Any success story always begins with the careful selection, proper identification of quality and purity of the materials and selection of methods required in the project. Therefore, in this chapter we are focusing on the executed plan of work which involves proper selection of drugs, their procurement and characterization, selection and optimization of suitable reactions for the synthesis of mutual prodrugs, characterization of the synthesized products, and \textit{in vitro} release studies of the synthesized mutual prodrugs for the colonic release. Each of these areas is studied in detail for the fruitful research outcome.

3.1 SELECTION OF DRUGS FOR SYNTHESIS OF MUTUAL PRODRUGS FOR COLON TARGETED DRUG DELIVERY

Research envisages the exploration of synthesis of the mutual prodrugs for the colonic delivering of the drugs. Therefore, it is necessary to have a look of the eligible candidates to pursue the selection of the drugs.

The drugs, which are required in the colon includes antibacterial, antiamoebic, anticancer and anti-inflammatory agents. They are required to treat various colonic diseases \textit{viz.} inflammatory bowel diseases (IBD, \textit{e.g.}, irritable bowel syndrome, ulcerative colitis, Crohn’s disease etc.), infectious diseases (\textit{e.g.} amoebiasis) and colon cancer. The inflammation of the colon is associated with all of them. However, all the drugs suffer with the problem of their insufficient concentration at site because of their early absorption. The treatment of these bacterial induced intestinal bowel diseases (IBD) is best accomplished by mutual prodrug concept as the purpose of getting control over bacterial population present in colon as well as the treatment of inflamed gut tissue can be successfully achieved.

For the present studies Antibacterial and Anti-inflammatory drugs were selected because of being curative agents for most prevalent colon disease namely intestinal bowel disease due to any reason. The rationale of selection of the antibacterial and anti-inflammatory are as following:
• The bacterial population can be controlled which is the main cause of all IBD by antibacterial as well as the treatment of inflammations due to such IBD by NSAIDs may be achieved simultaneously by their mutual prodrug formation.

• At present there is no effective anti-inflammatory agent is available. Anti-inflammatory therapy, at present, involves use of corticosteroids as all NSAIDs are absorbed in the stomach and they do not reach to colon.

• Corticosteroids must be used with caution under supervision of physician and shall be avoided if alternative drugs for inflammation are available.

• Most of the Antibacterial have free amino groups, although, it is important for their activity but they can be targeted to colon via formation of mutual prodrugs by azo bond formation with NSAIDs and amidation between amino group of antibacterial and carboxylic acid group of NSAID molecule.

• Most of the NSAIDs have free carboxylic acid groups, although, it is important for their activity but they can be targeted to colon via formation of mutual prodrugs by amidation between amino group of antibacterial and carboxylic acid group of NSAID molecule.

• Hydrolytic enzymes of stomach to ileum do not hydrolyze such mutual prodrugs as seen in the case with mutual prodrugs - sulfasalazine and mesalazine.

• Absorption of the NSAIDs primarily takes place in the stomach and followed with jejunum due to lipophilicity of the unionized form. Thus, they do not reach to the colon except 5-amino salicylic acid (BAN- Mesalazine; USAN- Mesalamine), as it is amphiphilic in nature. If the NSAIDs are targeted to the colon use of the corticosteroids may be avoided and if the carboxylic bond protected via formation of amide or ester linkage their potential hazard of being ulcerogenic can also be avoided. Therefore, formation of their mutual prodrugs may be beneficial.

• Antibacterial cover a wide variety of agents, they are not genotoxic and are comparatively safer to use, yet their selection is important. In sulfonamides, sulfadiazine and sulfamethoxazole are selected due to their lower toxicity. Trimethoprim is selected because of its useful selective toxicity against bacterial enzyme as compare to human enzyme and has a broad spectrum. In II generation quinolones, norfloxacin is selected as it has a broad spectrum and equivalent in potency to many of the fermentation derived antibiotics and also because of the fact that it is very much useful for the treatment of bacterial dysentery.
• NSAIDs, too, is a very large group, therefore, selection of the drug is also of paramount importance. Salicylic acid and Aspirin undoubtedly comes due to their good anti-inflammatory activity and if the slow absorption is affected may be beneficial for its antithrombotic effect to save heart attack for which a low dose is recommended. Indomethacin is selected as it is a highly potent analgesic with a high molecular weight. It is also used in the form of suppositories to treat the rectal inflammation. Thus, we can say it will be pertinent to use it with the antibacterials in the form of mutual prodrugs. Indomethacin is selected also due to its low dose, as advised by Friend (2005).

• Based on the above considerations the following drugs were selected for their delivery into the colon through mutual prodrug formation.
  - Sulfadiazine
  - Sulfamethoxazole
  - Trimethoprim
  - Norfloxacin
  - Salicylic acid
  - Aspirin
  - Indomethacin

After the selection of drugs to be delivered into colon it is very important to know about the profiles of their characteristics and the status of quality and purity. Their characteristics reported in the literature was collected and is described as the profile of individual drug, however, the literature available on these drugs is very bulky. Therefore, only the part of it selected on the basis of relevance was selected. The drugs were tested for its quality as per the practice prevailing in pharmaceutical sciences on the basis of their Pharmacopoeial monographs available in the pharmacopoeias. Hence, the characterization is described along with the profile.

