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HPLC and CMS Analysis of Clomipramine Metabolism: A Multi-Drug Study

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Abstract

Daily use of multiple medications is commonplace for many Americans. While pharmacists monitor prescriptions for drug interactions, consumers rarely check the possible side effects when using an over-the-counter product. In this fictional case study, Sam is prescribed clomipramine and ciprofloxacin, while also taking Tylenol PM.
All three medications are orally administered, and drug metabolism is catalyzed in the liver by cytochrome P450 enzymes (CYP450). CYP isoenzymes CYP1A2, CYP3A4 and CYP2C19 are responsible for clomipramine metabolism and formation of major bioactive metabolite desmethylclomipramine, while CYP2D6 hydroxylates clomipramine and metabolite into hydroxyclomipramine and hydroxydesmethylclomipramine, respectively. Multi-drug use can inhibit or induce metabolic activity. The extent to which activity is affected is determined by dose and ligand-enzyme binding strength. Ciprofloxacin is a known CYP1A2 inhibitor, while diphenhydramine inhibits CYP2D6. Quantification and visualization of metabolites formed from clomipramine, ciprofloxacin, and diphenhydramine in rat liver microsomes was performed using compact mass spectrometry and high-performance liquid chromatography. Chromatograms were analyzed for molecular weights of parent clomipramine and metabolized products. Data indicated a reduction ranging from 21.1% to 30.1% inhibition of clomipramine metabolism into desmethylclomipramine. It was concluded that CYP isoenzymes CYP1A2, CYP3A4, and CYP2C19 played a role in diminishing clomipramine metabolite formation. These results illustrate the need for research and education when administering multiple drugs.

Introduction

Adverse effects can stem from multi-drug use. Although medications are commonly checked by a pharmacist for drug/drug interactions, use of an over the counter or natural product often goes unchecked by the consumer. In this fictional case study, Sam is a college student who takes clomipramine for his depression and obsessive-compulsive disorder. After engaging in unprotected sex on homecoming weekend, Sam contracts chlamydia and is prescribed ciprofloxacin. He also takes Tylenol PM containing diphenhydramine to help him sleep.

Clomipramine, a 3-chloro derivative of imipramine, is an orally administered tricyclic antidepressant (TCA) used for the treatment of depression and obsessive-compulsive disorder.[1] Absorption occurs in the gastrointestinal system and the drug is metabolized hepatically via oxidation reactions catalyzed by cytochrome P450 enzymes (CYP450).[2] NADPH is needed as a coenzyme for flavoprotein cytochrome P450 reductase (POR) to assist in the electron transport to CYP450, as well as to other biological proteins.[3,4] CYP isoenzymes CYP1A2, CYP3A4 and CYP2C19 are responsible for clomipramine metabolism and formation of major bioactive metabolite desmethylclomipramine. Further N-demethylation by CYP1A2 produces didesmethylclomipramine, while CYP2D6 aromatically hydroxylates clomipramine and metabolite desmethylclomipramine into hydroxyclomipramine and hydroxydesmethylclomipramine, respectively.[5,6] Hydroxylation is essential for further conjugation and glucuronidation. Excretion is performed by the kidneys.

Clomipramine is functionally unique in that the primary mechanism of action for blocking reuptake of which monoamine is dictated by production of metabolites. In parent form, it is a higher affinity serotonin reuptake inhibitor, however as metabolite desmethylclomipramine, it is a stronger inhibitor of norepinephrine reuptake.[7] Because a mood response takes weeks to transpire, it is thought that susceptibility changes occur in the α_1, α_2 and β_1 adrenergic receptors (AR) in the cerebral cortex and hippocampus. A decreased sensitivity of the α_2ARs causes an increase in norepinephrine.[8,9] The elimination half-life of clomipramine is also affected by the form it is in. In original clomipramine form, the half-life is around 24 hours, but as active metabolite desmethylclomipramine the half-life is 96 hours.[10]

Caution must be exercised when taking clomipramine with other drugs. Six enzymes within the CYP450 class are responsible for 90% of drug metabolism,[11] with CYP3A4, CYP2D6, CYP2C1 CYP2C19, CYP1A2, and CYP2E1 being primarily accountable.[12,13] Drugs can interact with one or multiple CYP enzymes and can inhibit or induce activity. The extent to which activity is affected is determined by dose and ligand-enzyme binding strength.[14] Previous studies have shown clinically significant inhibition of CYP1A2 and CYP2C19 at usual therapeutic dose for tricyclic antidepressant, clomipramine.[13] This can lead to adverse drug-drug interactions and increased drug toxicity.

