

A series of studies by Sessink *et al*. in the 1990s used GC-MS for occupational CP exposure. By this point it was possible to reach 1 µg/L detection limits on a quadrupole GC-MS starting from only a 5 mL aliquot of urine, with LLE and TFAA derivatization similar to previously published methods [22]. The same GC method, adapted to an ion trap GC/MS, reached an LOD of 250 ng/L [56-58]. On an ion trap with tandem MS (GC-MS/MS), the same laboratory reported an LOD of 0.1 ng/L CP in urine [46]. Levels of 0.02-9.14 µg CP were found in the 24-hour urine of 20 of the 25 nurses studied who handled antineoplastics – including those who handled other drugs but not CP.

NMR spectroscopy has been used in a few scenarios for detection of CP and its byproducts despite its relative insensitivity. Gilard *et al*. reported in 1994 the kinetics of CP hydrolysis by ^{31}P NMR and elucidated structures of the products [40]. Joqueviel *et al*. carried out a similar study in 1997 for CBP degradation in urine [44]. In 1998 the same researchers analyzed four breast cancer patients' urine samples quantitatively for

CP and most of its phosphorylated metabolites and degradation products over a 48 h span [41]. Detection limits were 1-2 mg/L for each compound.

A 1995 *in vitro* study of CP metabolites used human liver fractions, and analyzed CP, CBP, KCP and NNM by GC and thermionic specific detection after LLE and derivatization [48]. The LODs for CBP and KCP were 150 and 140 µg/L respectively. The authors applied this method to urine samples of occupationally exposed workers but were unable to extract and derivatize the metabolites.

In 1998, Sottani *et al*. reported the first HPLC method with tandem MS (LC-MS/MS) for trace CP determination in urine samples from occupationally exposed workers. The method used LLE for sample cleanup and pre-concentration but required no derivatization. CP was found in the urine of 50% of workers studied, at levels from 0.1-2.1 µg/L, with an LOD of 50 ng/L [33]. The method was also applied to air samples and surface wipe samples, and to subsequent investigations over a 10-year span [34, 59]; later studies used SPE instead of LLE. On a newer model of MS/MS, the LOD in urine was reduced to 10 ng/L of CP.

The first LC-MS/MS analysis of both CP and metabolites in urine was reported by Kasel *et al*. in 2004. This method required no sample preparation other than dilution, with an LOD of 5 µg/L for CP and KCP, and 30 µg/L for CBP [45]. Urine samples from five chemotherapy patients were studied to validate the method.

In 2006, Buerge *et al*. reported detection of CP in surface waters (50-170 pg/L) and wastewater (0.3-11 ng/L) by LC-MS/MS, with ultratrace detection limits of 20 pg/L and 200-300 pg/L respectively [60]. The method used large SPE columns to extract the analytes from 1 L samples.

1.3.5. Estimating trace levels of metabolites in urine

Literature methods exist for analysis of CP and metabolites at clinical concentrations, and also for analysis of CP at trace occupational exposure concentrations. However, none of the trace analyses to date have included metabolites of CP at concentrations appropriate for an occupational exposure study. The expected concentrations of metabolites can thus only be extrapolated from clinical studies. It must be emphasized that metabolite levels in clinical studies are influenced by many factors; for example CP tends to induce its own metabolism at clinical doses [16], and CP metabolite levels are correlated with co-administration of other drugs in chemotherapy [18]. The ratio of metabolites to the parent drug may well be quite different at trace exposure levels in otherwise healthy workers.

Ratios of KCP and CBP to the parent drug in urine vary considerably in clinical studies. Joqueviel *et al*. reported KCP levels from 2-7% of the parent drug level, and CBP from 45-186%, in the 24-hour urine of 4 patients on two consecutive days each [41]. Kasel *et al*. anticipated 15% of the parent concentration for KCP and 5% for CBP; their results for KCP were 5-8% and CBP levels were below 1% of CP [45]. Notably, CBP levels in Joqueviel *et al*. were higher after a second day's infusion, no doubt because the first day's dose had not yet fully metabolized by that point. As trace occupational CP exposures derive from repeated events rather than a point exposure source, metabolite ratios may also be higher than in single-dose clinical studies.

1.3.6. Metabolites and monitoring exposure

Since CP is a prodrug, which is both clinically effective and toxic by way of its metabolites, an understanding of its metabolism is crucial to assessing its occupational risks. Early investigations into CP metabolism suggested that patients could be divided into "low carboxylators" who excrete more PM but less CP and CBP per dose, and "high carboxylators" who excrete less PM but more CP and CBP [61, 62]. This was taken as

evidence of polymorphic metabolism – in other words, some patients inherently would metabolize CP quite differently. However, this conclusion was retracted when a large part of this effect was attributed to degradation of CBP prior to sample analysis, and to the unsuitability of the method for quantifying PM [16, 18, 44].

Notwithstanding this error, the remaining variation in urine metabolite levels was attributed to individual differences in the expression of ALDH enzymes, or to metabolic induction resulting from other administered drugs [16, 18, 44]. An *in vitro* study with human liver fractions found large interindividual differences in CP metabolism, and assumed these differences would occur in occupationally exposed workers; however their analytical method was unable to extract metabolites from real urine samples to confirm this theory [63].

More recently, certain cytochrome P450 polymorphisms which are relevant to CP metabolism have been shown to be more prevalent in African Americans compared to Caucasians [64, 65]. The term *pharmacoethnicity* has been coined to describe ethnic diversity in drug response or toxicity.

Hedmer *et al*. analyzed trace concentrations of CP by monitoring cancer patients until their systematic CP levels were as low as in occupationally exposed workers. This study validated CP in urine as a more sensitive biomarker than in plasma, and therefore more appropriate for monitoring occupational exposure at a group level [47]. It was noted that a biomarker of exposure should measure the internal dose of the substance that has a harmful effect. CP levels in urine were found to reflect all exposure routes, but levels of CP alone in urine may over- or underestimate this internal dose.

Concentrations of the active metabolites OHCP and PM are found to be much more predictive of risk than the parent compound [11]. While OHCP and PM are too unstable for an LC-MS study without derivatization, KCP and CBP are significantly more stable.

The concentration of CP alone in urine represents only how much of the drug was *not* activated into toxic products. Since KCP and CBP both derive from metabolic activation to OHCP and subsequent inactivation, the quantification of these metabolites could provide additional and complementary information about toxicity and metabolism.

1.4. LC-MS of biological samples

Upon reviewing the chronology of detection methods for CP and its metabolites, a few trends emerge. First, sample preparation methods have become more efficient – in 1984, liquid-liquid extraction (LLE) of a full 24-hour urine sample with twice its volume in ethyl acetate was needed for an occupational exposure study [31]; in 1998, a 5 mL aliquot was cleaned up by solid-phase extraction (SPE) for the same purpose. One of the least wasteful extraction methods is online SPE, although this requires additional equipment which was not available for the present study. While offline SPE does require use of disposable cartridges, it uses less solvent than LLE and is more suited to batch automation [66].

Next, GC-based methods have been largely but not entirely supplanted by LC methods. It is reported that CP, KCP and other metabolites undergo cyclization at the elevated temperatures of the GC injector, resulting in a bicyclic structure (**Figure 1.6**) [67]. Ifosfamide (IF), a structural isomer of CP, is also a frequently used antineoplastic drug. The same cyclization applied to IF and 4-keto-IF would produce the same products as with CP and KCP. Thus even when the "auto-derivatized" product is stable enough to detect after GC, it obscures the difference between two drugs with different biochemical properties which may both be present in the same sample. The most successful GC methods for antineoplastics require chemical derivatization. Conversely, CP, KCP and CBP are all stable enough to reach the detector in an HPLC method without degradation.

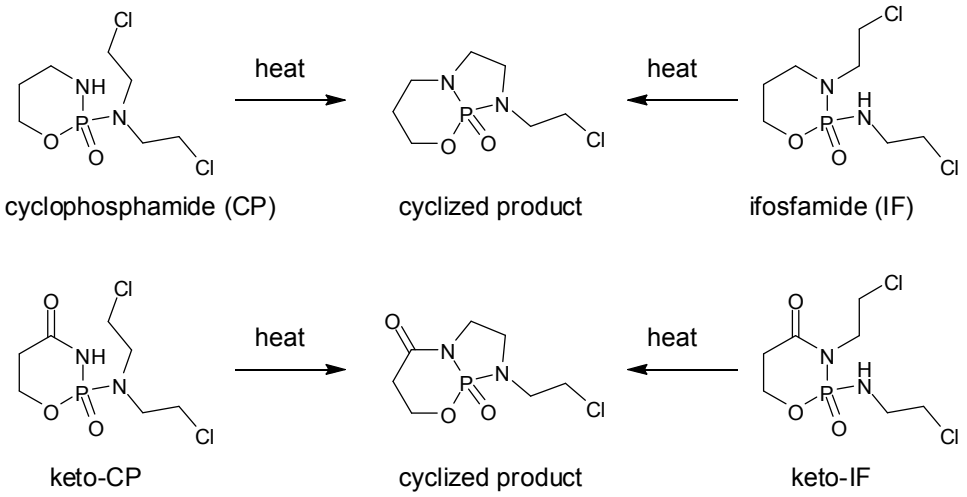

Figure 1.6: Heat-induced cyclization of CP & KCP, and their ifosfamide analogues, following injection on capillary GC. Adapted from [67].

A final trend to note is detection methods have become more sensitive and specific. CP and its metabolites are not well-suited to ultraviolet detection as they lack a useful chromophore. The most specific methods available to earlier studies included radiolabeled drugs [50], nitrogen-phosphorus detection [31], and colorimetric assays for alkylating activity [54]. NMR spectroscopy is very specific, but has poor sensitivity. Meanwhile, mass spectrometric methods for CP and metabolites have yielded significant improvements in detection limits. Furthermore, a shift to MS/MS instruments in both GC and LC experiments has produced improvements of over three orders of magnitude in detection limits [46, 56, 57].

MS methods are particularly suitable to analyses of multiple drugs, since each can be detected on a separate channel – a second dimension of specificity beyond the chromatographic separation, and a third in the case of MS/MS. In multiple reaction monitoring (MRM) mode, each detection channel represents the transition from a parent ion to a specific product ion after undergoing fragmentation reactions in a collision cell. In pharmacokinetic studies, LC-MS/MS enables rapid metabolite discovery by searching for common mass differences that result from biological transformations [68].

1.4.1. Choice of ion source

The gap between liquid chromatography and the mass analysis of gas-phase ions can be bridged using a number of different possible atmospheric pressure ion sources. Electrospray ionization (ESI), introduced in 1984, is today the default ion source of most commercial LC-MS units. The sample solution is pumped through a capillary set to a high voltage. A fine spray of charged droplets emerges, travelling towards a counterelectrode. As the solvent evaporates, a droplet shrinks and eventually breaks into smaller ones due to electrostatic repulsion forces on its surface. As this process repeats, sufficiently small droplets are able to emit gas-phase ions [69, 70]. Most LC-MS methods reported for CP and its metabolites used this ion source.

Atmospheric-pressure chemical ionization (APCI) is an alternative ion source in which the sample solution is vapourized (300-500°C), and a corona discharge needle then initiates ion-molecule reactions in the gas phase [71]. Many commercial LC-MS units include a dual APCI/ESI source or a secondary APCI source that can be installed in place of the ESI source. Analysis of CP in human plasma by LC-ACPI-MS has been reported, albeit with inferior detection limits to those of similar ESI methods [72]. A third ion source is atmospheric-pressure photoionization (APPI), in which the sample is vapourized similarly to APCI, but ionization is then initiated by a vacuum ultraviolet (VUV) lamp. The analyte can either be photoionized directly, or ionized as a result of photoinitiated reactions involving a dopant molecule and the solvent [71].

1.4.2. Effect of LC mobile phase in ESI-MS

The most common reversed-phase HPLC solvents are water, acetonitrile and methanol. Water alone has high viscosity and surface tension, which leads to low electrophoretic mobility of ions in solution. This leads to inefficient charge separation and poor spray formation. Furthermore, ions which are well-solvated in water are less likely to be emitted in the gas phase during the ESI process. Organic solvents such as methanol and

ACN have lower viscosity and surface tension, and less efficient solvation of ions. The result is a general trend towards better sensitivity when analytes elute in a high-percentage organic solvent [71].

In HPLC experiments with non-MS detection, a number of additives are typically used to optimize peak shapes, such as phosphate buffers, and the ion-pairing agent trifluoroacetic acid (TFA). Such HPLC additives are poor matches with ESI-MS due to ion suppression effects. Non-volatile buffers may contaminate the ion source rapidly, and ion-pairing reagents can block gas-phase emission of molecular ions [71]. Thus a balance of needs must be struck between chromatographic separation and mass spectrometric detection. The most typical additives in LC-MS are small organic acids below 0.1% concentration, or volatile ammonium salts below 10 mM [13].

A model by Enke *et al*. predicts that measured analyte response for ESI-MS is most proportional to concentration when the electrolyte concentration is below 1 mM. With high salt concentrations, the analyte response can be suppressed due to repulsive forces from the increased charge density, resulting in spreading of the spray, or alternately due to competition of ions at surface sites on the droplet [73].

In ESI-MS, it might be expected that acids are best analyzed in negative-ion mode under basic conditions, and that bases are best analyzed in positive-ion mode under acidic conditions, since the ions would be pre-formed in solution. However, in practice ESI in positive mode also produces abundant protonated molecules under basic or neutral conditions and deprotonated molecules under acidic or neutral conditions – in other words, “wrong-way-round ionization”. At the tip of the ESI sprayer, pH may differ by as many as 1-4 units from that of the bulk sample solution. Gas-phase ion-molecule reactions may also contribute to this process [71]. Thus pH control in LC-MS may be geared more to providing an efficient separation than to promoting ionization.

1.4.3. Matrix effects in ESI-MS

Matrix effects can be broadly defined as "interference from matrix components that are unrelated to the analyte" [74]. In LC-MS, this can mean enhancement or suppression of the MS signal due to co-eluting components, whether detected or undetected, from the sample. The mechanisms noted above for buffers and ion-pairing reagents are examples of how this can affect MS. While matrix effects cannot be completely eliminated, the challenge is to control variability; calibration standards and real samples should experience these effects to the same extent. An internal standard should also respond to matrix effects in the same way as the analyte. Failure to control for matrix effects is a frequent source of error in bioanalysis, and ESI-MS is particularly vulnerable [66, 75].

The MS ion source can be adversely affected by physical deposition of involatile components. These include inorganic salts, present in biological fluids or added during sample preparation; and endogenous biological macromolecules such as proteins [66]. One way to spare the ion source from this contamination is through chromatography – to divert the LC flow to waste while the matrix elutes. This can also be accomplished earlier in the analysis method, through offline or online sample preparation.

One way to gauge the extent of matrix effects in LC-MS is by post-column infusion. A splitter is placed after the HPLC column outlet but before the MS detector, and a constant flow of the analyte in question is infused. A clean solvent blank injected onto the column will show the base MS response of the infused analyte throughout the gradient program. Next, blank sample matrix (i.e. plasma, urine, wastewater) that has been subjected to the sample preparation method is injected for several additional runs. Regions of the chromatogram that show ion suppression or enhancement are then clearly visible by comparison. This type of validation was used successfully in a study of CP and CBP in plasma [76].

Following post-column infusion, a further refinement is to run a full scan LC-MS experiment and identify matrix ions of interest in the appropriate region of the chromatogram. These ions can be used as matrix markers for further assessments of the extraction or separation method [75]. If an extraction method is designed to separate the sample from the matrix, then its success can also be evaluated by preparing a matrix blank with the extraction method, spiking it with analyte and comparing the results with analytical standards [77].

1.5. Sample preparation for LC-MS

Typically, the main goal of sample preparation is to exchange sample matrix for clean solvent; in some cases a reduction in total sample volume (pre-concentration) is also accomplished. Certain analytes also require derivatization for LC-MS, although this is rarer than with GC-MS methods. Sample preparation can also protect the HPLC column, since some matrix components such as proteins can cause deterioration of the stationary phase and increases in back pressure [66].

1.5.1. Sample dilution

The simplest sample preparation is none at all, a "dilute-and-shoot" approach. If the instrument is sensitive enough, the targeted levels of analyte are high enough, and the matrix interference can be minimized, this method can be satisfactory and provide high sample throughput [77]. Urine and saliva, essentially aqueous systems, are amenable to simple dilution. Plasma samples may be subjected to protein precipitation by addition of acid or organic solvent and centrifugation, leaving the supernatant depleted of proteins [66].

Kasel *et al*. successfully used dilution for clinical concentrations of CP and metabolites in urine: a 100 µL aliquot of urine was spiked with internal standard and made up to 10 mL with methanol, a 100-fold dilution. The solution was vortexed and centrifuged, and the supernatant injected into the LC-MS/MS system (method LOD = 5 µg/L for CP and

KCP, 30 µg/L for CBP). The method was validated by preparing urine QC samples to compare with reference solutions in neat solvent at the same concentrations [45].