3.1.1 Sulfadiazine (Sulfapyrimidine)
Sulfadiazine is an antibacterial agent belongs to sulfonamide family. It has been used in the therapy of mild to moderate infections due to sensitive organisms. It is a synthetic pyrimidinyl sulfonamide derivative. It is a short-acting bacteriostatic.

3.1.1.1 Drug Profile
(a). Molecular Information

Chemical structure:

![Chemical Structure Image]

**Molecular Formula**  \( \text{C}_{10}\text{H}_{10}\text{N}_4\text{O}_2\text{S} \)

**Molecular Weight** 250.27

**Molecular Composition** 47.99% C, 4.03% H, 22.39% N, 12.88% O, 12.71% S

**Exact Mass** 250

**Chemical Name**  N\(^1\)-2-Pyrimidinylsulfanilamide
\( p\)-Amino-N-(2-pyrimidyl)benzenesulfonamide

**CAS Registry No.** 68-35-9

(b). Physical Properties

**Description**  White or slightly yellow powder, odourless.

**Melting Point**  253-254° C

**Solubility**  In water (0.08 mg/ml) at RT and in boiling water (16.7 mg/ml), sparingly soluble in alcohol and acetone, soluble in dil. mineral acids and alkali hydroxide solutions.

**pKa**  6.28 at 38°C

**Chemical Stability**  Stable in dry air and upto 100 °C temperature. Upon pyrolysis, it yields 2-aminopyrimidine and sulphur dioxide. In solution it undergoes acid-catalyzed hydrolysis.

(c). Spectral Characteristics:
UV-Visible Spectroscopy
UV $\lambda_{\text{max}}$ (0.1 M HCl): 215 nm (E$_{1\%}$ 1cm 548), 242 nm (E$_{1\%}$ 1cm 579); (0.1 M NaOH): 242 nm (E$_{1\%}$ 1cm 821), 254 nm ((E$_{1\%}$ 1cm 794); (Ethanol): 270 nm (E$_{1\%}$ 1cm 844)

Infrared Spectroscopy
See Fig. 3.1

H$^1$ NMR Spectroscopy
See Fig. 3.2

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Fig. 3.1: IR spectrum of Sulfadiazine
Fig. 3.2: $^1$H NMR spectrum of Sulfadiazine

(d). Biopharmaceutical and Toxicological Data

Pharmacokinetic and Metabolism

Sulfadiazine is absorbed readily from the GIT. Its protein binding affinity increased with the number of methyl substituents in the 2-pyrimidine ring. The metabolism of sulfadiazine in humans involves acetylation (in the liver), oxidation and hydrolysis. In addition to unchanged sulfadiazine, which accounted for over 50% of the products excreted, the following metabolites are identified in human urine: $N^4$-acetylsulfadiazine, sulfadiazine $N^4$-glucuronide, sulfadiazine sulfonate and sulfanilamide. The systemic toxicity of sulfadiazine is low, and relatively few side effects are associated with its use (Florey, 2005)

LD$_{50}$ in mouse is 1500 mg/kg.

(e). Therapeutic Category and Use

Sulfadiazine is used for the cure of infections caused by gram-positive bacteria. It is also found suitable for the treatment of *E. coli* infections in the urinary tract. It is also used prophylactically against rheumatic fever and meningococcal meningitis. It is used in combination with pyrimethamine against toxoplasmosis

**Dose:** Initial dose, 3 g; subsequent doses, upto 4 g daily, in divided doses.
3.1.1.2 Characterization of Sulfadiazine:

Its characterization can be done with the help of experimental based on its official monograph (The International Pharmacopoeia, 1981a; Indian Pharmacopoeia, 2007; United States’ Pharmacopoeia, 2004; British Pharmacopoeia, 1993b). The sulfadiazine procured was characterized on the basis of Pharmacopoeial monograph in I.P.

**EXPERIMENTAL**

(a). Procurement and Identification of Sulfadiazine:

Sulfadiazine was obtained as a gift sample from Vivek Pharmchem India Ltd. Jaipur. The obtained sample was a white crystalline and odorless powder. It was identified with the help of melting point, chemical test and volumetric method.

**Melting Point:** Melting point was determined by open capillary method in Toshniwal Melting Point Apparatus.

**Chemical Tests:** For the test of primary aromatic amines, about 5 mg of sulfadiazine in 10 ml of 1 M HCl was dissolved and 1 ml of this solution is diluted to 10 ml with water.

**Spectral Identification:** Sulfadiazine was identified by FTIR absorption spectra. The spectrum of the sulfadiazine was recorded by FTIR spectrophotometer (Shimadzu FTIR Model 8300). The recorded spectrum is shown in Fig. 4.1. The spectrum was matched with those shown in Fig. 3.1 taken from official texts.

(b). Tests for Purity and Assay:

The sample was tested for the tests of purity as described in I.P. using pharmacopoeial procedures. The tests conducted were Colour of Solution in 1 M sodium hydroxide; Heavy metals; Sulphated ash and Loss on drying. Assay was also conducted by pharmacopoeial procedure. The results of pharmacopoeial analyses are reported in Table 4.2.

3.1.2 Sulfamethoxazole

It is also a member of the sulfonamide family of antibiotics. It is an antibacterial sulfonamide which has been available in Germany since 1959. It is classified as an intermediate-acting sulfonamide effective against gram +ve, gram –ve bacteria, and some protozoans.