In this proposed study, Sam is taking clomipramine in conjunction with ciprofloxacin and diphenhydramine. Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic used to treat infections caused by bacteria. It is a known inhibitor of CYP1A2 and co-administration with other medications, such as clomipramine could lead to increased concentrations of unmetabolized drugs.[15,16] Other experiments confirmed CYP1A(2) inhibition[17] and showed a decrease in the N-demethylation of co-administered drug by CYP3A4 in human microsomes and by CYP3A2 in rat microsomes.[18] The activity appeared to be competitive in nature. Diphenhydramine is a common antihistamine medication used to treat allergies, cold symptoms, and insomnia. It is found in many popular over the counter medications. While diphenhydramine is a known CYP2D6 inhibitor,[19] research has identified it also as a high affinity substrate for CYP2D6, suggesting the inhibition is competitive. CYP1A2, CYP2C9, and CYP2C19 were also identified. Experiments in human liver microsomes confirmed activity of mentioned CYP isoenzymes in N-demethylation of diphenhydramine using P450 isozyme-specific inhibitors.[20] Competitive inhibition at CYP isoenzymes could cause an increase in unmetabolized clomipramine. This could lead to more serotonin production and cause serotonin syndrome (SS). SS is a serious condition that can be life threatening and includes symptoms such as diarrhea, agitation, sweating, and high blood pressure. Serotonin-norepinephrine reuptake inhibitors (SNRIs), such as clomipramine, are more likely than selective serotonin reuptake inhibitors (SSRIs) to cause SS.[21]

Major goals of the experiment included quantification, visualization and analysis of metabolites formed from clomipramine in the presence of ciprofloxacin and diphenhydramine, rat liver microsome, and cofactor NADPH using compact mass spectrometry. Minor goals included creating a fictional case study that was plausible, as well as bringing awareness to drug contraindications that may occur with over the counter in a college setting.

Methods and Materials

All experimental chemicals were purchased directly from Sigma Aldrich: clomipramine hydrochloride, ciprofloxacin hydrochloride, diphenhydramine, potassium phosphate, ammonium acetate, acetonitrile, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and rat liver microsomes. A Luna C18 reverse phase column (3 μm, 50 mm x 2 mm) was purchased from Phenomenex.
The following stock solutions were created with 50 mM potassium phosphate buffer: a 1.5 mM clomipramine solution, a 23.04 mM ciprofloxacin solution, and a 1.76 mM diphenhydramine solution. A microsomal suspension was created with rat liver microsomes and 50 mM potassium phosphate buffer. The NADPH regenerating system was provided courtesy of Dr. Myoung Lee. Ten samples were assembled according to Table 1, with six samples labeled control. The four samples of interest were created with drug(s), NADPH regenerating system, microsomal suspension, and 50 mM potassium phosphate buffer and contained clomipramine only, clomipramine and ciprofloxacin, clomipramine and diphenhydramine, and clomipramine, ciprofloxacin, and diphenhydramine. The final concentration of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and microsome was 1 mM, 5 mM, 0.5 U, and 0.2 mg/mL, respectively. After samples were incubated at 37°C for 1.5 hours, 250 μL methanol was added to arrest reaction. Samples were centrifuged at 15,000 g for 20 minutes and 10 μL of supernatant was extracted and mixed with 1 mL premade mass spectrometer solution.
Metabolized drug samples were processed in triplicates at an injection volume of 50 μL using Advion Expression S Compact Mass Spectrometer equipped with an electrospray ionizer (ESI) and a Phenomenex Luna C18 column. The run time was six minutes per sample with a mobile phase of 95:5 acetonitrile: water and 2 mM ammonium acetate and a 0.2 mL/min flow rate.