1.5.2. Liquid-liquid extraction

LLE can produce clean and concentrated samples, less prone to ion suppression. Ionizable analytes in the sample must first be converted to neutral forms by pH adjustment. Next, the sample is shaken with an immiscible organic solvent, and the organic layer is then dried and evaporated to be reconstituted in the final solvent for analysis. This process removes proteins, inorganic ions and polar organics [66].

Turci *et al*. used LLE to extract CP and IF from urine for LC-MS/MS. A 5 mL aliquot of urine was adjusted to pH 7 and extracted with ethyl acetate. The solvent was evaporated to dryness under nitrogen gas and reconstituted in 200 µL mobile phase, a 25-fold pre-concentration. This solution was centrifuged and filtered, and a 10 µL quantity was injected into the LC-MS/MS system (method LOD = 50 ng/L of CP in urine) [34]. It is not clear how well this method would work for KCP and CBP, as they are ionized at neutral and basic pH. An extraction at acidic pH may succeed in extracting them to the organic layer, but unfortunately CBP is unstable in an acidic environment.

Sessink *et al*. used LLE to extract CP, KCP, CBP and NNM from *in vitro* incubations with S-9 liver fractions prior to GC analysis. The sample was shaken with diethyl ether to remove excess CP, and the aqueous layer was then acidified with 6 M HCl and further extracted with ethyl acetate before derivatization. The extraction method was successful with liver fractions, but it failed to extract metabolites from urine samples of occupationally exposed workers [63]. While it is not clear why this method failed with urine, again it would be inadvisable to acidify strongly a solution of CBP for reasons of stability. Momerency *et al*. found that CBP could not be extracted with ethyl acetate or chloroform at pH 4 or 7 with any efficiency better than 11.1% [78].

1.5.3. Solid-phase extraction

SPE uses sorbent cartridges which are commercially available with a variety of stationary phases depending on the desired separation. First the dry cartridge is primed and conditioned with solvent, and then the sample is applied. The analyte partitions from the liquid to solid phase, and remains there during a wash step to remove contaminants; some methods also have a drying step at this stage. Next, the analytes are eluted from the cartridge with an organic solvent, which is usually evaporated down again and reconstituted in mobile phase for analysis [66].

The stationary phases available for SPE are typically silica-based and include common reversed-phase bonded groups such as octyl (C_8) and octadecyl (C_{18}), strong cation-exchange (SCX) groups such as sulfonic acids, strong anion-exchange (SAX) groups such as quarternary amines, and numerous other specialized functionalities. For improved selectivity, the wash solvent can be adjusted to be as strong as possible without eluting the analyte, and the elution solvent only just strong enough to elute the analyte [66]. The initial sample and conditioning solvent may also need adjustment in order to guarantee that the analyte does not elute during sample loading.

Sottani *et al*. used SPE to extract CP and the related drug trofosfamide from urine for LC-MS/MS. A 5 mL aliquot of urine was adjusted to pH 7.0 and loaded on a conditioned Varian Bond Elut C18 cartridge. After washing, elution and evaporation the residue was reconstituted in 200 µL mobile phase, for a 25-fold pre-concentration (method LOD = 10 ng/L) [59, 79]. While this method did not include metabolites, it was originally based on an SPE-GC-MS study that did include KCP, CBP and other metabolites at clinical levels [78]. The earlier study had found that CP, KCP and CBP could be extracted from plasma with efficiencies of 93.2%, 90.3% and 82.5% respectively on a Waters Millipore SEP-PAK C18 vacuum cartridge using pH 4 for sample adjustment and washing.

Another reported SPE method for CP and metabolites in plasma used dilute acetic acid for sample adjustment and washing, and acetonitrile for elution. The residues left after drying were frozen for later analysis. Recovery of CBP ranged from 60-70% after extraction. The authors took note of CBP instability, as it degraded in the autosampler at room temperature to 83.6% initial concentration after 3 h at pH 7.4, and to 31.7% after 24 h. Therefore samples were analyzed within 2 h of reconstitution in mobile phase [80]. It is unclear whether these test conditions also took the SPE method into account. The method as described would have incorporated a certain amount of acetic acid into the final SPE eluate, which may have left a highly acidic residue after solvent removal. Even at -70°C, CBP would be expected to under such acidic conditions.

Based on this study's target LOD for CP and metabolites, and on the available literature, a dilute-and-shoot method would be too insensitive by 2-4 orders of magnitude. When the conditions for dilute-and-shoot are not satisfied, an extraction method becomes necessary. Since LLE calls for mixing, centrifugation and extraction of large solvent volumes, it would be technically demanding to automate [66]. While SPE does require disposable cartridges, it has the advantages of requiring less solvent per extraction than LLE, being more time-efficient, and offering more fine-tuned separations of a wider variety of analytes based on the sorbent-solvent chemistry.

1.6. Aims and scope of the study

The experimental work undertaken to complete this thesis came about as a collaboration with the School of Environmental Health (SoEH) at UBC. A larger initiative at SoEH has set out to evaluate the risks of occupational exposure to antineoplastic drugs through interviews, biological and environmental monitoring, statistical analysis, exposure modelling and self-administered questionnaires. As part of the biological monitoring component, urine samples are to be collected from up to 200 health-care workers in British Columbia in order to assess exposure risks based on concentrations of CP and

metabolites. The scope of this thesis paper is limited to the analytical method development alone, and statistical treatment of the data is to be handled through SoEH.

To date, no study measuring occupational exposure to CP has successfully analyzed CP's metabolites in the same samples. The present study targets the parent drug CP, and two metabolites KCP and CBP which are inactive products of the active metabolite OHCP. Concentrations of the inactive prodrug CP in urine can only provide information on what the individual did not metabolize. Together, the three analytes in urine carry more information about the individual's total metabolism. While the interpretation of that data in terms of toxicity is beyond the boundaries of this study, it could provide a better understanding of how variations in metabolism affect the individual exposure risks of workers occupationally exposed to CP.

This study aimed to reach sufficient analytical sensitivity in KCP and CBP to detect these metabolites in urine samples with CP levels as low as 50 ng/L, which was the lowest level that Turci *et al*. detected and quantified [34]. If CBP and KCP levels are mostly above 5% of CP concentration, then a detection target of 2.5 ng/L for both metabolites in urine should suffice for this purpose.

2. Experimental

2.1. Chemicals and supplies

Cyclophosphamide (CP, CAS# 6055-19-2) was purchased from Sigma-Aldrich (MO, USA). 4-ketocyclophosphamide (KCP, CAS# 27046-19-1), carboxyphosphamide (CBP, CAS# 22788-18-7) and d_4-cyclophosphamide (d4-CP, CAS# 57154-94-6) were obtained from Niomech (Bielefeld, Germany). Acetic acid, ammonium acetate, ammonium hydroxide, phosphoric acid and formic acid were purchased from Fisher Scientific (CA, USA). Phosphate-buffered saline tablets were purchased from Sigma-Aldrich. Methanol (HPLC grade) was obtained from Fisher Scientific, and deionized water was generated in-house. Synthetic urine (Surine™) was purchased from Dyna-Tek Industries (KS, USA).

A Supelco Visiprep 24DL solid-phase extraction (SPE) vacuum manifold from Sigma-Aldrich was made available at SoEH to develop the extraction method. Disposable liner tips were used to reduce the possibility of contamination. SPE cartridges used in this study were Varian Bond Elut LMS (50 mg sorbent, 1 mL volume, 75 µm particle size) and Varian Bond Elut C18-INT (500 mg, 3 mL, 40 µm). Additional SPE cartridges tested in this study were Varian Bond Elut C18 (500 mg, 3 mL, 120 µm), Varian Bond Elut C18-OH (500 mg, 3 mL, 120 µm) and Agilent OPT SampliQ (150 mg, 6 mL). Breakthrough fractions and eluates for SPE were collected in borosilicate glass culture tubes (10 mL, 16 × 100 mm) from Chromatographic Specialties (ON, Canada).

HPLC columns used in this study were: 1) Supelco C_{18} Discovery (150 × 2.1 mm, 5 µm particle size), 2) Supelco C_8 Ascentis (100 × 2.1 mm, 3 µm particle size) with a Supelco C8 Discovery guard column (20 × 4.0 mm, 5 µm particle size), 3) Agilent XDB C_{18} (4.6 × 50 mm, 1.8 µm particle size), and 4) Agilent Eclipse Plus C_8 (30 × 2.1 mm, 1.8 µm).

2.2. Instrumentation

One LC-MS/MS system (Instrument #1) used in this study consisted of an API 3200 Series triple-quadrupole mass spectrometer (AB Sciex Instruments, ON, Canada), a Varian Star 9010 ternary pump (Varian Instruments, CA, USA) and a Varian Prostar 410 autosampler. Both the ternary pump and autosampler were made available by SoEH. The three devices were configured to communicate via contact closure. In addition, a Waters 6000A solvent delivery pump (Waters Associates, MA, USA) was used as a secondary pump in some configurations, to deliver a step gradient via the API 3200's integrated switching valve. The API 3200 includes three ion sources: electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photo-ionization (APPI). Beyond the initial investigations, solely the ESI source was used in this study. Data from this instrument was acquired and processed using AB Sciex's Analyst software package. Parameters for LC-MS/MS runs on this instrument are listed in **Appendix A** as configurations **1a** through **1h**.

Methods requiring infusion on the API 3200 used 1 to 5 mL Hamilton syringes (Hamilton Company, NV, USA) with the API 3200's integrated syringe pump, as well as an external Harvard 11Plus syringe pump when necessary. Additives were delivered with one syringe, and analytes with another; both flows were combined with the mobile phase using PEEK tubing and a PEEK 4-way cross (Upchurch Scientific, WA, USA).

Another LC-MS/MS system used in this study (Instrument #2) was also made available at SoEH. This consisted of an Agilent 6430 triple-quadrupole mass spectrometer (Agilent Corporation, MA, USA) equipped with an ESI source and an integrated Agilent 1200 SL series LC pump and autosampler. The binary pump was modified according to an Agilent application paper [81] to reduce delay volume. Data from this instrument was acquired and processed using Agilent's MassHunter software package. Parameters for these LC-MS/MS runs are listed in **Appendix A** as configurations **2a** through **2e**.

Method development for LC-MS/MS was carried out on Instrument #1 to evaluate its suitability for analysis of a large batch of urine samples. The method was then adapted to Instrument #2 in order to provide SoEH with a protocol to conduct the analysis in-house. SPE schemes were evaluated using only Instrument #1; the results should be applicable regardless of the detection method.

2.3. Stock, working standard and additive solutions

Stock standard solutions of each compound were prepared by dissolving solid reference substances in deionized water (CP) or methanol (KCP, CBP). These solutions were subsequently diluted to a 1 mg/L stock standard by micropipetting the appropriate amount into a volumetric flask and diluting with deionized water. The 1 mg/L stock was then used to prepare all subsequent dilutions for chromatography.

Additive solutions for optimization of MS conditions were prepared in 50:50 water:methanol. Solid ammonium acetate was dissolved first in deionized water to make a concentrated stock solution, and then diluted by micropipette to make two different solutions (5 mM and 215 mM) in volumetric flasks. Concentrated formic acid was transferred by micropipette to make a 106 mM (2%) solution in a volumetric flask.

2.4. Method development

2.4.1. Mass spectrometry

On Instrument #1, initial tests were performed with CP using the ESI, APCI and APPI sources. In light of this comparison, the present study focused exclusively on electrospray ionization.

For each of CP, CBP and KCP a full mass scan was taken in the first quadrupole (Q1), background-subtracted against a solvent blank (configuration **1a**). The molecular ion $[M + H]^+$ was then selected as the parent ion, and several fragments were chosen from the product ion spectrum. The analyte's MS parameters were optimized by infusion and

the MRM transition with the best signal was chosen. For CP, the transition at *m/z* 261 → 140 was selected. MS source and compound parameters were then optimized for the highest possible signal while infusing 1 µL/min of a 1 mg/L CP standard and 200 µL/min of 50:50 water:methanol (configuration **1bi**).

In order to detect the molecular ion of KCP and CBP more effectively than in neat solvent, the metabolites' MS parameters were optimized for the highest possible signal while infusing 10 µL/min of a 5 mM ammonium acetate solution, along with 1 µL/min of a 1 mg/L KCP or CBP standard and 200 µL/min of 50:50 methanol/water. Under these conditions the molecular ions $[KCP + H]^+$ at *m/z* 275 and $[CBP + H]^+$ at *m/z* 293 were selected. The optimal MS/MS transition for KCP was initially selected at *m/z* 275 → 63 (configuration **1bii**). The optimal transition for CBP was 293 → 221 (configuration **1biii)**.

Each analyte's signal was measured using the selected MS/MS parameters over an additive concentration range of 0-21.5 mM ammonium acetate and 0-106 mM formic acid by infusing a 1 mg/L solution of the analyte at 1 µL/min, various flow rates of the additive solutions, and 50:50 methanol/water mobile phase, for a total 200 µL/min solvent flow (configuration **1c**).

An optimal additive concentration of 0.5 mM ammonium acetate was selected. On Instrument #1 this was implemented by using 5 mM ammonium acetate as the "C" solvent in the gradient pump, and setting it to 10% regardless of the A & B compositions. Solvent C was adjusted in some experiments to pH 4 or 5 with acetic acid (details noted in **Appendix A)**.

On Instrument #2 which had only a binary pump, the optimal additive was approximated by using 1 mM ammonium acetate as the "A" solvent, resulting in an overall concentration of 0.1-1 mM over the course of the solvent gradient.

After additive optimization, the full mass spectra were revisited by injecting a standard solution of 50 µg/L each of CP, KCP and CBP onto the Supelco C_8 column (configuration **1d**). A mass spectrum was extracted at each analyte's retention time and background-corrected. Configuration **1d** was derived from the previous MS optimizations, and subsequently used for HPLC method development.

A full mass scan of the deuterated parent compound, d_4-CP, was also taken under the conditions of configuration **1a**, and again under the optimized configuration **1d**. All parameters other than mass were the same as for CP. The transitions at *m/z* 265 → 143 and 267 → 146 were compared as candidates for MS/MS, and the latter was selected for higher signal strength. This compound was obtained for use as an internal standard, but was not further included in the scope of the present study.

During some LC-MS/MS trials, the baseline noise for KCP and CBP was problematic. A second MS optimization was thus carried out in which the signal-to-noise ratio (S/N) of the metabolites was maximized rather than the signal alone. The Varian autosampler was configured to make repeated injections every 90 seconds of a 1 µg/L solution of KCP and CBP in 50:50 water:methanol while the source and compound parameters of the MS were varied. KCP was monitored at both *m/z* 275 → 63 and *m/z* 275 → 221; CBP was monitored at *m/z* 293 → 221 (configuration **1g**). Signal-to-noise for each MRM channel was measured as the peak height divided by the standard deviation of a 1 minute segment of background. After this procedure, the transition *m/z* 275 → 221 was selected instead for KCP in all subsequent experiments. The final MS parameters for Instrument #1, optimized for signal-to-noise, are listed in configuration **1h**.

On Instrument #2, an initial set of MS parameters was selected based on a rudimentary optimization carried out by the MassHunter software (configuration **2a**). Next, the column was removed and a solution of 200 µg/L CP, 500 µg/L KCP and 500 µg/L CBP in deionized water was infused at 20 µL/min. The MS source and compound parameters

were varied to optimize signal strength (configuration **2b**), and the results were used in all subsequent LC-MS/MS trials (configuration **2c**).

2.4.2. Liquid chromatography

The focus of chromatographic method development in the present study was to obtain good separation among analyte peaks and the matrix in a reasonable amount of time, to use solvent conditions optimized for MS, to minimize peak broadening effects, and to develop a protocol that would be reproducible and simple to automate in batches. It was also necessary to investigate carryover effects on both HPLC instruments, as this presented an obstacle to reaching target detection limits.

Chromatography was initially investigated on Instrument #1. After a preliminary comparison between the C_{18} Discovery (150 × 2.1 mm, 5 µm) and C_8 Ascentis (100 × 2.1 mm, 3 µm) columns from Supelco, the latter was selected for improved retention of the metabolites and lower column volume. A 0.5 µm precolumn filter was used to protect the column.

A solution containing 50 µg/L each of CP, KCP and CBP was analyzed using a series of isocratic methanol/water mobile phase compositions at 200 µL/min to determine if an adequate separation could be obtained without the use of a solvent gradient. After adjustment of the ammonium acetate additive to pH 5, configuration **1d** with 45% methanol was selected. The ammonium acetate concentration was also varied from 0.25-2.5 mM overall in a subsequent experiment, but the existing concentration of 0.5 mM was again selected as optimal.

Prior to exploring gradient chromatography, it was noted that the Varian pump lists a delay volume of 1 mL in its specifications, mainly contained in its damper and mixer components. In practice this is even larger due to solvent mixing in the damper. A higher flow rate can reduce the extent of the effect, but significantly higher rates gave too much

back pressure on the selected column and were detrimental to ESI sensitivity. Therefore a flow rate of 300 µL/min was chosen for gradient chromatography. A lower pH of 4 was also selected for the ammonium acetate additive in gradient chromatography to provide better retention of metabolites during the isocratic hold time effectively created by the delay volume. To visualize delay volume, the Varian pump and Supelco column were equilibrated with 57% methanol in deionized water at 300 µL/min, and then the pump was switched from deionized water to 5 mM ammonium acetate at a known time (t=0). The acetate ion was monitored by MS in negative-ion mode. An isotopic peak at *m/z* -60 was selected for this task, as the *m/z* -59 ion was too abundant and tended to saturate the detector.