3.1.2.1 Drug Profile

(a). Molecular Information

Chemical structure:
Molecular Formula \( \text{C}_{10}\text{H}_{11}\text{N}_{3}\text{O}_{3}\text{S} \)
Molecular Weigh 253.28
Molecular Composition 47.43% C, 4.38% H, 16.60% N, 18.94% O, 12.65% S
Exact Mass 253
Chemical Name \( \text{N}^1-(5\text{-methyl-3-isoxazolyl}) \) sulfanilamide
CAS Registry No. 723-46-6

(b). Physical Properties
- Description: Off-white crystalline powder; odorless.
- Melting Point 170 to 173 °C
- Solubility Soluble in acetone, sparingly soluble in \( \text{C}_{2}\text{H}_{5}\text{OH} \) (95%), very slightly soluble in water, in chloroform and in ether.
- pKa 5.60 at 25 °C
- Chemical Stability Stable in 0.4 N NaOH, but when refluxed in 0.4 N HCl, the molecule first cleaves to yield sulfanilic acid and 5-methyl-3-amino isoxazole and on extended heating in the HCl solution, 3 additional diazotizable products are formed.

(c). Spectral Characteristics:
- UV-Visible Spectroscopy When solution of sulfamethoxazole in
0.1 N NaOH was scanned between 350 to 215 nm, one maximum at 256-257 and one minimum at 224-225 nm were observed

Infrared Spectroscopy  See Fig. 3.3
H¹ NMR Spectroscopy  See Fig. 3.4

Fig. 3.3: IR spectrum of Sulfamethoxazole
(d). Biopharmaceutical and Toxicological Data

Metabolism

Sulfamethoxazole is metabolized to its N₄– acetyl derivative which is the major form found in human urine (Koechlin et al., Brandman and Engelberg 1960). The intact drugs along with three other metabolites which have not been completely identified are also present in lesser quantities in human urine. In human blood the sulfamethoxazole exists almost entirely as the intact drug.

LD₅₀ orally in mouse: 362 mg/kg.

(e). Therapeutic Category and Uses

It has been used since the 1960s in the management of various systemic infections in humans and other species. In combination with trimethoprim it is used mainly for the treatment of UTI. Against gonorrhea, meningitis and serious respiratory tract infection and prophylactically against susceptible meningococci, it has also been used. Sulfamethoxazole in combination with pyrimethamine is also used in the treatment of chloroquine-resistant *Plasmodium falciparum* malaria.

**Dose:** Initially 2 g, followed by 1 g twice a day.

3.1.2.2 Characterization of Sulfamethoxazole:

Characterization of sulfamethoxazole can be done with the help of experimental based on its official monograph (Indian Pharmacopoeia, 2007; United States’ Pharmacopoeia, 2004; British Pharmacopoeia, 1993c). The sulfamethoxazole procured was characterized on the basis of Pharmacopoeial monograph in I.P.

**EXPERIMENTAL**

(a). Procurement and Identification of Sulfamethoxazole:

Sulfamethoxazole was obtained as a gift sample from Vivek Pharmachem India Ltd. Jaipur. The sample was a white crystalline powder and odorless in nature. It was identified with the help of melting point, chemical test and its spectral analyses.

**Melting Point:** Melting point was found out by open capillary method in Toshniwal melting point apparatus.

**Chemical Tests:** For primary aromatic amines test, about 5 mg of sulfamethoxazole was dissolved in 10 ml of 1 M HCl and then 1 ml of it was diluted to 10 ml with water.

**Spectral Identification:** Sulfamethoxazole was identified by FTIR absorption spectra. The spectrum of the sulfadiazine was recorded by FTIR spectrophotometer (Shimadzu FTIR
Model 8300). The recorded spectrum is shown in Fig 4.2. The spectrum was matched with those shown in Fig. 3.4 taken from official texts.

(b). Tests for Purity and Assay:
The sample was tested for the tests of purity as described in IP using pharmacopoeial procedures. The tests conducted were Acidity; Colour of solution; Heavy metals; Related substances; Sulphated ash and Loss on drying. Assay was also conducted by pharmacopoeial procedure. The results of pharmacopoeial analyses are reported in Table 4.3.

3.1.3 Trimethoprim (Proloprim, Trimex)
Trimethoprim an antibacterial was developed in 1969, by George Hitchings and Gertrude Elion. It prevents tetrahydrofolic acid biosynthesis and results in bacteriostasis. The bacterial enzyme is sensitive to inhibition by trimethoprim by up to 40,000-fold lower concentrations than the mouse enzyme is. This shows useful selective toxicity of trimethoprim.

3.1.3.1 Drug Profile
(a). Molecular Information

Chemical structure:

![Chemical structure of Trimethoprim](image)

**Molecular Formula**  
$\text{C}_{14}\text{H}_{18}\text{N}_{4}\text{O}_3$

**Molecular Weight**  
290.32

**Molecular Composition**  
$57.92\% \text{ C}, 6.25\% \text{ H}, 19.30\% \text{ N}, 16.53\% \text{ O}$

**Exact Mass**  
290

**Chemical Name**  
2, 4-diamino-5- (3, 4, 5- tromethoxybenzyl) - Pyrimidine

**CAS Registry No.**  
738-70-5

(b). Physical Properties
Description: White to pale yellow crystalline powder; odorless.