Table I: Clomipramine Metabolism Trials

Tube	Clomipramine (µL)	NADPH (µL)	Microsomal Suspension (µL)	Potassium Phosphate buffer (µL)	Ciprofloxacin (µL)	Diphenhydramine (µL)	Total Volume (µL)
1	65	35	25	25	0	0	150
2	0	35	25	81.7	8.3	0	150
3	65	0	25	51.7	8.3	0	150
4	65	35	0	41.7	8.3	0	150
5	65	35	25	16.7	8.3	0	150
6	0	35	25	81.7	0	8.3	150
7	65	0	25	51.7	0	8.3	150
8	65	35	0	41.7	0	8.3	150
9	65	35	25	16.7	0	8.3	150
10	65	35	25	8.3	8.3	8.3	150

Conclusion

Clomipramine underwent both N-demethylation and aromatic hydroxylation during metabolism in rat liver microsomes, *in vitro*. Chromatograms created using compact mass spectrometry and high-performance liquid chromatography showed a reduction in metabolite desmethylclomipramine formation both in the presence of ciprofloxacin and diphenhydramine.
When clomipramine and ciprofloxacin were co-administered, production of desmethylclomipramine fell by 28.6%. When clomipramine and diphenhydramine were co-administered, production of desmethylclomipramine fell by 21.1%. When clomipramine was metabolized with both ciprofloxacin and diphenhydramine, desmethylclomipramine formation was reduced by 30.1%, for an additive effect of 1.5%. It was concluded that ciprofloxacin impacted the N-demethylation of clomipramine in CYP isoenzymes CYP1A2 and CYP3A4 and diphenhydramine altered desmethylclompramine formation in CYP1A2 and CYP2C19.

Discussion

Ten samples were injected in triplicate and measured using compact mass spectrometry and high-performance liquid chromatography. The four samples of interest contained clomipramine only, clomipramine and ciprofloxacin, clomipramine and diphenhydramine, and clomipramine, ciprofloxacin, and diphenhydramine. The remaining six samples were controls. All samples were subjected to ESI leading to protonation of drug and metabolites for the addition of +1 to the molecular weight. Chromatograms were analyzed for presence of unmetabolized clomipramine (315.9 g/mol), the N-monodemethylated metabolite desmethylclomipramine (301.9 g/mol), and N-didemethylated metabolite didesmethylclomipramine (287.9 g/mol). Only desmethylclomipramine was shown as measurable. The molecular weight of didesmethylclomipramine was included in total ion chromatogram, but not the filtered results.

In the three trials run with clomipramine only, clear peaks were seen at filtered molecular markers 315.9 g/mol (clomipramine) and 301.9 g/mol (desmethylclomipramine). The unmetabolized clomipramine was found at average retention time 3.15 minutes, while its metabolized counterpart was found at average retention time 3.72 minutes. The longer retention time was due to the decrease in polarity caused by the N-demethylation of clomipramine. This is reflected in higher LogP value found in Table 2. Formation of desmethylclomipramine was measured at 0.963% on average with a standard deviation of 0.074%.

In the three trials run with clomipramine and ciprofloxacin, a clear peak was seen at filtered molecular marker 315.9 g/mol (clomipramine). Peaks seen for molecular marker 301.9 g/mol (desmethylclomipramine) were defined, but presence of ciprofloxacin caused additional peak at earlier retention time of 0.78 minutes. The unmetabolized clomipramine was found at average retention time 3.13 minutes, while its metabolized counterpart was found at average retention time 3.66 minutes. Formation of desmethylclomipramine was measured at 0.688% on average with a standard deviation of 0.062%. Clomipramine when metabolized with ciprofloxacin had a reduced metabolism by 28.6%

In the three trials run with clomipramine and diphenhydramine, clear peaks were seen at filtered molecular markers 315.9 g/mol (clomipramine) and 301.9 g/mol (desmethylclomipramine). The unmetabolized clomipramine was found at average retention time 3.19 minutes, while its metabolized counterpart was found at average retention time 3.75 minutes. Formation of desmethylclomipramine was measured at 0.76% on average with a standard deviation of 0.13%. Clomipramine when metabolized with diphenhydramine had a reduced metabolism by 21.1%.

In the three trials run with clomipramine, ciprofloxacin, and diphenhydramine, clear peaks were seen at filtered molecular markers 315.9 g/mol (clomipramine) and 301.9 g/mol (desmethylclomipramine). The unmetabolized clomipramine was found at average retention time 3.21 minutes, while its metabolized counterpart was found at average retention time 3.78 minutes. Formation of desmethylclomipramine was measured at 0.673% on average with a standard deviation of 0.14%. Clomipramine when metabolized with ciprofloxacin and diphenhydramine had a reduced metabolism by 30.1%.