The best gradient conditions within the limitations of this LC pump are reported as configuration **1f**, in which a very sharp 0.5 min gradient begins at the moment of injection from 20% to 75% methanol, followed by a 3 min isocratic hold and equilibration back to 20% methanol for 11.5 min.

An alternate configuration for Instrument #1 was designed both to bypass the MS source during matrix elution and to bypass the delay volume. In this arrangement, the Varian pump and Waters pump are each configured to deliver a constant isocratic mix, and the ABI 3200's built-in 10-port valve is switched according to a timetable set in the Analyst software. The sample is loaded using the Waters pump from the autosampler onto the column. In the "load" valve position, the column outlet goes directly to waste for matrix diversion while the analytes remain on the column. In the "elute" valve position, the Varian pump connects to the column inlet, and elutes the analytes for MS detection.

The dual-pump setup effectively created a step gradient from one isocratic composition to the other, and afforded more precise control of solvent composition with less delay volume than the Varian pump operated alone. A larger injection volume of 40 µL was adopted for this method as it did not cause a significant increase in peak width. This

method (configuration **1h**) was subsequently used for analysis of liquid fractions during SPE method development.

Gradient chromatography was considerably simpler on Instrument #2, which is designed for lower flow rates. After a pump modification suggested by Agilent [81], the pump had an internal delay volume of only 200 µL.

The Agilent XDB C_{18} column (4.6 × 50 mm, 1.8 µm) supplied with the LC-MS/MS system was tested with CP, KCP and CBP, and compared to the Supelco C_8 column already used on Instrument #1. Again, the C_8 column showed better retention for metabolites than C_{18}; therefore the C_8 was used to develop an LC method (configuration **2a**).

Agilent Masshunter also provides an automatic delay volume reduction feature, which was enabled in configurations **2a** through **2c**; however, it was found to complicate trace analysis by increasing carryover. The solvent delay introduced by disabling this feature was offset by starting the gradient 1 minute earlier (configuration **2d**).

An additional Agilent Eclipse Plus C_8 column (30 mm × 2.1 mm, 1.8 µm) was made available at a late stage of method development, and an alternate method was designed for this column as well (configuration **2e**).

To assess carryover in the Agilent autosampler, a 20 µg/L standard solution of CP was analyzed twice, followed by two deionized water blanks. Next, the 1 mg/L standard of CP was analyzed twice, followed by two more deionized water blanks. Finally, the autosampler and column were equilibrated first with 90% isopropanol and then again with mobile phase before analyzing two more blanks.

2.4.3. Matrix effects

Under isocratic LC conditions (configuration **1e**), an assessment of matrix effects for a "dilute-and-shoot" scenario was carried out by post-column infusion of a standard solution containing 50 µg/L each of CP, KCP and CBP, at a rate of 5 µL/min. The MS/MS signal for each analyte was monitored while injecting onto the column 1) a 90:10 water:methanol solvent blank, and 2) 1% synthetic urine in 80:20 water:methanol. The resulting chromatograms were compared with retention times of CP, KCP and CBP for the same LC method.

2.4.4. SPE method development

A protocol for CP extraction developed by Sottani *et al*. [79] was used as the starting point for SPE method development. The literature method used Varian Bond Elut C18 cartridges, which contain a highly endcapped silica-based sorbent functionalized with octadecyl groups for retention of nonpolar compounds. In this original method, the cartridge was conditioned with 3 mL of methanol. A 5 mL aliquot of urine was adjusted to pH 7.0 using phosphate buffered saline solution (PBS), spiked with internal standard, and then loaded onto the cartridge. The sorbent was washed with 1 mL of PBS and air-dried, and the analytes eluted with ethyl acetate. After evaporation the residue was reconstituted in 200 µL mobile phase, and 20 µL were injected onto the LC-MS/MS system.

For the present study, initial SPE method development was carried out instead on Varian Bond Elut LMS cartridges, which are based on a styrene divinylbenzene polymer. This phase is also designed to retain compounds of low polarity. A mock urine sample was prepared from the 1 mg/L stock solutions, diluted in synthetic urine, for total concentrations of 1 µg/L of CP, and 20 µg/L each of KCP and CBP. The literature SPE method was tested and then adapted to suite the present study's needs (**Table 2.1**, scheme **A**).

SPE scheme	Conditioning	Loading	Washing	Elution
A (literature) [79]	MeOH (2 mL), then 40 mM PBS (2 mL)	Sample (5 mL) + 40 mM PBS (2 mL)	40 mM PBS (2 mL), dry under vacuum	Ethyl acetate (5 mL)
B (acid variation)	MeOH (2 mL), then 50 mM phosphoric acid (2 mL)	Sample (5 mL) + 50 mM phosphoric acid (2 mL)	50 mM phosphoric acid (2 mL), dry under vacuum	Ethyl acetate (5 mL)
C (weaker acid)	MeOH (2 mL), then 1 mM phosphoric acid (2 mL)	Sample (5 mL) + 1 mM phosphoric acid (2 mL)	1 mM phosphoric acid (2 mL), dry under vacuum	Ethyl acetate (5 mL)
D (adapted for acid sensitivity)	MeOH (2 mL), then 50 mM phosphoric acid (2 mL)	Sample (5 mL) + 1 M phosphoric acid (0.2 mL), *mixed right before loading*	1 mM phosphoric acid (2 mL), then 100 mM PBS (1 mL), dry under vacuum	Ethyl acetate (5 mL)
E (elution solvent adjusted)	MeOH (2 mL), then 50 mM phosphoric acid (2 mL)	Sample (5 mL) + 1 M phosphoric acid (0.2 mL), *mixed right before loading*	1 mM phosphoric acid (2 mL), then 100 mM PBS (1 mL), dry under vacuum	Methanol (5 mL)

Table 2.1: Summary of SPE methods tested. PBS = phosphate-buffered saline solution, pH = 7.0. Schemes A-C were only tested as far as the washing steps; schemes D-E were tested completely.

The first step in evaluating an SPE method was to assess whether analytes stay on the sorbent during loading, or break through prematurely. The cartridge was conditioned as required, with the last 1 mL of conditioning solvent collected as a blank. The mock urine sample's pH was adjusted (**Table 2.1**, "Loading" column), and then 10 mL of the sample was loaded, collecting aliquots of the liquid eluted as *breakthrough fractions*. A vial of

pre-treated sample was also set aside as a control. The fractions and control were each analyzed by LC-MS/MS (configuration **1h**). In some trials, the LC method ran at a lower flow rate (configuration **1i**) due to increased column back pressure. The literature SPE method (scheme **A**) was then adapted to use phosphoric acid instead of PBS to improve metabolite retention (scheme **B**).

Next, the stability of CBP in acid was evaluated in order to design a method that would avoid degradation during extraction. A vial was prepared containing 20 µg/L of CBP and 2.5 mM phosphoric acid in water, along with a control vial that did not contain acid. The samples were stored at 4°C overnight (17.5 h) and then analyzed. Similar tests were carried out with a pH 4 potassium hydrogen phthalate buffer (5 mM) and a pH 4.3 ammonium acetate solution (5 mM). The samples were analyzed by LC-MS/MS (configuration **1h**). In order to correct for matrix effects, additional control vials were prepared in which the acid or buffer was only added immediately before analysis.

In order to evaluate CBP retention under acidic conditions independently from CBP degradation, the breakthrough fractions were taken again using a weaker 1 mM phosphoric acid concentration for equilibration and sample pH adjustment (scheme **C**). A portion of the pH-adjusted sample was set aside as the control. Each collected fraction, as well as the control, was then buffered by withdrawing 800 µL with a micropipette and adding it to 800 µL of 5 mM PBS (pH 7.0) in an HPLC vial. The fractions were then analyzed on the same day by LC-MS/MS (configuration **1h**).

Four additional sorbent types (Varian Bond Elut C18, C18-INT, C18-OH, and Agilent OPT) were screened by taking breakthrough fractions of the mock urine sample using scheme **A**, to investigate whether analytes could be retained at neutral pH. The C18-INT cartridges were selected at this stage as they showed less KCP and CBP in the breakthrough fractions.

In scheme **D**, phosphoric acid was added to the mock sample only moments before loading. The cartridge was washed first with a weaker 1 mM phosphoric acid solution, and then with 1 mL of 100 mM PBS before drying under vacuum.

Since scheme **D** was the first method which showed sufficient stability and retention for CBP, the elution step was also tested for this method. After the conditioning, loading, washing and drying steps, five 1 mL portions of ethyl acetate were passed through the cartridge and collected separately. Each was evaporated to dryness under a stream of nitrogen, and then reconstituted in 200 µL of 100 mM ammonium acetate, vortexed for 1 minute, and transferred to an HPLC vial with a 250 µL insert, for LC-MS/MS analysis. Due to poor elution of the metabolites, the same procedure was also tested with great success using methanol as the elution solvent (scheme **E**).

3. Results and discussion

Method development was focused on four key experimental stages – sample handling, sample preparation by solid-phase extraction, separation by liquid chromatography, and detection by tandem mass spectrometry – in order to devise a protocol for trace analysis of cyclophosphamide and metabolites in urine. The details of mass spectrometry are reported first, since MS was used to detect the analytes during all other stages of method development. Next, the liquid chromatography methods are presented, and limitations of the available instruments are also noted and discussed. The resulting LC-MS/MS methods are evaluated with respect to matrix effects and to the target detection levels for the analytes. Factors affecting stability of the analytes in urine samples are discussed, and taken into consideration as a part of method development for solid-phase extraction. Finally, the proposed methods are summarized and situated in the context of the goals of this study.

3.1. MS method development

Most of the MS method development was carried out on Instrument #1. When CP was examined using all three ion sources of the ABI MS, the ESI source gave superior signal strength by more than an order of magnitude compared to APPI and APCI. Since the detection limit of CP is critical for this method, ESI was selected and the other ion sources were not investigated further. The mass spectra and optimal parameters are presented here, followed by details of ion fragments used for MS/MS detection.

3.1.1. Optimization of MS parameters

In neat 50:50 water:methanol on Instrument #1, the $[CP + H]^+$ ion at *m/z* 261 showed lower intensity compared to $[CP + Na]^+$ at *m/z* 283 (**Figure 3.1a**). Both KCP and CBP showed very poor intensity for the molecular ion compared to the sodiated peak under the same conditions (**Figure 3.2a**, **Figure 3.3a**). Although these $[M + Na]^+$ peaks were strong for all three analytes, repeatability of sodiated peaks would be expected to be poor

due to a dependence on solvent conditions and on the origin of sodium ion impurities [71]. Therefore it was preferable to analyze the molecular ion $[M + H]^+$ in all cases. With addition of ammonium acetate to the mobile phase, all analytes showed improved intensity in Q1 for $[M + H]^+$. This justified an investigation into the optimal additive conditions to observe the molecular ion.

Formic acid and ammonium acetate were considered as solvent additives since both are volatile and commonly used in LC-ESI-MS methods. For all three analytes, ammonium acetate showed significant signal enhancement (**Figure 3.4**). Formic acid caused no enhancement for CP and moderate enhancement for CBP and KCP (**Figure 3.5**). For CP and CBP, 0.25-0.5 mM ammonium acetate was optimal; for KCP, 0.5-1 mM was optimal. An overall additive concentration near 0.5 mM was therefore ideal. After this optimization, the molecular ion was the most abundant peak in all three spectra, and the sodiated ion was less prominent (**Figure 3.1b**, **Figure 3.2b**, **Figure 3.3b**).

During later chromatography experiments, the extracted ion chromatograms for KCP and CBP showed high background noise that had not manifested without a solvent gradient. Since the MS parameters were selected to maximize signal intensity during a constant syringe infusion, the optimization procedure may have favoured conditions where the highest signal had a large noise component. Baseline noise improved considerably after the second MS optimization based on S/N. Several parameters were revised (configuration **1h**), including lower values for temperature, ion spray voltage and gas flows.

On Instrument #2, the MassHunter software provided an automatic optimizer for MS parameters. These conditions were useful enough to proceed with LC method development, however, a manual optimization via syringe infusion produced far superior MS results. Although parameters were again selected for high signal intensity, this did not lead to any problems with baseline noise on the Agilent MS.

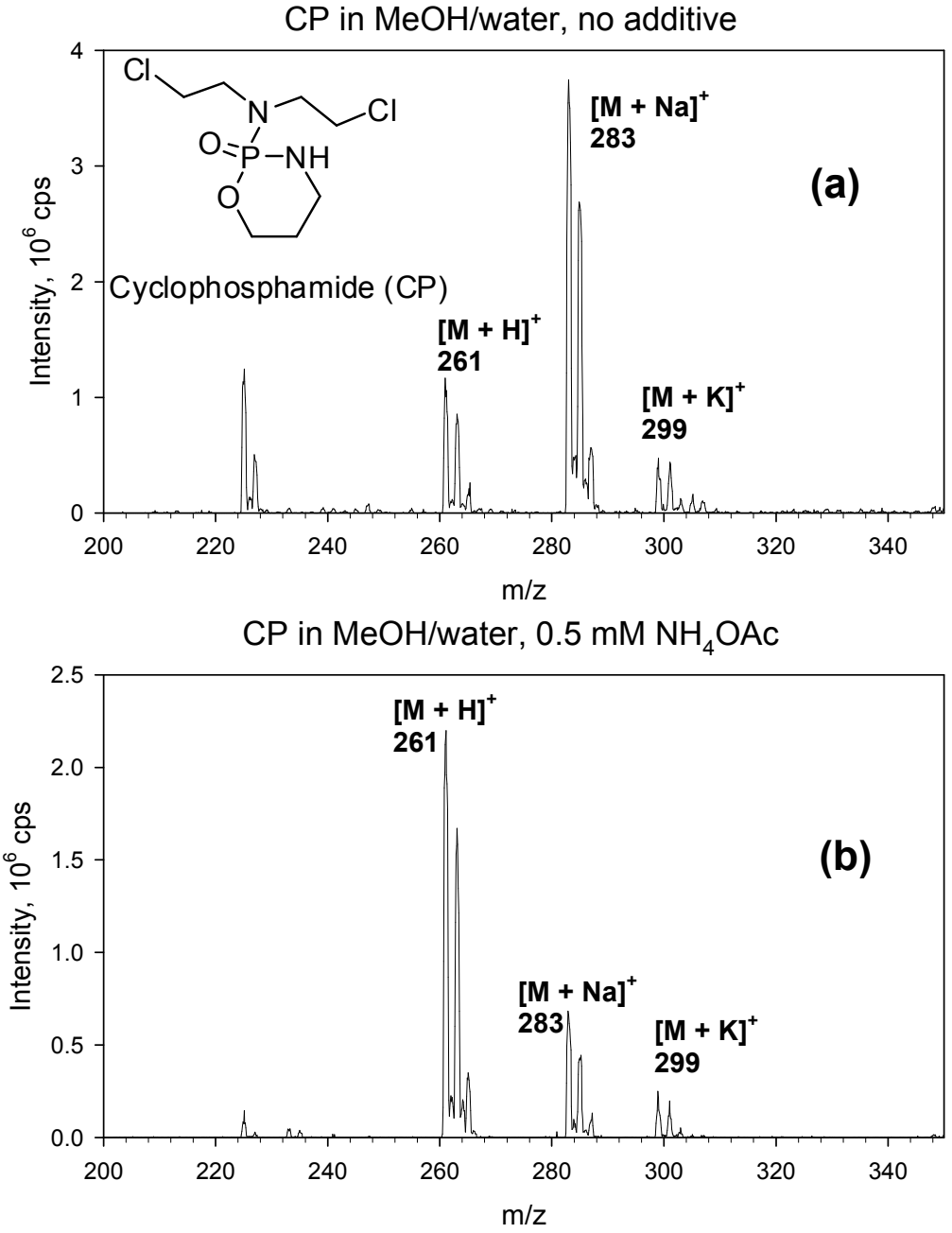

Figure 3.1: Q1 Mass spectra of cyclophosphamide (CP), background-corrected, in (a) neat 50:50 water:methanol by infusion (configuration **1bi**) and (b) 55:45 water:methanol with 0.5 mM ammonium acetate during isocratic chromatography (configuration **1d**).