Melting Point
201 °C

Solubility
95% Ethanol (0.81 g/100 ml), Methanol (1.21 g/100 ml), Chloroform (1.82 g/100 ml), very slightly soluble in water.

pKa
6.6 at 25 °C

Chemical Stability
Stable in dry air.

(c). Spectral Characteristics:

UV-Visible Spectroscopy
The UV spectrum of trimethoprim in the region of 400 to 230 nm exhibits one maximum at 287±2 nm (ε = 7.4 x 10³, absorptivity = 25.5) and a minimum at 257 nm.

Infrared Spectroscopy
See Fig. 3.5

H¹ NMR Spectroscopy
See Fig. 3.6

Fig. 3.5: IR spectrum of Trimethoprim
(d). Biopharmaceutical and Toxicological Data

Metabolism
Five major metabolites of trimethoprim have been isolated: Metabolite I = 2, 4-diamino-5- (4-hydroxy-3, 5-dimethoxybenzyl)-pyrimidine; Metabolite II = 2, 4-diamino-5- (α-hydroxy-3, 4, 5- trimethoxybenzyl) –pyrimidine; Metabolite III (a and b), a = 2, 4-diamino-5- (3, 4, 5- trimethoxybenzyl) –pyrimidine-1-oxide, and b = 2, 4-diamino-5- (3, 4, 5- trimethoxybenzyl) –pyrimidine-3-oxide, and Metabolite IV = 2, 4-diamino-5- (3-hydroxy-4, 5- dimethoxybenzyl) –pyrimidine. Metabolite IV is excreted twice as much as Metabolite I. The N-1 and N-3-oxide forms of metabolite III are produced equally. Metabolite II is a minor metabolite. Metabolite II and III are unconjugated in both plasma and urine, whereas Metabolites I and IV are conjugated and excreted in the urine as glucuronides (Florey, 2005).

Toxicity
Those who might be under folate stress such as the elderly, pregnant woman, the chronically ill and the malnourished, must be treated more carefully.

(e). Therapeutic Category and Uses
It is frequently used for the oral treatment of UTIs as a single agent clinically. It is potentially useful against many microorganisms. It is most commonly used in a 1:5 fixed concentration ratio with the sulfamethoxazole for oral management of UTIs, shigellosis, otitis media, traveler’s diarrhea, MRSA, *Legionella* infection, and bronchitis. **Dose**: 200 mg twice daily.
3.1.3.2 Characterization of Trimethoprim:
Trimethoprim can be characterized with the help of experimental based on its official monograph (The International Pharmacopoeia, 1981a; Indian Pharmacopoeia, 1996b; United States’ Pharmacopoeia, 1990a; British Pharmacopoeia, 1993b). The trimethoprim procured was characterized on the basis of pharmacopoeial monograph in I.P.

EXPERIMENTAL

(a). Procurement and Identification of Trimethoprim:
Trimethoprim was procured as a gift sample from Vivek Pharmachem India Ltd. Jaipur. The sample was a yellowish white powder and odorless. It was identified with the help of melting point and its spectral analyses.

Melting Point: Melting point was found out by open capillary method in Toshniwal melting point apparatus.

Spectral Identification: (A). Trimethoprim was identified by FTIR absorption spectra. The spectrum of the trimethoprim was recorded by FTIR spectrophotometer. The recorded spectrum is shown in Fig 4.3. The spectrum was matched with those shown in Fig. 3.4 taken from official texts.

(B). About 25 mg of drug was dissolved in 25 ml of ethanol (95%) and 2.0 ml of it was diluted to 100 ml with 0.1 M sodium hydroxide. The solution so obtained was scanned from 230 to 360 nm.

(C). About 25 mg was dissolved in 5 ml of 0.005 M H$_2$SO$_4$, then heated, and 2 ml of a 1.6% w/v solution of KMnO$_4$ in 0.1 M NaOH was added. It was heated to boiling and 0.4 ml of formaldehyde solution was added to the boiling solution. 1 ml of 0.5 M H$_2$SO$_4$ was then poured, and heated the above to boiling. It was then cooled and filtered. Now, 2 ml of chloroform was added to the filtrate and shake vigorously. The chloroform layer was examined under UV light at 365 nm.

(b). Tests for Purity and Assay:
The sample was tested for the tests of purity as described in I.P. using Pharmacopoeial procedures. The tests conducted were Clarity and Colour of Solution in 5% w/v solution in a mixture of chloroform, methanol and water (10:9:2); Heavy metals; Related substances; Sulphated ash and Loss on drying. Assay was also conducted by Pharmacopoeial procedure. The results of Pharmacopoeial analyses are reported in Table 4.4.

3.1.4 Norfloxacin
Norfloxacin is a synthetic antibacterial drug discovered in the late nineteen-seventies. It is also known as the first member of the second-generation quinolones or 6-fluoro quinolones. Presence of fluorine atom and a piperazine moiety are responsible for its higher potency than nalidixic acid. It has a broad spectrum and equivalent in potency to many of the fermentation derived antibiotics.