Initial trials did not indicate a hydroxylation product; however, a subsequent trial was run with clomipramine only. In the trial run with clomipramine only, clear peaks were seen at filtered molecular markers 315.9 g/mol (clomipramine), 317.9 g/mol (hydroxydesmethylclomipramine), and 331.9 (hydroxyclomipramine). The unmetabolized clomipramine was found at retention time 1.78 minutes, while its metabolized counterparts hydroxydesmethylclomipramine and hydroxyclomipramine were found at retention time 1.78 and 3.63 minutes respectively. Although, the retention times are the same for both clomipramine and hydroxydesmethylclomipramine, this is also reflected in the LogP values.

Goals of the experiment were met as a reduction in clomipramine metabolism was show with both ciprofloxacin and diphenhydramine present. The co-administration of clomipramine, ciprofloxacin and diphenhydramine only showed an additive effect of 1.5%, when compared to taking clomipramine with ciprofloxacin. It can be concluded that ciprofloxacin reduced clomipramine metabolism by 28.6% through inhibition of CYP enzymes CYP1A2 and CYP3A4 and that diphenhydramine reduced clomipramine metabolism by 21.1% through inhibition of CYP enzymes CYP1A2 and CYP2C19. Additional experiments should be conducted measuring metabolites formed from aromatic hydroxylation of clomipramine alone and in the presence of other drugs.

Figure I: The Clomipramine Metabolism Pathway:

The flowchart shows the possible products of clomipramine metabolism catalyzed by cytochrome P450 isoenzymes. Cofactor NADPH is oxidized by cytochrome P450 reductase to NADP in the enzymatic process. CYP isoenzymes CYP1A2, CYP3A4 and CYP2C19 are responsible for formation of desmethylclomipramine, which can be N-demethylated by CYP1A2 to form didesmethylclomipramine or hydroxylated by CYP2D6 to form hydroxydesmethylclomipramine. Other routes of metabolism include aromatic hydroxylation of clomipramine by CYP2D6 to form hydroxyclomipramine. Hydroxylation is essential for further conjugation and glucuronidation. Excretion performed by the kidneys.

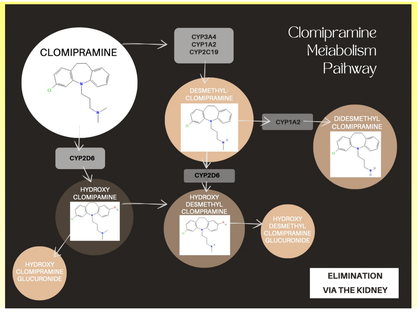

Table 2: Compound Names, Molecular Weights, and LogP Values

Compound Name	Compound Molecular Weight (g/mol)	LogP
Clomipramine	314.9	3.63-4.53
N-Desmethylclomipramine	300.9	4.7
N-Didesmethylclomipramine	286.9	4.2
2-Hydroxyclomipramine	330.9	4.8
8-Hydroxyclomipramine	330.9	4.51
2-Hydroxydesmethylclomipramine	316.9	5.5
8-Hydroxydesmethylclomipramine	316.9	4.4
Ciprofloxacin	331.3	0.28
Diphenhydramine	255.4	3.44

Experimental Results

Clomipramine Only

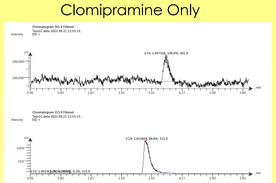

Figure 2: MS Chromatogram of the N-Demethylation of Clomipramine

The figure shows the metabolism of clomipramine with NADPH regenerating system, rat liver microsomes, and buffer. Defining peaks are seen at molecular weight of both parent compound (315.9 g/mol) and desmethylclomipramine (301.9 g/mol) at retention times 3.14 and 3.74 minutes respectively.