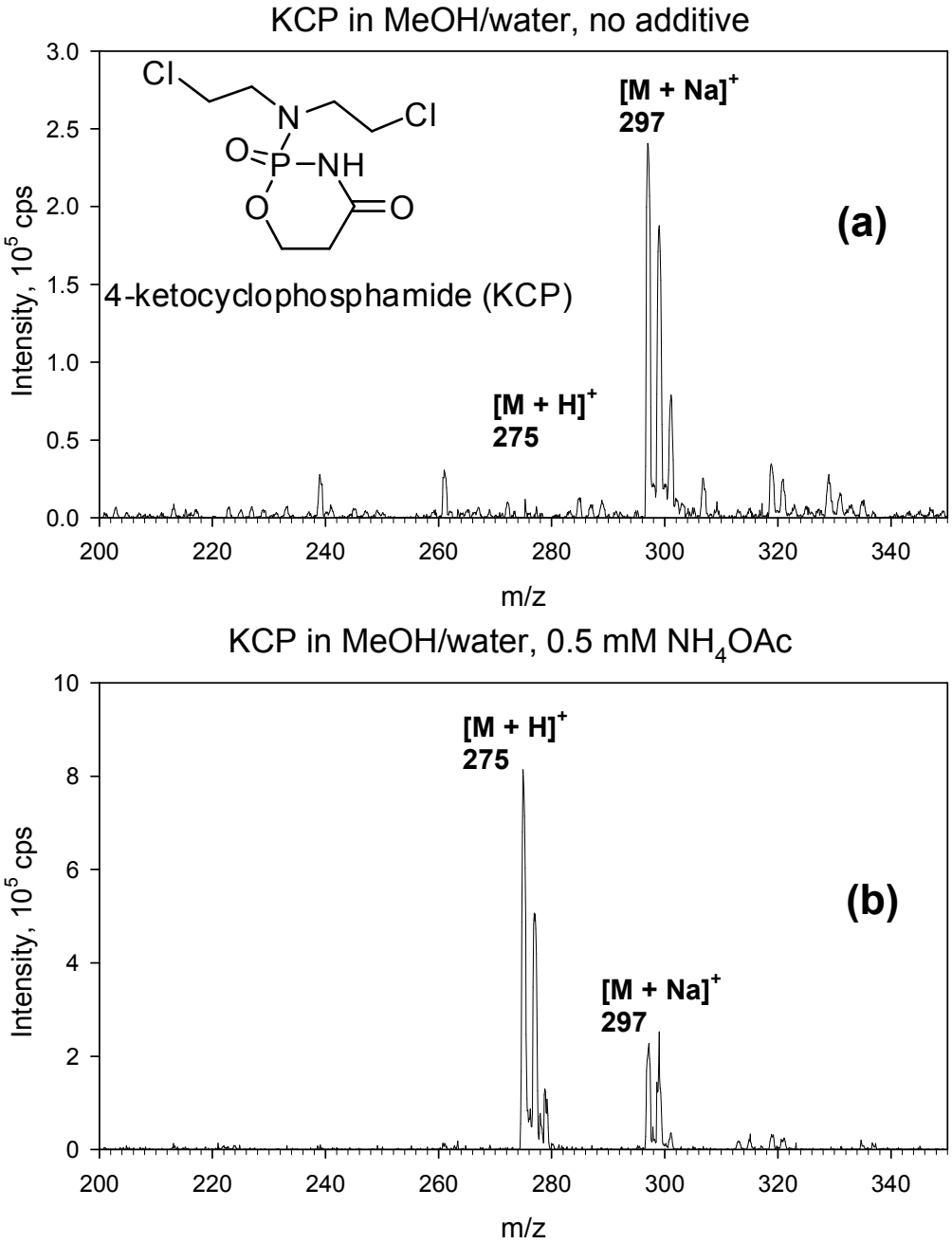

Figure 3.2: Mass spectra of 4-ketocyclophosphamide (KCP) in Q1, background-corrected, in (a) neat 50:50 water:methanol by infusion (configuration **1bii**) and (b) 55:45 water:methanol with 0.5 mM ammonium acetate during isocratic chromatography (configuration **1d**).

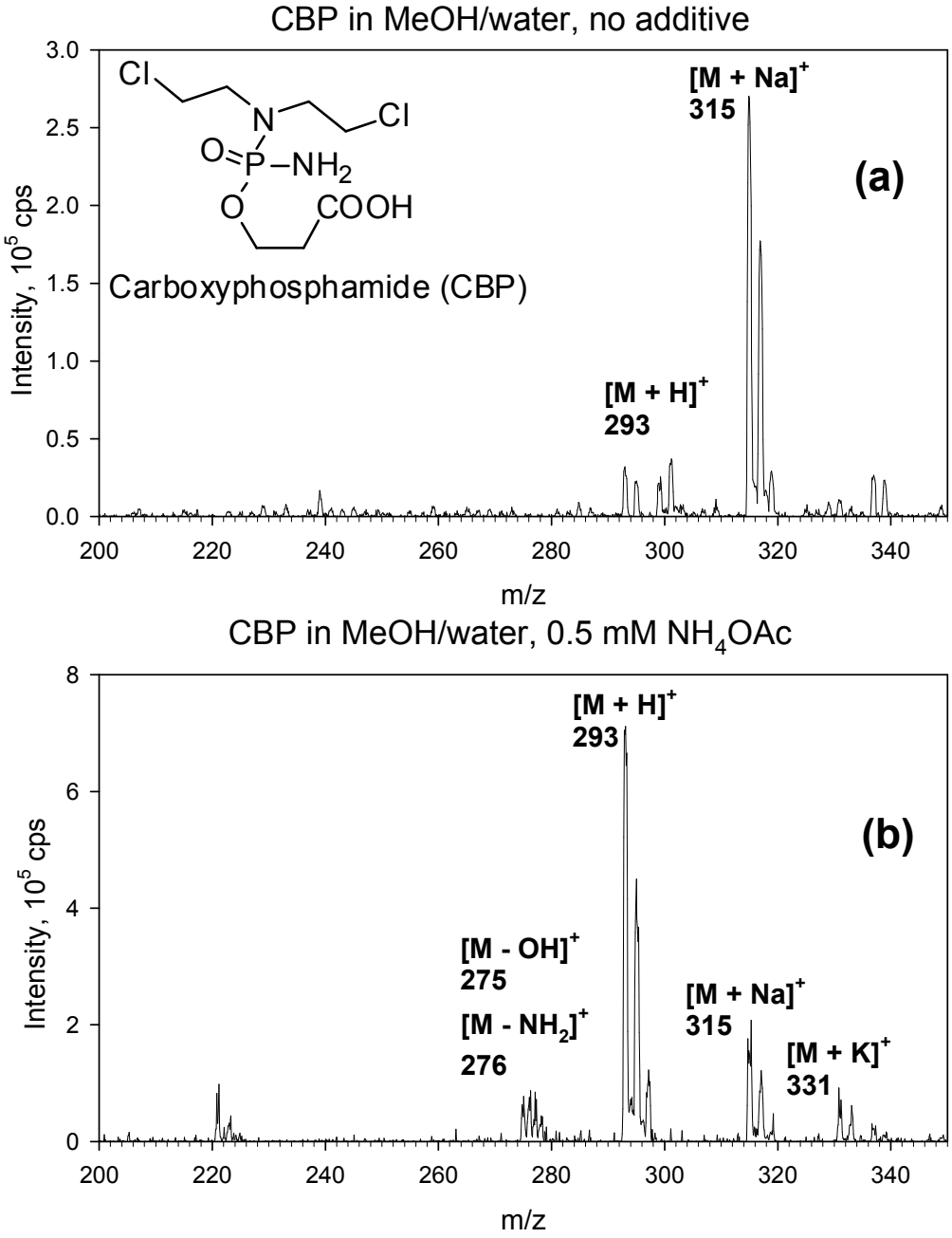

Figure 3.3: Q1 mass spectra of carboxyphosphamide (CBP)in Q1, background-corrected, in (a) neat 50:50 water:methanol by syringe infusion (configuration **1biii**) and (b) 55:45 water:methanol with 0.5 mM ammonium acetate during isocratic chromatography (configuration **1d**).

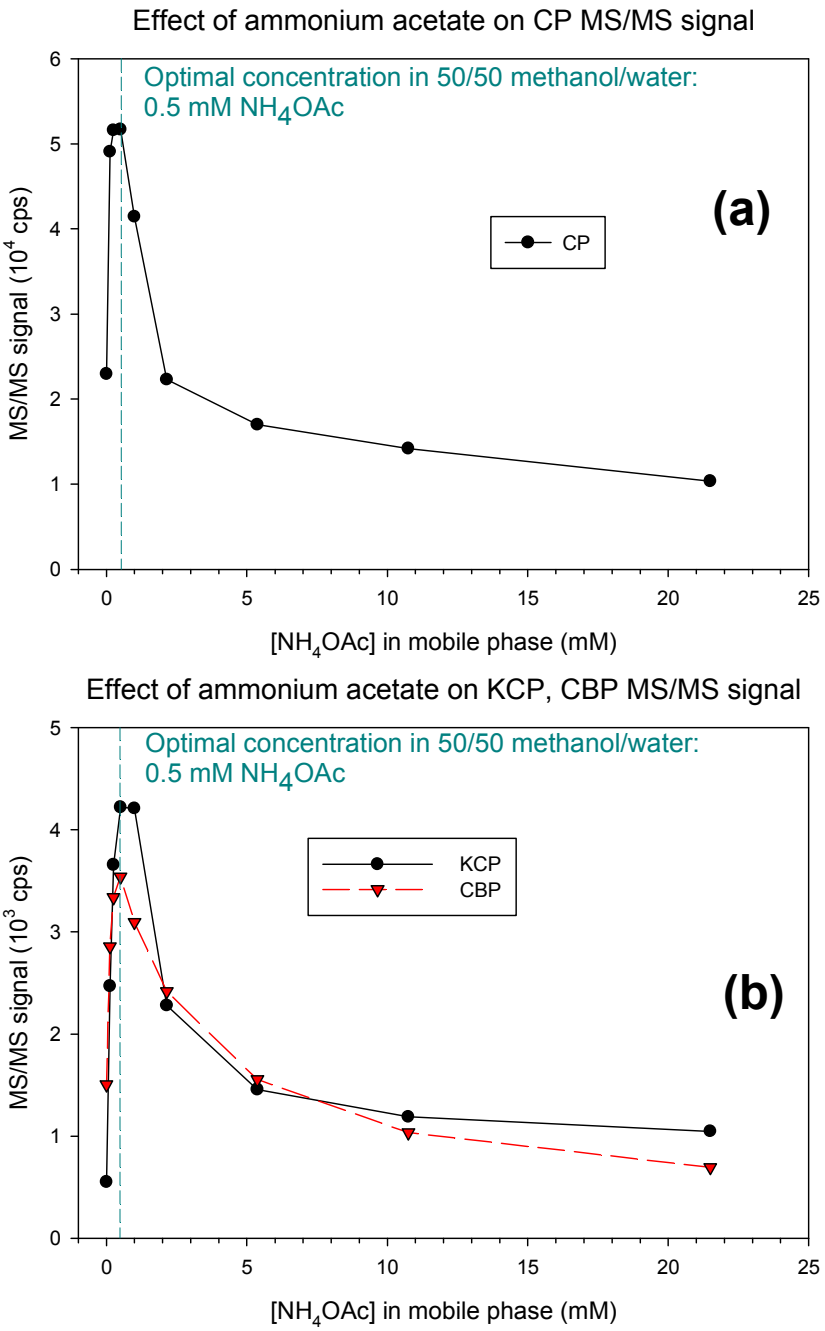

Figure 3.4: Investigation of ammonium acetate additive for ESI-MS (configuration **1c**). Analytes were infused at 1 ng/min.

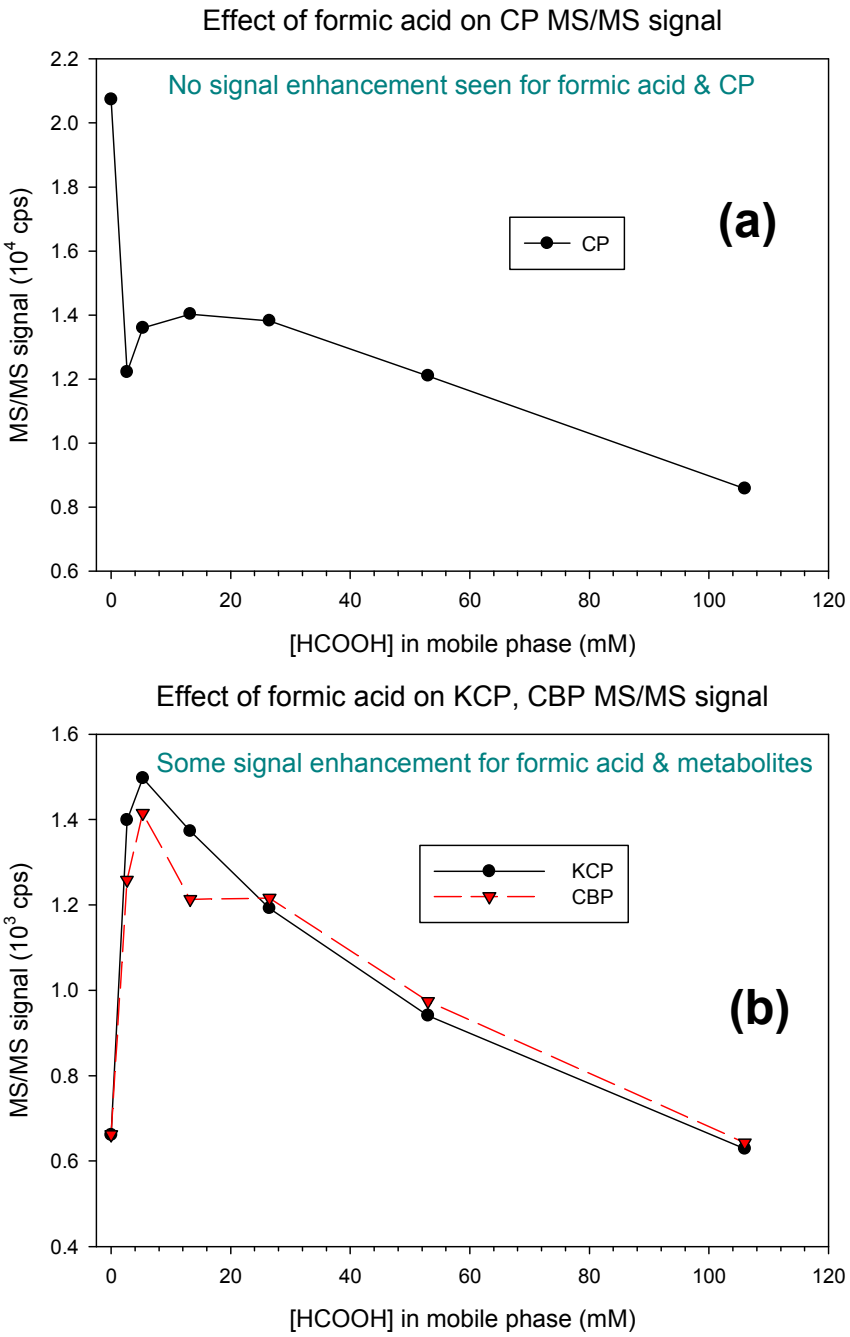

Figure 3.5: Investigation of formic acid additive for ESI-MS (configuration **1c**). Analytes were infused at 1 ng/min.

3.1.2. MS/MS transitions

The CP fragment at *m/z* 261 → 140 is known to represent cleavage of the P–N bond and neutral loss of the ring with dehydrogenation of the C–N bond (**Figure 3.6a**) [82]. The *m/z* 63 fragment originally used for KCP most likely represents the chloroethyl fragment after C–N cleavage; this same fragment is also seen from CP and CBP, especially at higher collision energies.

An MS/MS fragment *m/z* 277 → 221 is reported for KCP's metabolic precursor OHCP (**Figure 3.6d**), via ring-opening tautomerization to aldophosphamide and fragmentation of the phosphate C–O bond [82]. This could also be the structure of the CBP fragment at *m/z* 293 → 221 (**Figure 3.6c**) via fragmentation of the P–O bond with neutral loss of acrylic acid. However, the mechanism of arriving at the same ion from KCP (**Figure 3.6b**) is less clear. Since KCP has no ring-opened tautomer, fragmentation to *m/z* 221 would require both the phosphate C–O and the amide P–N bonds to fragment with redistribution of protons.

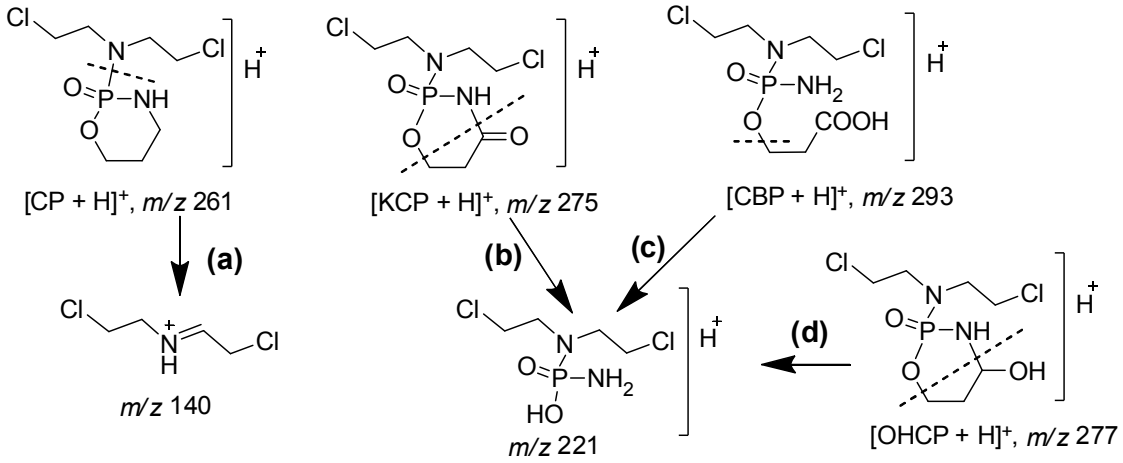

Figure 3.6: MS/MS fragments utilized in this study for (a) CP, (b) KCP, and (c) CBP; structures are based on fragments reported in the literature for CP and (d) OHCP [82].