3.1.4.1 Drug Profile

(a). Molecular Information

Chemical structure:

![Chemical Structure of Norfloxacin](image)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>C₁₆H₁₈FN₅O₃</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>319.34</td>
</tr>
<tr>
<td>Molecular Composition</td>
<td>60.18% C, 5.68% H, 5.95% F, 13.16% N, 15.03% O</td>
</tr>
<tr>
<td>Exact Mass</td>
<td>319</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>1-ethyl-6-fluoro-1, 4-dihydro-4-oxo-7-</td>
</tr>
<tr>
<td></td>
<td>(1-piperazinyl)- 3-quinolinecarboxylic acid</td>
</tr>
<tr>
<td></td>
<td>3-Quinolinecarboxylic acid, 1-ethyl-6-fluoro-1,</td>
</tr>
<tr>
<td></td>
<td>4- dihydro-4-oxo-7- (1-piperazinyl)</td>
</tr>
<tr>
<td>CAS Registry No.</td>
<td>70458-96-7</td>
</tr>
</tbody>
</table>

(b). Physical Properties

Description: White to light yellow crystalline powder, odourless.

Melting Point 142° C

Solubility Ethanol (1.90 mg/ml), Acetone (5.10 mg/ml)
mg/ml), Chloroform (5.50 mg/ml), Glacial acetic acid (340.00 mg/ml), very slightly soluble in water and CH$_3$OH.

**pKa$_1$ (Carboxylic acid)**

6.34 ± 0.06 at 25°C

**pKa$_2$ (protonated piperazine N)**

8.75 ± 0.07 at 25°C

**Chemical Stability**

Norfloxacin solution in 2 N HCl after heating at 100°C led to the formation of a decarboxylated degradate, whereas no change was noted in 1 N NaOH after 15 h at 100°C. Norfloxacin solutions at pH 5.2 are stable for at least 36 months at 23°C and 30°C. It solutions can rapidly become discolored upon contact with stainless steel because of the formation of metal complexes. Solution stability was found optimum at pH 5.5 for an ionic strength of 0.20

**(e). Spectral Characteristics:**

**UV-Visible Spectroscopy**

UV $\lambda_{max}$ (0.1 N NaOH): 274 nm ($E_{1\%}$ 1cm 1109), 325 nm ($E_{1\%}$ 1cm 437), 336 nm ($E_{1\%}$ 1cm 425)

**Infrared Spectroscopy**

See Fig. 3.7

**H$^1$ NMR Spectroscopy**

See Fig. 3.8
Norfloxacin is quickly but incompletely absorbed from gastrointestinal tract when administered orally. Following single dose administration to fasting healthy volunteers, it is readily absorbed to give peak levels in serum and urine between 1 and 2 hours (Ozaki et al. 1981, Eandi et al. 1983). Norfloxacin is 10-15% bound to plasma protein. About 30% of the administered dose was recuperated from urine as unmetabolized drug. The serum half-life reported by these different authors is approximately 3 hours.

**Metabolism**

Norfloxacin is metabolized in man, to six active metabolites (Ozaki et al. 1981). The metabolites were present in quantities equal to or less than 1% of the intact drug. They are less active than the parent drug.

**Therapeutic Category and Uses**

Norfloxacin is administered mainly for management of uncomplicated UTIs (including cystitis) caused by susceptible *Enterobacter cloacae, Escherichia coli, Klebsiella pneumonia, Staphylococcus epidermidis, Staphylococcus saprophyticus*. Usually, it is reserved for
treatment of complicated UTIs caused by multidrug resistant bacteria. It is used to treat
gonorrhea, bacterial gastroenteritis and skin infections.

**Dose:** 400 mg (after every 12 h) for the duration of 3 days in the management of UTI but in
severe UTIs the treatment is continued for 10-21 days

**3.1.4.2 Characterization of Norfloxacin:**

Its characterization can be done with the help of experimental based on its official monograph
(The International Pharmacopoeia, 1981a; Indian Pharmacopoeia, 2007; United States’
Pharmacopoeia, 2004; British Pharmacopoeia, 1993b). The norfloxacin procured was
categorized on the basis of pharmacopoeial monograph in I.P.

**EXPERIMENTAL**

**(a). Procurement and Identification of Norfloxacin:**

Norfloxacin was procured as a gift sample from Vivek Pharmachem India Ltd. Jaipur. The
sample was a light yellow crystalline powder and odorless in nature. It was identified with the
help of melting point, chemical test and its spectral analyses.

**Melting Point:** Melting point was verified by open capillary method in Toshniwal melting
point apparatus.

**Spectral Identification:**

Norfloxacin was identified by using UV and FTIR spectroscopic. The scanning of UV-Visible
Spectrum was performed in a 0.0005% w/v solution in 0.1 M NaOH in the range 230 to 360
nm. FTIR Spectrum of norfloxacin was recorded by FTIR spectrophotometer (Shimadzu
FTIR Model 8300). The spectrum obtained as a mineral oil dispersion of material previously
dried. The recorded spectrum is shown in Fig. 4.4. This spectrum was matched with those
shown in Fig. 3.7 taken from official texts.

**(b). Tests for Purity and Assay:**

The sample was tested for the tests of purity as described in I.P. using pharmacopoeial
procedures. The tests conducted were Heavy metals; Related substances; Sulphated ash and
Loss on drying. Assay was also conducted by pharmacopoeial procedure. The results of
pharmacopoeial analyses are reported in Table 4.5.