Clomipramine and Ciprofloxacin

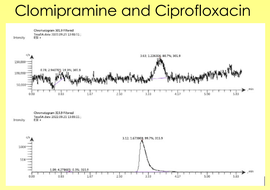

Figure 3: MS Chromatogram of the N-Demethylation of Clomipramine in the Presence of Ciprofloxacin

The figure shows the metabolism of clomipramine with NADPH regenerating system, rat liver microsomes, and buffer when taken with ciprofloxacin. Defining peaks are seen at molecular weight of both parent compound (315.9 g/mol) and desmethylclomipramine (301.9 g/mol) at retention times 3.12 and 3.63 minutes respectively

Clomipramine and Diphenhydramine

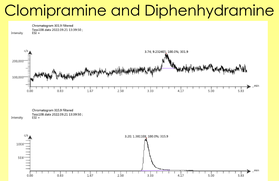

Figure 4: MS Chromatogram of the N-Demethylation of Clomipramine in the Presence of Diphenhydramine

The figure shows the metabolism of clomipramine with NADPH regenerating system, rat liver microsomes, and buffer when taken with diphenhydramine. Defining peaks are seen at molecular weight of both parent compound (315.9 g/mol) and desmethylclomipramine (301.9 g/mol) at retention times 3.20 and 3.74 minutes respectively

Clomipramine, Ciprofloxacin, and Diphenhydramine

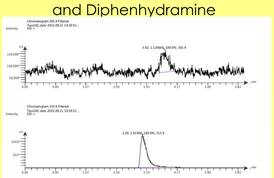

Figure 5: MS Chromatogram of the N-Demethylation of Clomipramine in the Presence of Ciprofloxacin and Diphenhydramine

The figure shows the metabolism of clomipramine with NADPH regenerating system, rat liver microsomes, and buffer when taken with both ciprofloxacin and diphenhydramine. Defining peaks are seen at molecular weight of both parent compound (315.9 g/mol) and desmethylclomipramine (301.9 g/mol) at retention times 3.14 and 3.74 minutes respectively

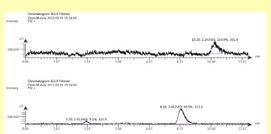

Figure 6: MS Chromatogram of N-Demethylation of Clomipramine in Baseline Study

The figure shows the metabolism of clomipramine with NADPH regenerating system, rat liver microsomes, and buffer. Defining peaks are seen at molecular weight of both parent compound (315.9 g/mol) and desmethylclomipramine (301.9 g/mol) at retention times 8.38 and 10.25 minutes respectively. The baseline study was run at a flow rate of 0.1mL/min, accounting for the longer retention times.

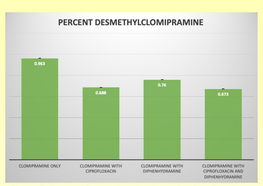

Figure 7: Percent Desmethylclomipramine Bar Graph

The bar graph shows the percent production of desmethylclomipramine from clomipramine to be 0.963% (st. dev.-0.00074) from clomipramine only, 0.688% in the presence of ciprofloxacin (st. dev.-0.000624), 0.76% in the presence of diphenhydramine (st. dev.-0.00132), and 0.673% in the presence of ciprofloxacin and diphenhydramine (st. dev.-0.00142)

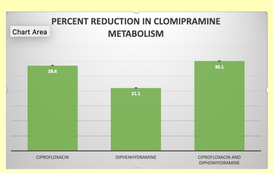

Figure 8: Percent Reduction in Clomipramine Metabolism Bar Graph

The bar graph shows a 28.6% reduction in clomipramine metabolism when co-administered with ciprofloxacin (st. dev.- 0.06%), a 21.1% reduction in metabolism when co-administered with diphenhydramine (st. dev.- 0.13%), and a 30.1% reduction in metabolism when co-administered with ciprofloxacin and diphenhydramine (st. dev– 0.14%).

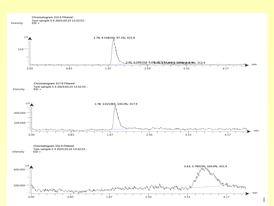

Figure 2: MS Chromatogram of Hydroxylation of Clomipramine and Desmethylclomipramine

The figure shows the metabolism of clomipramine with NADPH regenerating system, rat liver microsomes, and buffer. Defining peaks are seen at molecular weights of parent compound (315.9 g/mol), hydroxydesmethylclomipramine (317.9 g/mol), and hydroxyclomipramine (331.9 g/mol) at retention times 1.78, 1.78, and 3.63 minutes respectively.

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