Under optimized additive conditions a $[CBP - OH]^+$ ion appeared at *m/z* 275 in the Q1 spectrum of CBP (**Figure 3.3b**), at about 10% the abundance of the molecular ion. $[CBP - OH]^+$ is isomeric to $[KCP + H]^+$, and can also produce similar fragments. Thus a small peak appeared on the *m/z* 275 → 221 MRM channel for KCP, whenever CBP eluted in a chromatogram – a "crosstalk" effect (e.g. **Figure 3.12**, inset). The same effect was seen on both MS instruments. In contrast, there was no crosstalk between CP and either metabolite.

The MRM transition for KCP at *m/z* 275 → 63 also showed the crosstalk effect, albeit to a lesser extent (e.g. **Figure 3.10**, inset). Although this ion was selected when optimizing MS parameters based on signal strength, it was later found to show high background noise under chromatographic conditions. This ion also tended to show "ghost" peaks in chromatography, possibly due to impurities from degradation of the analyte standards. A better signal-to-noise ratio was achieved with the *m/z* 275 → 221 ion, which showed no extraneous peaks other than the crosstalk with CBP.

The d_4-CP obtained from Niomech was deuterated entirely on one of the two chloroethyl side chains. This asymmetric deuteration was confirmed by MS/MS based on the respective fragments for $[CH_2CH_2Cl]^+$ and $[CD_2CD_2Cl]^+$ at *m/z* 265 → 63 and *m/z* 265 → 67. The CP fragment at *m/z* 261 → 140 (**Figure 3.7a**) appears as two distinct peaks in d_4-CP, at *m/z* 265 → 144 and *m/z* 265 → 143 (**Figure 3.7c-d**). These peaks are consistent with the reported dehydrogenation of this fragment, since the loss of ^2D from one side chain would lead to *m/z* 143, and the loss of ^1H from the other side chain would lead to *m/z* 144. However the *m/z* 144 fragment was also observed to be twice as abundant, which suggests that other mechanisms may be involved.

If this isomer of d_4-CP is to be used as an internal standard, the choice of MS/MS peak should be carefully considered. Due to the natural 3:1 isotope ratio of ^{35}Cl and ^{37}Cl, all samples of CP will contain some $^{37}Cl_2$-CP at *m/z* 265. This molecular ion will also

fragment to *m/z* 144 (**Figure 3.7b**), presenting an isobaric interference with the internal standard's *m/z* 265 → 144 ion. However, the fragment at *m/z* 265 → 143 is unique to d_4-CP, since there is no mechanism to produce that fragment mass from $^{37}Cl_2$-CP.

An alternative molecular ion for d_4-CP is at *m/z* 267, corresponding to ^{37}Cl-d_4-CP, which has no isobaric interference with the most abundant isotopic peaks of unlabeled CP. This molecular ion has analogous fragments at *m/z* 146 and 145 (**Figure 3.7e-f**). The *m/z* 267 → 146 fragment was ultimately selected as the best interference-free ion for d_4-CP, as it showed better signal strength than *m/z* 265 → 143.

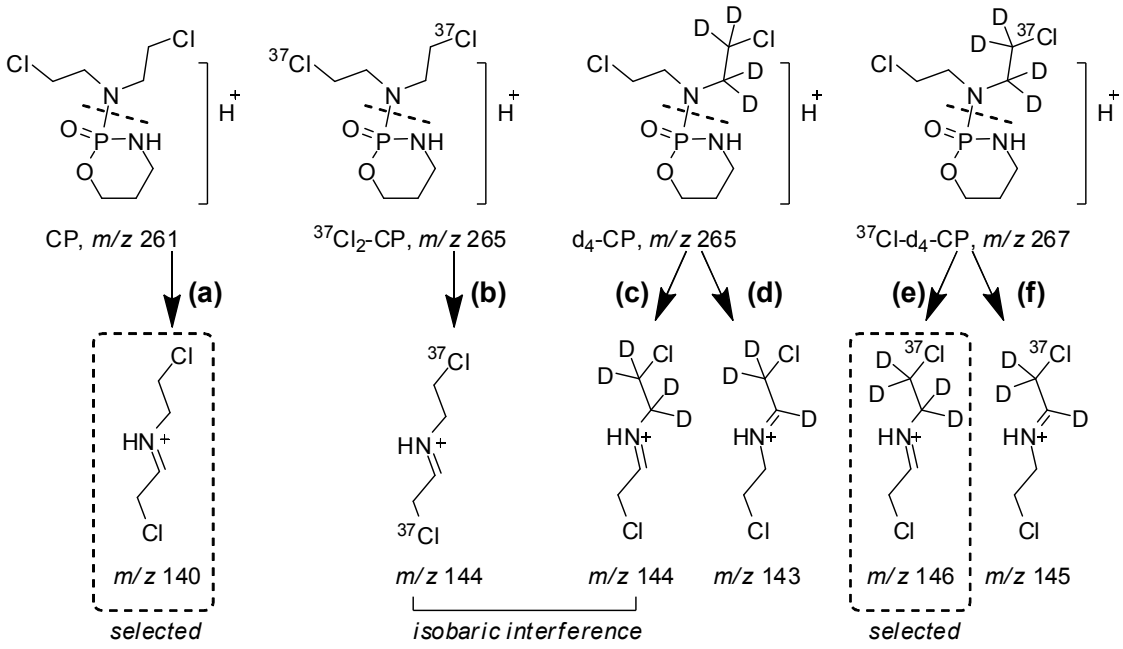

Figure 3.7: Isobaric interferences between CP and d_4-CP. The selected MS/MS transition for CP (a) is also seen in its $^{37}Cl_2$ isotopomer (b), and this interferes with analysis of d_4-CP (c). The alternative fragment (d) is interference free, as are the fragments of ^{37}Cl-d_4-CP (e, f).

3.2. Chromatographic method development

Both isocratic and gradient liquid chromatography were evaluated to separate analytes from each other and from the matrix. Overall the methods used on Instrument #1 were intended to produce the best available separation on older HPLC equipment that was not designed for the low flow rates typical to LC-MS. Method development on Instrument #2 was considerably more straightforward, as the system was designed to work as an integrated unit. Both instruments experienced some degree of sample carryover with CP.

3.2.1. Isocratic chromatography

In isocratic runs on Instrument #1, the ammonium acetate additive helped to stabilize retention times and signal strength for all three analytes compared to neat solvent. The pH of aqueous ammonium acetate solutions is close to 7; at this pH, CBP would be completely deprotonated and KCP partly ionized. Therefore it is not surprising that CBP eluted much earlier than either CP or KCP with neutral ammonium acetate as an additive. Retention of both metabolites was improved by adjusting the additive pH to 5 with acetic acid.

Any increase in the total additive concentration above 0.5 mM tended to reduce signal strength without providing any noticeable improvements to the chromatography. Meanwhile, at lower additive concentrations than 0.5 mM, the retention times were less stable from run to run. Based on this optimization, a concentration of 0.5 mM ammonium acetate was used as a guideline for all LC-MS methods.

Under these conditions, 45% methanol was selected (configuration **1d**) as the best balance between sufficient retention of the first peak (CBP) and reasonable elution time for the final peak (CP). In the chromatogram (**Figure 3.8**), peaks for CBP and KCP are well enough resolved to distinguish between genuine KCP and the false peak from crosstalk with CBP (inset).

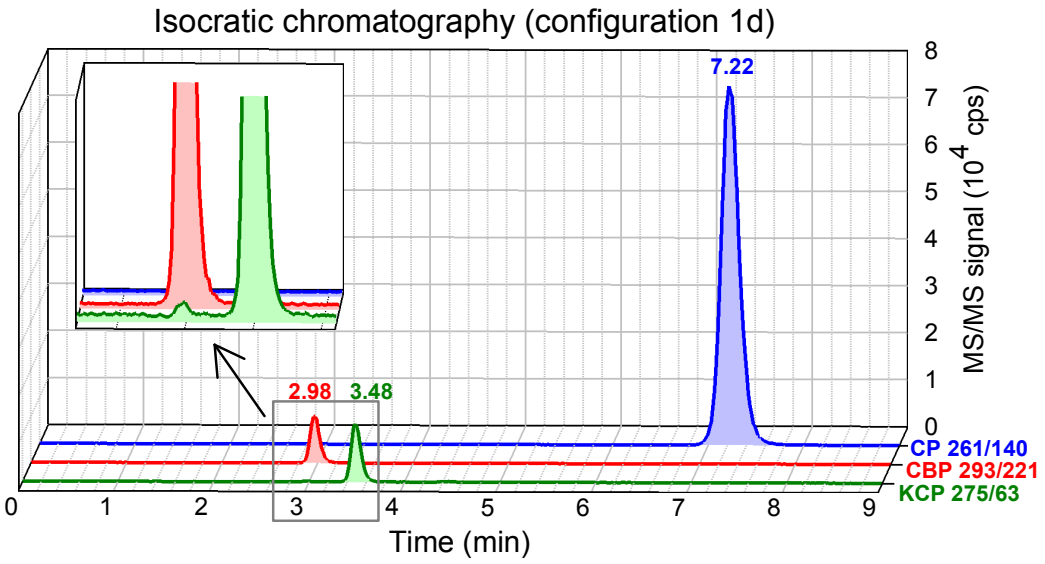

Figure 3.8: Chromatogram, isocratic conditions (configuration **1d**). 500 pg each of CP, KCP and CBP injected on Supelco C_8 Ascentis column. Inset: KCP is well-resolved from the false peak due to crosstalk from CBP.

The isocratic chromatogram shows typical features of the "general elution problem" – the peak for CP is delayed by several minutes after the metabolites, and is significantly broadened. If the mobile phase organic composition is decreased, the analytes separate better from the matrix, but at the cost of broadening the CP peak and increasing run time unacceptably. At higher organic composition the metabolites elute too close to the matrix, rendering the method unsuitable for urine samples.

Another issue affecting isocratic chromatography with biological samples is that of late-eluting matrix components [75]. Even if the last analyte of interest has eluted within 8 minutes of injection, other matrix components may be trapped on the column and elute at erratic times after a new injection has started. This can adversely impact reproducibility. In a well-designed gradient method, any late-eluting components will wash off the column at high organic composition before re-equilibration.

3.2.2. Gradient chromatography

An ideal gradient for this experiment would start with a low percentage of organic solvent to keep CBP and KCP on the column while the highly polar matrix components elute. However, once this is accomplished, it is best to elute these compounds with a rapid increase in organic composition. The higher percentage of organic solvent provides better ESI-MS sensitivity [71], while the fast gradient helps to reduce peak broadening.

As noted earlier, the Varian Star 9010 ternary pump used with Instrument #1 had a stated delay volume of 1 mL. The guard column and analytical column had a calculated internal volume of 0.60 mL. When the total delay was visualized using an acetate tracer ion, it was observed that an abrupt solvent change programmed into this pump at t=0 did not begin to reach the end of the LC column until at least 1.2 mL had passed through it (**Figure 1.1**). From 1.2-2.1 mL, the tracer signal gradually increased towards equilibrium, but did not fully stabilize until over 3 mL of solvent had exited the pump.

At flow rates optimal for ESI (200-300 µL/min), this delay volume was not acceptable for a gradient, as it introduced an undesired isocratic hold time into every method. On the LC columns available to us, a long isocratic hold at low organic composition tended to elute CBP and KCP as broad peaks with poor MS sensitivity, as any increase in the organic fraction of the mobile phase did not take effect until after the hold.

Within the limitations of this LC pump, the best gradient was obtained by starting at 20% methanol (configuration **1f**), which was sufficiently weak that all analytes were retained on the column for the entire delay volume. Based on the delay volume experiment, the sharp gradient to 75% methanol from 0 to 0.5 min would have eluted from the column as a broad gradient from 4 to 7 min. This is reflected in the chromatogram, as the first peak (CBP) eluted at 6.9 min (**Figure 3.10**). The genuine peak for KCP was baseline-resolved from the CBP crosstalk peak (inset).

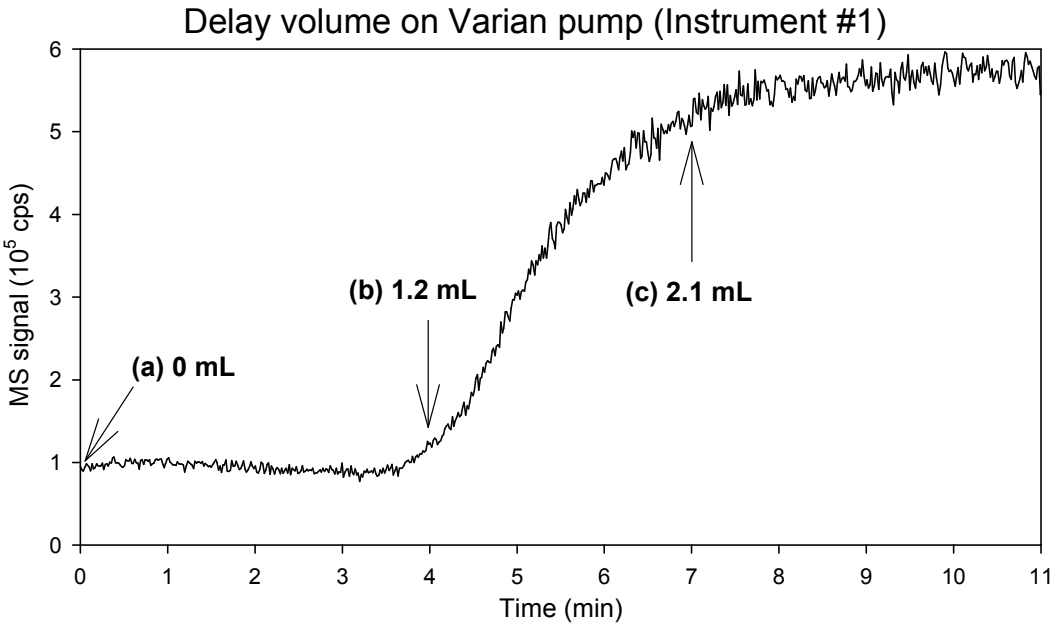

Figure 3.9: Delay volume on Varian gradient pump (Instrument #1) at 300 µL/min, visualized by monitoring the acetate isotopic peak at *m/z* -60 as a tracer ion. Ammonium acetate was introduced into the mobile phase at (a) 0 min; the tracer signal did not visibly increase until (b) 4 min, and was still not at equilibrium by (c) 7 min.

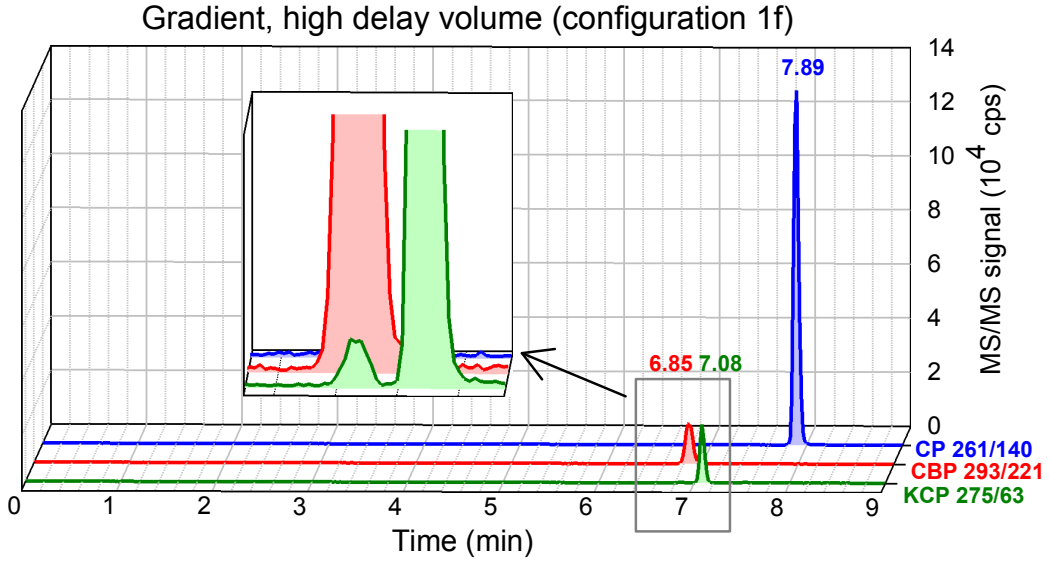

Figure 3.10: Chromatogram, gradient conditions with high delay volume (configuration **1f**). 500 pg each of CP, KCP and CBP injected on Supelco C_8 Ascentis column. Inset: KCP is baseline-resolved from the false peak due to crosstalk from CBP.