**3.1.5 Salicylic Acid (O-hydroxybenzoic acid)**

Salicylic acid was first obtained in 1838 from salicin, a glycoside present in most willow
poplar bark. Interestingly, Hippocrates prescribed chewing willow bark for pain relief in the
fifth century AD. In 1860, Kolbe synthesized salicylic acid from sodium phenoxide and
carbon dioxide by a method that inexpensively produced large quantities. Derivatives of salicylic acid began to receive medical attention shortly thereafter. Sodium salicylate was employed as an antipyretic/antirheumatic agent in 1875, and the phenyl ester was used in 1886. Since then, numerous derivatives of salicylic acid have been synthesized and evaluated pharmacologically.

3.1.5.1 Drug Profile

(a). Molecular Information

Chemical structure:

![Chemical structure](image)

- **Molecular Formula**: C$_7$H$_6$O$_3$
- **Molecular Weight**: 138.12
- **Molecular Composition**: 60.87% C, 4.38% H, 34.75% O
- **Exact Mass**: 138
- **Chemical Name**: O-hydroxybenzoic acid
- **CAS Registry No.**: 69-72-7

(b). Physical Properties

- **Description**: Colourless acicular crystals or white, crystalline powder; odourless.
- **Melting Point**: 157-159 °C
- **Solubility**: 1g dissolves in 460 ml water, in 2.7 ml alcohol, 4 ml chloroform, 3 ml ether, 135 ml benzene.
- **pKa**: 2.4 at 25 °C
- **Chemical Stability**: Stable in dry air.

(c). Spectral Characteristics:

- **UV-Visible Spectroscopy**: UV $\lambda_{max}$ [salicylic in ethanol (4 mg %)]: 210, 234, 303 nm (molecular extinction coefficient 8342, 5466, 3591)
Infrared Spectroscopy  See Fig. 3.9
H¹ NMR Spectroscopy  See Fig. 3.10

Fig. 3.9: IR spectra of Salicylic acid
(d). Biopharmaceutical and Toxicological Data

Metabolism
Acetyl salicylic acid is rapidly hydrolyzed to salicylic acid by ubiquitous esterases. The apparent half-life of salicylic acid varies from 2.4 to 19 hours, because of two easily saturable salicylic acid biotransformation pathways. It is removed from the body by five parallel and competing pathways: renal excretion; conjugation with glycine to form two glucuronides, SAG and SPG, and hydroxylation to yield salicyluric acid; and conjugation of the carboxyl or hydroxyl group to form gentisic acid.

Toxicity
All salicylates cause severe toxicity (salicylism), symptoms are CNS stimulation, with vomiting, hypernea, hyperactivity, hyperthermia, and even convulsions. They quickly turn to depression, respiratory failure, and collapse.

(e). Therapeutic Category and Uses
Salicylic acid is slightly antiseptic and exerts a marked keratolytic action, so it is used externally on skin. It is locally beneficial in the management of warts, corns, and fungous infections. Also applied in 2 to 20% concentration as collodion, lotions, ointments and as a 10 to 40% concentration in plasters. Due to its keratolytic properties, it is used topically in the cure of hyperkeratic and scaling skin conditions (Martindale, 1989). It is often used in conjunction with many other agents, such as benzoic acid, coal tar, and resorcinol.

3.1.5.2 Characterization of Salicylic Acid:
Characterization of salicylic acid can be done with the help of experimental based on its official monograph (The International Pharmacopoeia, 1981a; Indian Pharmacopoeia, 2007; United State’s Pharmacopoeia, 2004; British Pharmacopoeia, 1993b). The salicylic acid procured was characterized on the basis of pharmacopoeial monograph in I.P.

EXPERIMENTAL
(a). Procurement and Identification of Salicylic acid:
Salicylic acid was procured as a gift sample from Vivek Pharmachem India Ltd. Jaipur. The obtained sample was a white crystalline powder and odourless. It was identified with the help of melting point, chemical test and its spectral analyses.

Melting Point: Melting point was found out by open capillary method in Toshniwal melting point apparatus.
**Chemical Tests:** A: Dissolve 0.2g in 5 ml of water and FeCl₃ solution was added to it. The colour so obtained was noted.

**Spectral Identification:** Salicylic acid was identified by using UV and FTIR spectrophotometer. For UV analysis, the sample was diluted in 95% ethanol and absorbance is measured at 303 nm. FTIR Spectrum of salicylic acid was recorded by FTIR spectrophotometer (Shimadzu FTIR Model 8300). The recorded spectrum is shown in Fig. 4.5. This spectrum was matched with those shown in Fig. 3.9 taken from official texts.

(b). **Tests for Purity and Assay:**

The sample was tested for the tests of purity as described in I.P. using pharmacopoeial procedures. The tests conducted were Clarity and Colour of Solution in Ethanol; Clarity of solution in alkali; Chloride; Sulphate; Iron; Heavy metals; Sulphated ash and Loss on drying. Assay was also conducted by pharmacopoeial procedure. The results of pharmacopoeial analyses are reported in Table 4.6.

3.1.6 **Aspirin (Acetyl Salicylic Acid; ASA)**

Aspirin is one of the world’s safest and least pricey pain relievers with over 100 years of proven and effective treatment for a variety of diseases. In the 5th century B.C., Hippocrates-“The father of modern medicine” is said to have used ground willow bark to relieve aches and pains. Willow bark contains salicin, the basis of a class of drugs called salicylates (http://www.aspirin.org/prof01.html). Later on German chemist Felix Hoffman in 1897, developed a less-irritating medicine for the treatment of arthritis of his father, and synthesized acetylsalicylic acid (ASA) and thus the aspirin was born. Thus, it can be regarded as the first ever prodrug prepared even before the conceptualization of the theory. The profile of the drug is given as under.

3.1.6.1 **Drug Profile**

(a). **Molecular Information**

**Chemical structure:**

![Chemical structure of aspirin](http://www.aspirin.org/prof01.html)
Molecular Formula: C₉H₈O₄
Molecular Weight: 180.15
Molecular Composition: 60% C, 4.48% H, 35.52% O
Exact Mass: 180
Chemical Name: 2-(Acetyloxy) benzoic acid
Acetylsalicylic acid
2-Acetoxybenzoic acid
CAS Registry No.: 50-78-2

(b). Physical Properties

Description: Colourless crystals or crystalline white powder; odourless.
Melting Point: 142 °C
Solubility: 1g dissolves in 300 ml water at 25 degrees, in 100 ml water at 37 degrees, in 5 ml alcohol, 17 ml CHCl₃, 10-15 ml ether
pKa: 3.49 at 25 °C
Chemical Stability: Easily hydrolyzed to salicylic acid and acetic acid by mineral acid and alkali or in moist air. Decomposed by boiled water or when dissolved in solutions of NaOH and Na₂CO₃.

(c). Spectral Characteristics:

UV-Visible Spectroscopy: UV λ_max (0.1 N H₂SO₄): 229 nm (E₁% 1cm 484); (Chloroform): 277 nm (E₁% 1cm 68)
Infrared Spectroscopy: See Fig. 3.11
H¹ NMR Spectroscopy: See Fig. 3.12
Fig. 3.11: IR spectrum of Aspirin

Fig. 3.12: $^1$H NMR spectrum of Aspirin

(d). Biopharmaceutical and Toxicological Data
Pharmacokinetics
Following i.v administration, the $t_{1/2}$ of aspirin in the human was found to be only 15 minutes (Rowland et al. 1967) and only 20% of the *in vivo* hydrolysis takes place in blood and most of it seems to be hydrolyzed in liver by esterases. Once absorbed, aspirin is rapidly converted to salicylic acid and follows the metabolic pathway of the latter.

**Toxicity**

Severe poisoning symptoms include hyperthermia, tachypnea, respiratory alkalosis, metabolic acidosis. In severe life-threatening toxicity, expected symptoms are delirium, hallucinations, convulsions, coma and respiratory arrest.

$LD_{50}$ – 500 mg/kg of body weight

**(e). Therapeutic Category and Uses**

Aspirin possesses number of properties like analgesic; antipyretic and anti-inflammatory thus make it the most often recommended drug. Gastrointestinal bleeding may occur on repeated use of aspirin. Vomiting, diarrhea, vertigo and hallucination may observe on administration of its larger doses. Average dose is 0.3-1 g; Doses of 10-30 g can be lethal. Apart from the traditional applications of acetyl salicylic acid, it is also prescribed for preventive measures for heart attacks; Pancreatic Cancer; Ovarian Cancer; Atrial Fibrillation; cataract etc. (Reynold, 1996b; Drugs Facts and Comparisons, 1997a; Roberts and Morrow, 2001a; Hason, 2000a).

3.1.6.2 Characterization of Aspirin:

Aspirin is one of the best document drugs; therefore, its characterization can be done with the help of experimental based on its official monograph (The International Pharmacopoeia, 1981a; Indian Pharmacopoeia, 2007; United States’ Pharmacopoeia, 2004; British Pharmacopoeia, 1993b). The aspirin procured was characterized on the basis of pharmacopoeial monograph in I.P.

**EXPERIMENTAL**

(a). Procurement and Identification of Aspirin:

Aspirin was procured as a gift sample from Vivek Pharmachem India Ltd. Jaipur. The obtained sample was a white crystalline powder and odourless powder. It was identified with the help of melting point, chemical test and its spectral analyses.

**Melting Point:** Melting point was verified by open capillary in Toshniwal melting point apparatus.

**Chemical Tests:** A: About 0.5 g of drug was boiled with 10 ml of NaOH solution for 3 min. It was cooled and added 10 ml of dil. $H_2SO_4$. A crystalline white ppt. was obtained. The odor
of the solution was noted. The crystalline precipitate was filtered, dissolved in about 2 ml of water and FeCl₃ solution was added to it. The colour obtained was noted.

B: In the filtrate obtained in above test, 3 ml of 95% C₂H₅OH and 3 ml of H₂SO₄ was added and heated gently. The odor so produced was noted.

**Spectral Identification:** Aspirin was identified by using UV and FTIR spectrosopes. The scanning of UV-Visible Spectrum was performed by liquefying 20 mg of sample in 20 ml of PBS (pH 7.4) and scanning the above solution in the UV region. FTIR Spectrum of the aspirin was recorded by FTIR spectrophotometer (Shimadzu FTIR Model 8300). The recorded FTIR spectrum is shown in Fig. 4.6. The spectrum was matched with those shown in Fig. 3.11 taken from official texts.

(b). **Tests for Purity and Assay:**

The sample was tested for the tests of purity as described in IP using pharmacopoeial procedures. The tests conducted were Clarity and Colour of Solution in Ethanol; Clarity of solution in alkali; Chloride; Sulphate; Arsenic; Heavy metals; Readily carbonisable substances; Salicylic acid; Sulphated ash and Loss on drying. Assay was also conducted by pharmacopoeial procedure. The results of pharmacopoeial analysis are reported in Table 4.7.