Overall this gradient method improved upon the isocratic method by providing ample separation from the matrix and by narrowing the CP peaks significantly. It would also be adaptable to situations where late-eluting interferences are problematic, by adding a wash segment at higher organic composition. However, the delay volume also necessitated a minimum re-equilibration time of 11 minutes after each run, and this time increases with the additional internal volume of an autosampler. As this would not have been ideal for a large batch of samples, a number of options were considered to reduce the delay and the total run time.

It was not possible to reduce delay volume by hardware modification, as the same components which imparted this delay volume were also responsible for stabilizing the pump pressure. Any attempt to bypass some of this volume resulted in massive pressure changes with each pump stroke. One way to skip this delay volume is to set up a delayed injection program; however, since the autosampler could synchronize only by contact closure with the Analyst software, there was no way to program such a method with reliable timing.

The dual-pump arrangement with a switching valve (configuration **1h**) produced a step gradient that bypassed the delay completely. The entire experiment was complete in 10 min, and re-equilibration was incorporated into the run time via the switching valve. This method also provided better matrix separation and less broadening than the isocratic method (**Figure 3.11**). Unfortunately the separation between CBP and KCP only barely sufficed to resolve genuine KCP from the CBP crosstalk peak (inset). Nonetheless, this configuration was used for analysis during SPE method development due to its faster turnaround time.

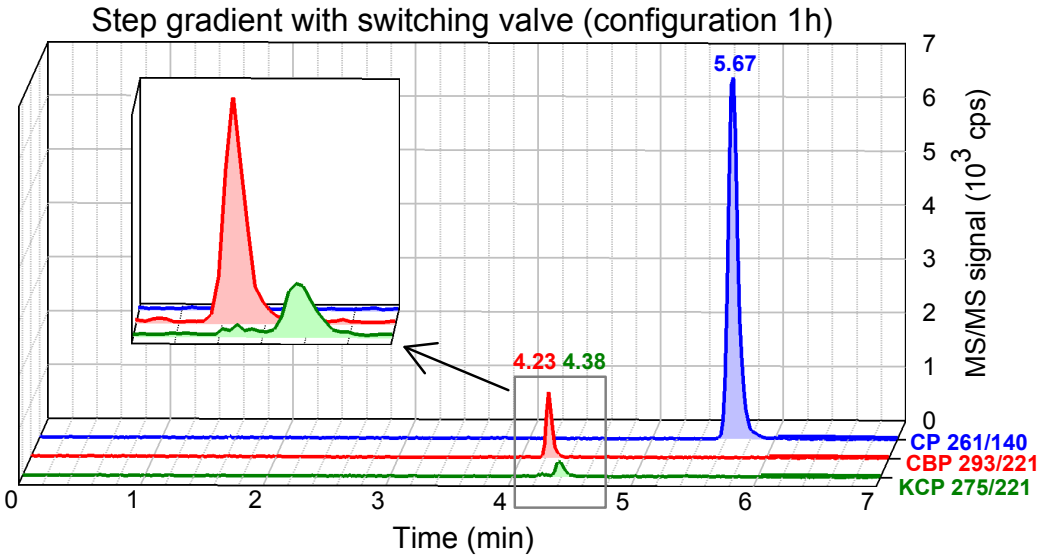

Figure 3.11: Chromatogram, step gradient with switching valve (configuration **1h**). 40 pg each of CP, KCP and CBP injected on Supelco C_8 Ascentis column. Inset: KCP elutes quite close to the false peak from CBP crosstalk.

On Instrument #2, delay volume was much less problematic. As the Agilent binary pump uses high-pressure mixing, its mixer and damper components play a very minimal role in stabilizing pump pressure, and the manufacturer provides instructions to bypass them. Following removal of the mixer and damper as described by Agilent [81], the total pump delay volume becomes 200 µL plus the flow path through the autosampler. Under certain conditions such as higher flow rates, or UV detection, this modification can produce visible irregularities in the baseline. This configuration did not cause any pressure fluctuations that were significant enough to affect the MS signal. Therefore it was possible to apply a rapid gradient to higher organic composition without any additional hardware.

The lowest delay volume on Instrument #2 was achieved using an automatic delay volume reduction feature, which excludes the autosampler from the flow path after analytes have eluted onto the column. Ultimately this feature was disabled in order to provide the autosampler with a better solvent rinse cycle between injections, which

helped to mitigate carryover problems. Even with the feature disabled, delay volume was not large enough to present any problems in method development.

Since the gradient composition with respect to time could be controlled more precisely on this instrument, it was not necessary to acidify the ammonium acetate additive for retention of the metabolites on the Supelco C_8 column (configuration **2d**). A short initial isocratic hold at 25% methanol was sufficient to separate CBP from the matrix, and a fast gradient to 60% methanol eluted CBP followed by KCP. Subsequently, a slower gradient to 75% methanol was used to reduce the lag time before CP eluted, and to improve the peak shape for CP. A final gradient to 95% methanol was used to wash out the column before re-equilibration. All three analytes had eluted after 4 min (**Figure 3.12**), and the total run time including re-equilibration was 9.5 min. The crosstalk of CBP and KCP (inset) was of no consequence since the peaks were fully resolved by LC.

An Agilent C_8 column was also made available for testing. Due to its 1.8 μm particle size, this column had higher back pressure and was operated at a lower flow of 250 μL/min. The mobile phase timetable was revised to start at 10% methanol for retention of CBP (configuration **1e**), followed again by a sharp rise to 60% methanol and a slower rise to 75%. An isocratic hold was added here to allow CP to elute fully before the 95% methanol wash. All analytes eluted within 3 min (**Figure 3.13**), and separation of CBP from KCP was again sufficient to avoid problems with signal crosstalk (inset). The total run time of 9.5 min could have possibly been reduced further, since the smaller column volume required less time to re-equilibrate.

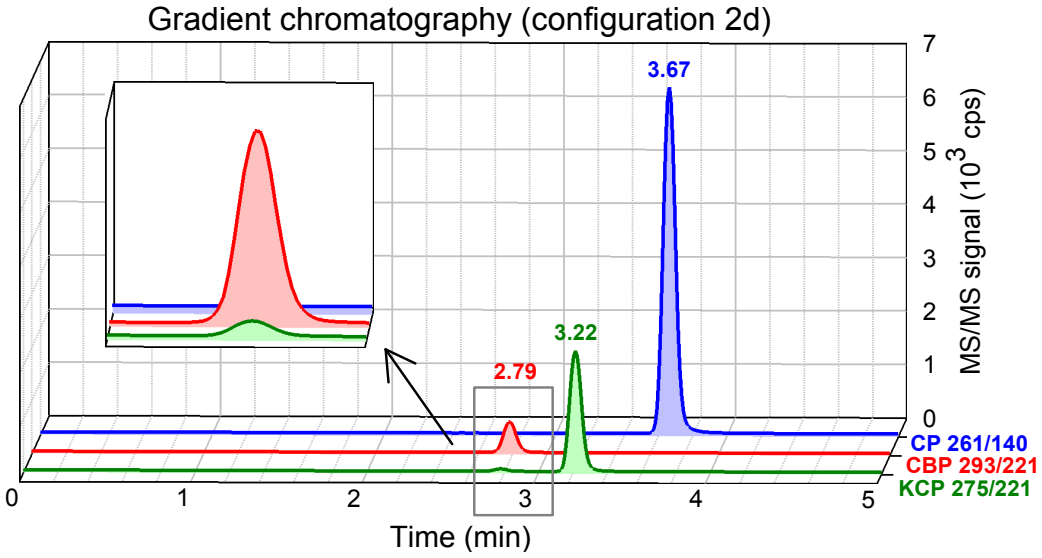

Figure 3.12: Chromatogram, gradient conditions on Instrument #2 (configuration **2d**) . 400 pg of CP and 1000 pg each of KCP & CBP injected on Supelco C_8 Ascentis column. Inset: Crosstalk from CBP is visible but does not interfere with KCP detection.

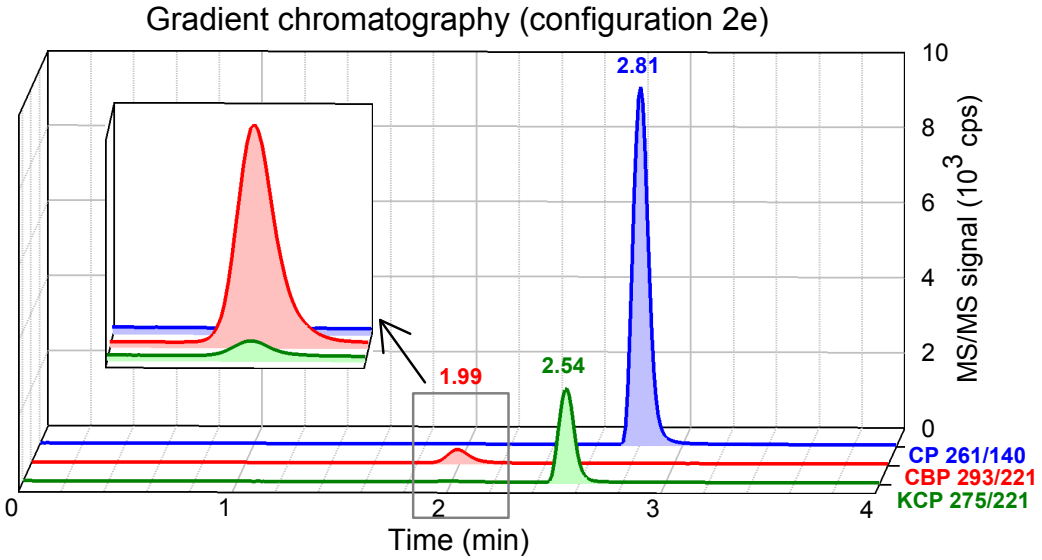

Figure 3.13 Chromatogram, gradient conditions on Instrument #2 (configuration **2e**). 400 pg of CP and 1000 pg each of KCP & CBP injected on Agilent Eclipse Plus C_8 column. Inset: Crosstalk from CBP is visible but does not interfere with KCP detection.

3.2.3. Carryover and contamination

The Varian autosampler (Instrument #1) showed considerable carryover for CP even after several blank solvent injections, and required frequent cleaning as a result. According to its user manual, the autosampler is factory-tested to meet a carryover specification of <0.5%. This would typically suffice for UV detection, which has lower sensitivity and a smaller dynamic range compared to MS/MS detection, but presents a problem with trace analysis. For comparison, the Agilent LC (Instrument #2) specifies <0.01% carryover, and was designed to be integrated with an MS/MS system.

One possible source of autosampler contamination was from prior use. In earlier experiments outside of our laboratory, the Varian autosampler had been used with considerably higher concentrations of both CP and the unrelated drug methotrexate (MTX), for LC-UV method development. In the present study, the MRM transition for MTX (*m/z* 455 → 308) was monitored in addition to CP while examining possible sources of carryover. After the autosampler needle was cleaned by sonication, MTX was detectable in six consecutive blank injections – even though MTX had never been handled in our laboratory.

The most repeatable procedure to reduce carryover and contamination on the Varian autosampler was to prepare a series of at least three deionized water vials with the septum removed, and make an injection from each in sequence. After the final vial, at least 15 injections of further blanks from vials with proper septa could be made without any false positives for CP. The CP contaminant peaks tended to recur after enough repeated blank injections, and were again eliminated with another series of wash vials. Thus, if Instrument #1 were used for trace analysis of CP in real samples, the protocol would require criteria to screen out false positives and reacquire data for the affected samples.

In contrast, the Agilent HPLC (Instrument #2) was much more robust with respect to carryover. One CP standard used to assess carryover (20 µg/L) was selected to be greater than the upper bound of typical CP concentrations in the urine of occupationally exposed workers. Deionized water blanks injected after this standard did not show any measurable carryover. A second standard (1 mg/L) was selected to represent the higher CP concentrations that would be analyzed for other experiments in the SoEH lab such as extracts from surface wipe samples. Chromatograms for the 1 mg/L standard showed significant peak tailing; subsequent blanks had an elevated baseline signal and exhibited carryover of CP. After a wash of 90% isopropanol through the autosampler and column, and re-equilibration to the mobile phase, it was once again possible to observe clean blanks.

The 0.01% carryover specification would suggest carryover of <2 ng/L for the 20 µg/L standard, and <100 ng/L for the 1 mg/L standard. Since the LOD for CP was close to 2 ng/L on this instrument, these results are consistent with the Agilent specification. Based on anticipated CP concentrations, there should be minimal difficulties with carryover between samples and standards for this experiment. However, carryover problems are expected if CP samples over 4 orders of magnitude above the LOD are analyzed regularly on the same apparatus. Therefore, any laboratory in which CP is handled over a large concentration range should consider setting a maximum allowable concentration per instrument, using dilutions when necessary. Alternately, cleaning procedures could be established to avoid carryover problems.

3.3. Evaluation of LC-MS/MS methods

3.3.1. Matrix effects

Post-column infusion provided a means to visualize matrix effects in synthetic urine. Since the analytes were added after the column at a constant rate, the chromatograms show a baseline signal that represents how the MS signal can vary independently of the actual analyte concentration. Both mobile phase composition and other components injected on the column can enhance or suppress this signal. A matrix effect can be identified in any region where the matrix blank chromatogram deviates from the neat solvent blank. Although there was no available opportunity to assess either the SPE method or any of the gradient methods using post-column infusion, the isocratic LC-MS/MS method is evaluated here as a proof of concept.

For the matrix blank, a dilution of 1% Surine was selected to match a literature "dilute-and-shoot" method for CP and metabolites [45]. For each analyte, the solvent blank injection shows a relatively constant signal (**Figure 3.14**). The 1% Surine injections display ion suppression in the region of 1.2-2.2 min for CP and KCP, and 1.2-1.8 min for CBP. In a reference chromatogram, the first peak (CBP) does not begin to elute until 2.8 min, by which point there is no visible matrix suppression.

As Surine is a proprietary brand of synthetic urine, information on its composition is not available. Typical organic compounds in urine include urea, creatinine, hippuric acid, citric acid, glucuronic acid, uric acid and creatine [83]. Abundant inorganic constituents include chloride, sodium, potassium, ammonia, calcium, magnesium, carbonate and various forms of sulphur and phosphorus. The non-volatile inorganic components would be expected to cause the most ion suppression in ESI-MS, and will tend to elute unretained with the solvent peak in reversed-phase chromatography.

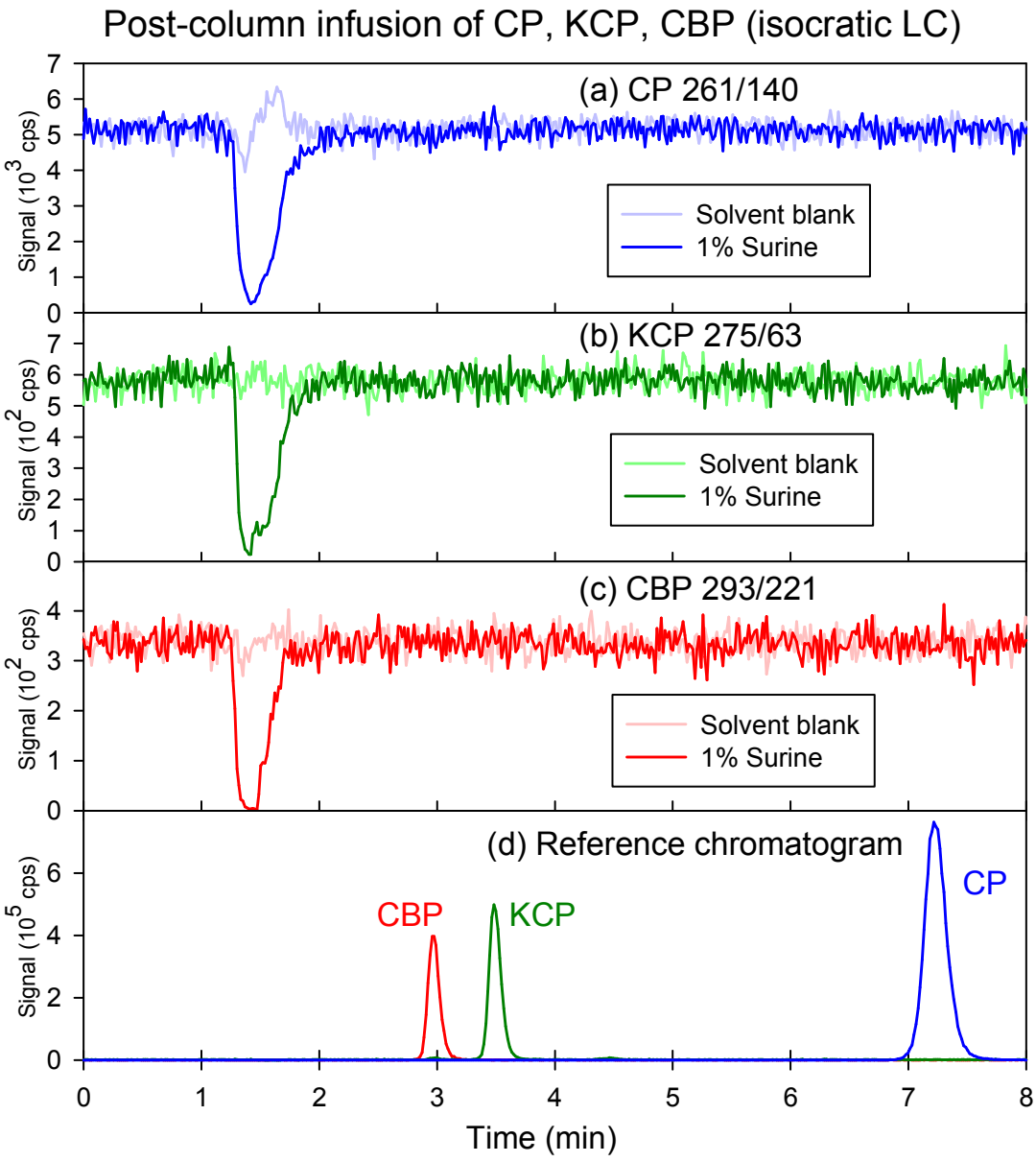

Figure 3.14: Post-column infusion for visualization of matrix effects during isocratic LC-MS (configuration **1e**). Analytes were infused at a rate of 250 pg/min. Extracted ion chromatograms are shown for (a) CP, (b) KCP, and (c) CBP following injections of a solvent blank and a 1% Surine matrix blank. Regions of ion suppression due to matrix effects were well-separated from (d) retention times of all analytes.

Post-column infusion was quite useful in confirming that all analyte peaks were sufficiently separated from the matrix components causing ion suppression. Over the course of several repeated runs, there was no sign of any late-interfering matrix effects; however, it may well be that Surine is not designed to contain any such compounds. A similar assessment would have to be carried out with the genuine urine samples in order to validate these results.

A dilute-and-shoot method was ultimately ruled out for this study as it would not have achieved the target detection limits. An assessment of matrix effects must ultimately represent the complete sample handling and analysis protocol. Therefore, after an extraction method is developed, a post-extraction matrix blank should be tested for matrix effects in a similar manner.

3.3.2. Limits of detection

For each configuration and analyte, the signal-to-noise ratio (S/N) was calculated using peak height and the standard deviation of adjacent background noise. The limit of detection (LOD) was estimated as the concentration of analyte required to produce a S/N of 3, and the limit of quantitation (LOQ) as the concentration required for a S/N of 10 (**Table 3.1**).

Due to limitations of instrument availability, it was not possible to test the selected LC-MS methods with a full range of calibration standards in order to calculate more rigorous values of LOD and LOQ. The values reported here are derived from method development experiments in which a small number of standard solutions were used repeatedly over the span of several days. Any degradation of analytes in the standards may have led to overestimates of LOD and LOQ, especially in the case of CBP.

Although the values in **Table 3.1** represent only standards made in neat solvents, they are useful in determining how the method must be adapted for urine samples of

occupationally exposed workers. The present study aimed to detect CP in urine at levels as low as 50 ng/L, as well as KCP and CBP as low as 2.5 ng/L. The LOD for CP is low enough that minimal sample preparation would be required – even a simple 10-fold dilute-and-shoot method might succeed.

However, based on the results for KCP and CBP, the sample preparation should include not only matrix cleanup but also pre-concentration. If sample volume were reduced by a factor of 25, then by configuration **2d** the method LOQ would be 0.17 ng/L for CP, 0.33 ng/L for KCP and 2.3 ng/L for CBP. Since Sottani et al. achieved a factor of 25 for CP in urine by SPE [79], this literature method was used as a starting point for development of an extraction protocol.

	CP		**KCP**		**CBP**	
Configuration (Appendix A)	*LOD (ng/L)*	*LOQ (ng/L)*	*LOD (ng/L)*	*LOQ (ng/L)*	*LOD (ng/L)*	*LOQ (ng/L)*
1d	4.1	14	96	321	68	226
1f	4.2	14	17	57	74	246
1h	1.5	5.0	31	103	12	39
2d	1.3	4.2	2.5	8.2	17	57
2e	3.0	9.9	8.1	27	18	59

Table 3.1: Instrument limits of detection (LOD) and quantitation (LOQ), estimated as the analyte concentration required to produce S/N of respectively 3 and 10.

3.4. Sample handling and preparation

Results are presented here for the pH sensitivity of CBP, and examined in the context of establishing a protocol for sample collection, handling and extraction. Preliminary SPE method development is reported, and further refinements are discussed.

3.4.1. Stability of analytes and urine samples

Based on the literature review and the observations of this study, CBP is considerably less stable than CP and KCP. All three overnight stability tests (17.5 h) for CBP revealed significant degradation under acidic conditions at 4°C. Based on peak areas, the residual concentration of CBP was 25% of its original value in a solution of phosphoric acid (1 mM). In solutions of pH 4 KHP buffer (5 mM) and pH 4.3 ammonium acetate (5 mM), the residual percentages of CBP were 28% and 38%.

These results are also comparable with the literature. When CBP was stored at pH 5.5 for 24 h, residual percentages were 45% at 25°C and 86% at 8°C as measured by ^{31}P NMR [44]. Since the stability of CBP decreases with falling pH from 8 to 5.8 [18], one would expect even faster degradation at pH 4. For CBP stored in urine at pH 7 for 24 h, the residual percentage was 89% at 25°C; and 93% at the same pH for 9 d at 8°C [44].

Based on these values, any CBP present in sufficiently acidic SPE extracts may degrade unacceptably during the hours or days leading to analysis, even if they are refrigerated or kept in a cooled autosampler. This was a particularly troublesome consideration when working with Instrument #1, as the Varian autosampler was not equipped with a cooling feature.

Since normal human urine pH ranges from 4.6 to 8.0 [84], the variable decomposition of CBP is also a concern for collection and storage of the samples. A number of clinical studies are now known to have underestimated rates of urinary excretion for CBP and its ifosfamide analogue due to incorrect sample storage temperatures, or to excessively long

storage periods before analysis [16, 18, 44]. One recommended protocol is to collect urine samples on ice in 6-8 h periods and freeze at -80°C as soon as possible, for no longer than 2 months prior to analysis [44]. An alternate or complementary approach could be to stabilize samples at collection time with pH 7 buffer to limit the rate of CBP degradation; this is only acceptable if the buffer does not interfere with subsequent sample preparation and analysis.

Aside from the analytes, urine itself also degrades during storage. A study of urine pH stability showed that at 25°C, a sample could shift from pH 7.0 to 8.5 in one day, and to pH 9.0 after a second day [85]. At 4°C, the pH shifted from 7.0 to 8.0 in 9 d, and at -20°C from 7.0 to 7.5 in 9 d. In particular, bacterial decomposition of acidic urea to ammonium carbonate can increase the pH and the buffering capacity of urine. A significant increase in urine pH would also be of concern, since KCP is reported to degrade at pH 7.8 and above [41].

3.4.2. SPE method development

In order to adapt the literature extraction method for CP in urine (Scheme **A**) [79], it was necessary to examine whether KCP and CBP could be extracted under the same SPE conditions. Ideally when the entire pre-treated sample has passed through the cartridge, and the matrix components have been removed with a wash, all analytes should still be on the cartridge. A poorly retained analyte will partially or completely break through the cartridge during these steps, removing it from the system prematurely. Although liquid fractions collected from the loading and washing steps would usually be discarded, they may be analyzed for breakthrough as part of method development (**Figure 3.15**).

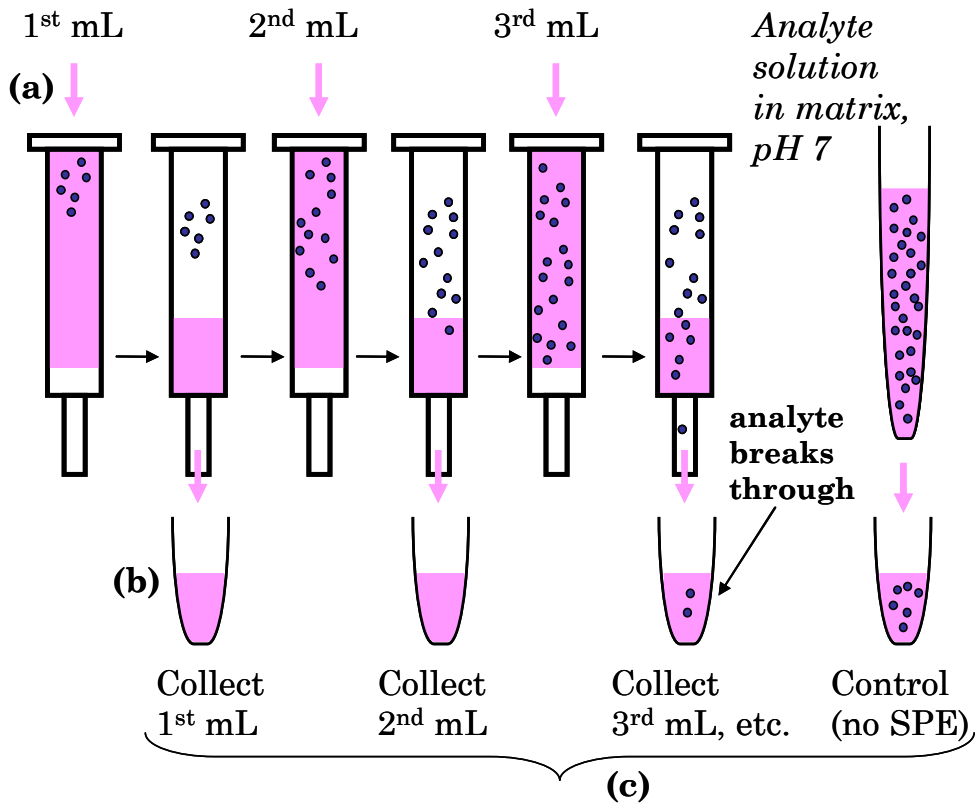

Figure 3.15: Schematic of breakthrough behaviour in SPE. After the cartridge is conditioned and equilibrated, (a) the analyte solution is applied in aliquots, and (b) each fraction is collected separately. Fractions are (c) analyzed by LC/MS, and compared to the control solution. The analyte depicted is poorly retained at pH 7, and migrates as more solution is loaded; it breaks through after the 3rd mL.

In the literature method, a total of 9 mL was applied to the column during loading and washing: 5 mL urine, pre-treated with 2 mL of pH 7 buffer, followed by another 2 mL of pH 7 buffer as wash solvent. Thus for breakthrough fraction analysis, a 10 mL volume of pre-treated mock urine sample was selected as a proxy to measure breakthrough from both loading and washing steps. The last 1 mL of buffer from the conditioning step was also collected as a blank to account for any carryover or contamination in the SPE manifold. Once an analyte breaks through, it will continue to appear in subsequent fractions; thus for expediency, only the first and last 1 mL fractions were analyzed in most of these trials. Breakthrough percentage is reported here as the percentage of peak area for an analyte in a given fraction compared to the control sample.

Since breakthrough fractions contain most of the matrix components, the LC-MS methods for analyzing these fractions (configurations **1h**, **1i**) included diversion of LC flow to waste while the matrix eluted. A precolumn filter was also placed between the autosampler and column to remove any particulates deriving from the matrix or the SPE sorbent. However, these steps did not provide sufficient protection to the LC column, which experienced increased back pressure during a number of analysis runs. In some cases, peak shapes degraded after repeated analysis of these fractions. These difficulties also contributed to poor resolution between the genuine KCP peak and the false peak from MS/MS crosstalk with CBP. Nonetheless, the same method development steps would have been followed even if the KCP measured in breakthrough fractions had been a false positive.

Scheme **A** was first tested on the Varian Bond Elut LMS cartridges. The breakthrough fractions suggested near-complete retention of CP, as the 10^{th} mL had only slightly higher peak area than the blank (**Table 3.2**). However, CBP was quite poorly retained, showing 11.5% breakthrough in the 1^{st} mL, and 80.6% in the 10^{th} mL. KCP showed a smaller but measurable breakthrough as well by the 10^{th} mL.

As noted earlier, at pH 7 CBP would be mostly ionized and KCP partly ionized. At neutral pH these metabolites would not retain as well as CP on a nonpolar sorbent. In light of this, scheme **B** used 50 mM phosphoric acid for pH adjustment rather than a neutral buffer. Breakthrough fractions were analyzed the next day and appeared not to contain CP, KCP or CBP; however, CBP was also undetectable in the control vial. Further stability tests confirmed the degradation of CBP in acid. Thus, the absence of CBP in breakthrough fractions could not be taken as evidence of its successful retention.

The concentration of phosphoric acid was decreased to 1 mM in scheme **C**. Both the control and breakthrough fractions were immediately mixed with a 5 mM PBS buffer after collection, to stabilize the pH and thereby avoid CBP degradation. A second control

was also prepared 5 h later, immediately before analysis, to determine if CBP had still degraded. The 10th mL fraction showed less than 1% breakthrough for all three analytes. The first control had 88% residual concentration of CBP compared to the second, and there was no evidence of KCP or CP degradation. Since CBP had only slightly degraded in the control, the absence of CBP in these breakthrough fractions was a conclusive indication of successful retention.

a) CP breakthrough: % peak area at pH=7 versus control					
Fraction	*Bond Elut LMS*	*Bond Elut C18*	*Bond Elut C18-INT*	*Bond Elut C18-OH*	*Agilent OPT SampliQ*
Blank	0.6	0.8	2.7	0.6	1.2
1st mL	2.6	0.7	0.4	0.1	0.3
10th mL	0.8	0.4	0.5	0.2	1.3
b) KCP breakthrough: % peak area at pH=7 versus control*					
Fraction	*Bond Elut LMS*	*Bond Elut C18*	*Bond Elut C18-INT*	*Bond Elut C18-OH*	*Agilent OPT SampliQ*
Blank	0.1	0.5	0.2	0.3	0.4
1st mL	0.2	0.2	0.1	0.1	3.9
10th mL	8.4	5.5	0.7	4.6	11.3
c) CBP breakthrough: % peak area at pH=7 versus control					
Fraction	*Bond Elut LMS*	*Bond Elut C18*	*Bond Elut C18-INT*	*Bond Elut C18-OH*	*Agilent OPT SampliQ*
Blank	0.0	0.1	0.1	0.1	0.2
1st mL	11.5	0.4	0.1	0.1	43.4
10th mL	80.6	57.1	8.0	58.4	97.8

Table 3.2: SPE breakthrough fractions. Each cartridge was conditioned, equilibrated with blank pH 7 buffer, and then loaded with a 10 mL mock urine sample containing CP, KCP and CBP. The 1st and 10th mL fractions were analyzed, as well as the blank, and the original sample as a control. Results are reported as % peak area compared to the control sample.
***** *Some of the KCP peak area may have derived from CBP crosstalk due to poor LC resolution; the results are still reported here for completeness.*

At this stage of method development, four additional sorbent types were made available; the breakthrough results using scheme **A** are reported in **Table 3.2**. By far, the Varian Bond Elut C18-INT cartridges showed superior retention, as CBP showed only 8% breakthrough in the 10th mL fraction; KCP and CP had <1% breakthrough. Based on these results, method development continued on C18-INT instead of the Bond Elut LMS cartridges. Although the C18 and C18-INT sorbents have the same octadecyl bonded group, Varian advertises that the C18-INT has lower carbon loading and has been designed to differentiate between metabolites.

Since acidic conditions were shown to retain CBP on a nonpolar sorbent, but at the cost of degradation, scheme **D** was designed to minimize exposure to acid. The washing step used 1 mM phosphoric acid to reduce the overall concentration of acid in the cartridge without causing breakthrough, and then 100 mM PBS to buffer any remaining acid to pH 7 before the drying step. Based on observed results for the C18-INT cartridge, 1 mL of pH 7 buffer was not expected to cause significant breakthrough. Using this method, breakthrough fractions again suggested good retention, but neither CBP nor KCP could be detected in the ethyl acetate extract. If both compounds were ionized prior to the drying step, this may have impaired their ability to elute in an aprotic solvent.

After changing the elution solvent to methanol (scheme **E**), all three analytes were visible in the 1st mL extract. The 4th and 5th mL extracts both contained CP but had no peaks for KCP or CBP. This suggested CP was not fully eluted with 5 mL of methanol. An alternative explanation was that the shared SPE equipment had introduced CP contamination into methanol extracts. A number of diagnostic runs suggested that blank methanol samples, evaporated to dryness under nitrogen, contained CP after reconstitution in aqueous solution.

3.4.3. Limitations and alternatives

Due to constrained instrument availability, a number of matters were left unaddressed in SPE method development. A separate manifold was configured for solvent evaporation in a segregated fumehood in order to address the question of CP carryover in the SPE method, but the opportunity to use it for experiments was not available. Similarly, a method was devised to assess post-extraction recovery and matrix effects, but these steps were not completed.

Since scheme **E** would require urine to be acidified before loading on the cartridge, there is a question of whether this method is compatible with the proposal to stabilize collected urine samples at neutral pH with a buffer additive. Since urine itself has a variable buffering capacity, one would require a stronger buffer than urine in order to correct its pH consistently across a large variety of samples. If this sample is then re-acidified for extraction, a yet-stronger concentration of acid would have to be added to surmount the buffering properties of urine and the additive. It may ultimately be simpler and more reproducible to search for the best possible SPE method that can accept samples at pH 7.

Based on the assessment of the C18-INT cartridge (**Table 3.2**), at least 92% of a 5 mL urine sample would be retained at pH 7 after loading and washing. Despite the detectable breakthrough, the total recovery from this cartridge by scheme **A** may have been sufficient to attain the experimental goals without any acidification.

A proper internal standard can help to correct for low or variable recovery, providing one can assume that it experiences the same extent of MS ion suppression or enhancement as the analyte, and has similar retention behaviour in chromatography and extraction [68]. An ideal choice for CP would be the isotopically labelled d_4-CP, as noted earlier. It would not be advisable to use d_4-CP as an internal standard for the metabolites, as its retention behaviour would differ greatly, thus introducing systematic errors into the analysis. The synthesis of labelled metabolites d_4-KCP and d_4-CBP has been reported in

the literature [86], and one study used d_8-CBP (synthesized in-house) as an internal standard [76]; however, these compounds are not commercially available at present.

Strong anion exchange (SAX) was also considered as an alternate stationary phase for SPE, but the opportunity to implement it was not available. Agilent provided SampliQ-SAX cartridges to test, along with a technical note describing their retention behaviour [87]. The stationary phase is a divinylbenzene polymer with a quaternary ammonium cation on a side chain, exhibiting both reversed-phase character and strong retention of deprotonated acids.

A typical SPE scheme on an SAX cartridge would accept sample buffered to pH 7, and the same pH would be used for the washing step. Neutral compounds would elute with methanol, while acidic compounds would remain on the cartridge until eluted with acidified methanol. Although this last elution step might present a conflict with CBP's acid sensitivity, it could easily be remedied by having an appropriate base or buffer in the collection vial.

Ultimately, SPE method development was intended to provide a technique for sample volume reduction and solvent exchange, in order to achieve or surpass detection targets of 50 ng/L for CP and 2.5 ng/L for KCP and CBP in urine. The best LC-MS/MS methods were developed on Instrument #2, which was subsequently not available for testing SPE methods. If the extraction methods outlined here can be executed with high recovery and reproducibility, then the detection targets are likely to be reached in combination with Instrument #2. Although a great deal of work was done with SPE, no final protocol can be reported here; thus it is not possible to report a method detection limit that includes an extraction method.

3.5. Summary

To reiterate, this study aimed to design a protocol for analysis of trace levels CP and metabolites in the urine of occupationally exposed hospital personnel. Method development provided insight into the necessary procedures for sample handling, sample preparation by SPE, separation by HPLC, and detection by MS/MS. Although a final validated protocol could not be developed due to instrument availability, the work completed for this project is presented here nonetheless.

Since the mechanism of CP's toxicity relies on metabolic activation, the detection of CP and its metabolites in the urine of hospital workers would provide unique data on repeated low-level exposures to CP, carrying more information about the individual's total metabolism than CP alone. Nonetheless, a recent multi-year study showed that improved personal protection measures have led to a strong downwards trend in urine CP levels among health-care personnel [36]. Since the detection targets were selected based on past studies, there is no guarantee that any real samples will contain detectable levels of CP and metabolites. While this would be a positive outcome from an occupational health perspective, it would not necessarily indicate the absence of risk.

4. Concluding remarks

4.1. Sample handling and preparation

The necessary conditions for handling urine samples were considered for cyclophosphamide (CP) and its metabolites 4-ketocyclophosphamide (KCP) and carboxyphosphamide (CBP). Stability of the analytes was studied under a variety of pH conditions, and CBP was observed to decompose in acidic aqueous solutions. Based on this and other literature data, it is proposed that collected urine samples be stored on ice from the moment of collection, and then frozen at -80°C when possible, in order to assure stability of both the analytes and the urine itself. Sample preparation should take place within two months of collection, and should be followed up immediately with analysis.

Solid-phase extraction (SPE) was explored as a means to exchange the sample solvent and reduce sample volume. Previously developed SPE methods for CP [78, 79] were not able to retain the metabolites on several non-polar sorbents at pH 7. Metabolites were extracted with greater success by selecting a sorbent with reduced carbon loading and by acidifying the sample immediately prior to application on the SPE cartridge. After sample loading, the cartridge was buffered to avoid degradation of CBP under acidic conditions, and analytes were eluted with methanol. All three analytes were retained on the cartridge under these conditions, and were observed to elute in the organic phase.

4.2. Analytical methods

An analytical method was optimized for CP, KCP and CBP on two different LC-MS/MS instruments. For an efficient trace analysis of these compounds in urine, it was critical to use liquid chromatography equipment with low sample carryover and low delay volume. Post-column infusion was used to demonstrate that the ion suppression resulting from a mock urine matrix could be separated chromatographically from the analyte peaks.

Estimated instrument limits of quantitation (LOQ) for CP, KCP and CBP in neat solvent were respectively 4.2, 8.2 and 57 ng/L on the Agilent 6430 MS (configuration **2d**). These parameters were sufficient to meet a quantitation target of 50 ng/L CP in urine, but fell short of the 2.5 ng/L target for KCP and CBP in urine. Further development is needed to establish if the SPE method can bridge the gap between the quantification target and the instrument LOQ.

4.3. Future work

4.3.1. Completion and validation of method development

The SPE methods explored here should be validated by assessing recovery for each analyte. The acidic extraction method with buffering (scheme **E**) should be compared with the neutral pH method (scheme **A**) on C18-INT cartridges in order to determine whether the additional retention afforded by acidic sample loading justifies the risk of degradation. An alternate SPE method should also be explored using strong anion-exchange cartridges, which are expected to provide superior retention of acidic metabolites. In addition, the consistency of the extraction method would be improved greatly if a suitable internal standard is used with KCP and CBP.

The LC-MS/MS method should be revisited to assess limits of detection and quantitation using lower concentrations of the analytes via a calibration curve. A final determination of matrix effects in post-extraction urine blanks should be carried out by post-column infusion, and the results should be used to ensure that matrix components are diverted to waste during the LC program.

After these details of the analytical method are finalized, the entire protocol would require testing for intraday consistency, using real urine samples spiked for quality control. Furthermore, the sample collection and storage protocols should be tested to ensure that all analytes are sufficiently stable from the moment of collection through to the time of analysis.

Once these steps are complete, the protocol should be ready for application towards the biological monitoring of health-care workers by analysis of urine samples. The resulting data is to be applied towards risk assessment of occupational exposure to antineoplastic drugs, and for comparison of contamination levels to identify potential determinants of exposure.

4.3.2. Supplementary research

Further investigations, beyond the scope of the present study, are worth consideration to refine the analytical method and further our understanding of occupational exposure to antineoplastic drugs.

The present understanding of CP metabolism in humans is based largely on clinical studies, where patients received high doses of CP in conjunction with other drugs. If an occupational exposure study is able to measure the metabolites at trace concentrations, these findings should be examined from a pharmacokinetic and toxicological perspective in order to establish a better correlation between exposure levels and detectable biomarkers in urine.

While SPE is a powerful and flexible extraction method, it adds additional experimental time per sample and demands the use of disposable sorbent cartridges. An online extraction and chromatography system would automate the extraction procedure, improve method consistency and reduce total analysis time.

Additional metabolites such as dechloroethyl-CP may also merit analysis in an exposure monitoring study. The inclusion of degradation products of CP and its metabolites could allow for a more complete exposure assessment.

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Appendix A: Instrument configurations

Instrument #1: ABI 3200 MS/MS with Varian HPLC and Varian autosampler

Source parameters: GS1 = nebulizer gas (psi), GS2 = turbo gas (psi), TEM = turbo gas temperature (°C), CUR = curtain gas (psi), IS = ion spray voltage (V), CAD = collision gas (no units provided)
Compound parameters: DP = declustering potential (V), EP = entrance potential (V), CE = collision energy (V), CXP = collision cell exit potential (V)
Solvents: A = deionized water, B = methanol, C4 = 5 mM ammonium acetate buffered to pH 4 with acetic acid, C5 = same as C4, but at pH 5

Configuration	Detailed parameters
1a (full scan)	Mobile phase: 200 µL/min 50:50 A:B Analyte infusion: 1 mg/L solution of CP in 50:50 water:methanol, 1 µL/min Scan type: Q1 MS, mass range = 50 to 350 amu, 1 s scan time Source: TEM = 475, IS = 5000, CUR = 10, GS1 = 55, GS2 = 65 Compound: DP = 50, EP = 4
1bi (CP optimization)	Mobile phase: 200 µL/min 50:50 A:B Scan type: MRM, 1 s dwell time, transition 261 → 140 *Final optimized parameters:* Source: TEM = 475, IS = 5000, CUR = 10, GS1 = 55, GS2 = 65, CAD = 6 Compound: DP = 50, EP = 4, CE = 30, CXP = 3

Configuration	Detailed parameters
1bii (KCP optimization)	Mobile phase: 200 µL/min 50:50 A:B Additive infusion: 10 µL/min of 5 mM ammonium acetate Analyte infusion: 1 mg/L solution of KCP in 50:50 water:methanol, 1 µL/min Scan type: MRM, 1 s dwell time, transition 275 → 63 *Final optimized parameters:* Source: TEM = 550, IS = 5000, CUR = 10, GS1 = 55, GS2 = 50, CAD = 4 Compound: DP = 45, EP = 4, CE = 45, CXP = 2
1biii (CBP optimization)	Mobile phase: 200 µL/min 50:50 A:B Additive infusion: 10 µL/min of 5 mM ammonium acetate Analyte infusion: 1 mg/L solution of CBP in 50:50 water:methanol, 1 µL/min Scan type: MRM, 1 s dwell time, transition 275 → 63 *Final optimized parameters:* Source: TEM = 550, IS = 5000, CUR = 20, GS1 = 55, GS2 = 55, CAD = 5 Compound: DP = 40, EP = 4, CE = 21, CXP = 4.5
1c (additive optimization for CP, CBP, KCP)	Mobile phase: 200 µL/min (<u>total</u> after additive) 50:50 A:B Additive infusion: 0 to 50 µL/min of i) 5 mM ammonium acetate, ii) 215 mM ammonium acetate, ii) 106 mM formic acid Analyte infusion, scan type, source, compound: Same as **1bi**, **1bii**, **1biii** for CP, KCP and CBP respectively

Configuration	Detailed parameters
1d (isocratic LC with flow injection)	Mobile phase: 200 µL/min 45:45:10 A:B:C5 Injection loop: 10 µL HPLC column: Supelco C_8 Ascentis (100 × 2.1 mm, 3 µm) with Supelco C_8 guard column (20 × 4.0 mm, 5 µm) Scan type: MRM transitions 261 → 140, 275 → 221, 293 → 63; dwell time 333 ms each Source: TEM = 525, IS = 5000, CUR = 10, GS1 = 55, GS2 = 55, CAD = 6 Compound: DP = 45, EP = 4 (global setting); CE = 30, CXP = 3 (CP); CE = 45, CXP = 2 (KCP); CE = 21, CXP = 4.5 (CBP)
1e (post-column infusion)	Same as **1d**, plus: Post-column infusion: 5 µL/min of standard solution containing 50 µg/L each of CP, KCP and CBP in 90:10 water:methanol
1f (gradient on Varian pump)	Mobile phase timetable: 300 µL/min 70:20:10 A:B:C4 (initial), gradient to 15:75:10 (0-0.5 min), isocratic hold (0.5-3.5 min), return to initial (3.5 min), end time 15 min Injection loop, column, scan type, source, compound: Same as **1d**
1g (new optimization based on S/N)	Mobile phase: 300 µL/min 35:55:10 A:B:C4 Analyte solution: 1 µg/L KCP and CBP in 50:50 water:methanol Autosampler: Inject 10 µL every 1.5 min while optimizing MS parameters Scan type: MRM transitions 275 → 221, 293 → 63, 293 → 221; dwell time 333 ms each *Final optimized parameters*: See **1h**

Configuration	Detailed parameters
1h (step gradient via valve switching)	Loading phase: 200 µL/min 20% methanol with 0.5 mM ammonium acetate adjusted to pH 5 (0-2 min and 7-10 min), delivered by Waters pump Elution phase: 300 µL/min 55% methanol with 0.5 mM ammonium acetate adjusted to pH 5 (2-7 min), delivered by Varian pump Mobile phase timetable: Loading phase (0-2 min), elution phase (2-7 min), elution phase (7-10 min), end time 10 min Autosampler: 40 µL injection volume HPLC column: Supelco C_8 Ascentis (100 × 2.1 mm, 3 µm) with Supelco C_8 guard column (20 × 4.0 mm, 5 µm) Scan type: MRM transitions 261 → 140, 275 → 221, 293 → 221; dwell time 333 ms each Source: TEM = 400, IS = 4000, CUR = 10, GS1 = 40, GS2 = 50, CAD = 3 Compound: EP = 4 (global setting); DP = 40, CE = 30, CXP = 3 (CP); DP = 40, CE = 30, CXP = 2 (KCP); DP = 25, CE = 35, CXP = 7.5 (CBP)
1i (lower flow rate)	Same as **1h**, except: Elution phase: 250 µL/min 60% methanol with 0.5 mM ammonium acetate adjusted to pH 5 (2-7 min), delivered by Varian pump

Instrument #2: Agilent LC-MS/MS with integrated pump and autosampler

Source parameters: Temp = gas temperature (°C), Gas = gas flow (L/min),

Neb = nebulizer (psi), Cap = capillary potential (V)

Compound parameters: Frag = fragmentation potential (V), CE = collision energy (V)

Solvents: A = deionized water + 1 mM ammonium acetate, B = methanol

Configuration	Detailed parameters
2a (LC method development, Supelco column)	Mobile phase timetable: 300 µL/min 75:25 A:B (0-1.2 min), gradient to 40:60 (1.2-1.22 min), then gradient to 25:75 (1.22-2.2 min), isocratic hold (2.2-6.5), end time 6.5 min, post time 3 min Autosampler: 5 µL injection volume, 4°C Automatic delay volume reduction: enabled HPLC column: Supelco C_8 Ascentis (100 × 2.1 mm, 3 µm) with Supelco C_8 guard column (20 × 4.0 mm, 5 µm), temperature 25°C Scan type: MRM transitions 261 → 140, 275 → 221, 293 → 221; dwell time 200 ms each Source: Temp = 350, Gas = 6, Neb = 25, Cap = 4000 Compound: Frag = 144, CE = 19 (CP); Frag = 125, CE = 20 (KCP); Frag = 99, CE = 17 (CBP)
2b (MS parameter optimization for CP, KCP, CBP)	Mobile phase: 300 µL/min 40:60 A:B Column removed, no autosampler injection Analyte infusion: Solution of 200 µg/L CP, 500 µg/L KCP and 500 µg/L CBP in deionized water, 20 µL/min Scan type, source, compound: Initially same as **2a**, then each parameter was optimized based on signal strength *Final optimized parameters:* See **2c**

Configuration	Detailed parameters
2c (LC-MS trials)	Mobile phase timetable: Same as **2a** Autosampler: 20 µL injection volume, 4°C Automatic delay volume reduction: enabled HPLC column: Supelco C_8 plus guard, temperature 25°C Scan type: Same as **2a** Source: Temp = 350, Gas = 12, Neb = 55, Cap = 1750 Compound: Frag = 110, CE = 19 (CP); Frag = 125, CE = 15 (KCP); Frag = 99, CE = 15 (CBP)
2d (no auto delay volume reduction)	Mobile phase timetable: 300 µL/min 75:25 A:B (0-0.7 min), gradient to 40:60 (0.7-0.72 min), then gradient to 25:75 (0.72-1.7 min), then gradient to 5:95 (1.7-3.5), end time 6.5 min, post time 3 min Autosampler: 20 µL injection volume, 4°C Automatic delay volume reduction: disabled HPLC column: Supelco C_8 plus guard, temperature 25°C Scan type, source, compound: Same as **2c**
2e (change of LC column)	Mobile phase timetable: 250 µL/min 90:10 A:B (0-0.7 min), gradient to 40:60 (0.7-0.72 min), then gradient to 25:75 (0.72-1.7 min), isocratic hold (1.7-3 min), gradient to 5:95 (3-3.5 min), isocratic hold (3.5-6.5 min), end time 6.5 min, post time 3 min Autosampler: 20 µL injection volume, 4°C Automatic delay volume reduction: disabled HPLC column: Agilent Eclipse Plus C_8 (30 mm × 2.1 mm, 1.8 µm), temperature 25°C Scan type, source, compound: Same as **2c**