### 3.1.7 Indomethacin

Indomethacin is the member of class aryl acetic acids of derivative of indole acetic acid. It is more potent NSAID but used with restrictions due to its adverse effects. However, it is still a lead compound for researchers due to its potency and excellent anti-inflammatory properties. It is one of the model drug used for the colon delivery experiments due to its potentials as anti-inflammatory properties. Friend (2005) in his review foresees the utilization of this drug in low dose embedded in polymeric prodrug in future.

#### 3.1.7.1 Drug Profile

(a). **Molecular Information**

**Chemical structure:**
Molecular Formula \( \text{C}_{19}\text{H}_{16}\text{ClNO}_4 \)

Molecular Weight 357.80

Molecular Composition C- 63.71% H- 4.51% Cl- 9.91% O- 17.89% N- 3.91%

Exact Mass 357

Chemical Name \[1-(4\text{-chlorobenzoyl})-5\text{-methoxy-2-methylindol-3-yl}] \text{acetic acid} \]

CAS Registry No. 53-86-1

(b). Physical Properties

Description: White to pale yellow, Crystalline powder; Odourless.

Melting Point 155 °C for Polymorph-I and 162 °C for Polymorph-II

Solubility Soluble in CHCl₃; sparingly soluble in C₂H₅OH (95%) and in ether; practically insoluble in water

pKa 4.5 at 25 °C

Chemical Stability Stable in neutral or slightly acidic media; decomposed by strong alkali.

(c). Spectral Characteristics:

UV-Visible Spectroscopy UV max (ethanol): 230, 260, 319 nm (molecular extinction coefficient 20800, 16200, 6290)
Infrared Spectroscopy \hspace{1cm} \text{See Fig. 3.13}

H$^1$ NMR Spectroscopy \hspace{1cm} \text{See Fig. 3.14}

Fig. 3.13: FTIR Spectra of Indomethacin
Pharmacokinetics
Indomethacin is rapidly and almost completely absorbed from the GIT after oral intake. The peak concentration in plasma is achieved within 2 h in the fasting subject. The plasma concentration requisite for an anti-inflammatory action have not been absolutely determined but are probably less than 1 \( \mu \text{g/ml} \). Steady-state concentrations in plasma after long-term administration are approximately 0.5 \( \mu \text{g/ml} \). 90% of indomethacin is bound to plasma proteins. Its concentration in the cerebro spinal fluid is low but in synovial fluid it is equivalent to that in plasma within 5 h of administration.

Metabolism:
Indomethacin is biotransformed principally to inactive metabolites, formed by O-demethylation (about 50%), conjugation with glucuronic acid (about 10%), and N-deacylation. About 10% - 20% of the drug is excreted through urine remain unchanged, in part by tubular secretion. The t\(_{1/2}\) in plasma is not constant, perhaps due to enterohepatic cycling, but average t\(_{1/2}\) is about 3 h.

LD\(_{50}\) in male mice, rats 13 mg/kg

(e). Therapeutic Category and Uses
Because of the high incidence and severity of side effects associated with long-term administration, Indomethacin is not commonly used for therapy as an analgesic or antipyretic. However, it has proven to be useful as an antipyretic in certain settings (e.g., Hodgkin's disease) when the fever has been refractory to other drugs. Indomethacin is also efficient in dealing osteoarthrosis. It is found very effective in the treatment of acute gout, although it is not uricosuric. Patients with Bartter's syndrome have been treated successfully with Indomethacin, as well as with other inhibitors of prostaglandin synthetase. The results are frequently dramatic; however, the condition of the patients may deteriorate rapidly when therapy is discontinued, and the long-term therapy necessary to control the disease requires administration of a drug that is better tolerated. Indomethacin has at least two uses in obstetrics and neonatal medicine. It can be used as a tocolytic agent to suppress uterine contractions in women with preterm labor and cardiac failure in neonates caused by a patent ductus arteriosus may be controlled by the administration of Indomethacin. (Reynold 1996d; Hason 2000c; Drugs Facts and Comparisons 1997c and Roberts and Morrow 2001c)
Dose: Orally, 50 to 200 mg daily, in divided doses, with food and as suppositories, 100 mg at night and in the morning if required. Maximum combined oral and rectal dose, 150 to 200 mg daily.

3.1.7.2 Characterization of Indomethacin:
Indomethacin is one of the well-studied drugs; therefore, its characterization can be done with the help of experimental based on its official monograph (The International Pharmacopoeia, 1981c; Indian Pharmacopoeia, 2007; United States’ Pharmacopoeia, 2004; British Pharmacopoeia, 1993c). The indomethacin procured was characterized on the basis of pharmacopoeial monograph in I.P.

EXPERIMENTAL

(a). Procurement and Identification of Indomethacin:
Indomethacin was gained as a gift sample from Vivek Pharmachem India Ltd. Jaipur. The obtained sample was a white crystalline powder and odourless powder. It was identified with the help of melting point, chemical test and its spectral analyses.

Melting Point: Melting point was found out by open capillary method in Toshniwal melting point apparatus.

Chemical Tests: The sample (0.1 g) was dissolved in 10 ml of ethanol (95%) by heating gently. Mixture of 25% w/v solution of hydroxylamine hydrochloride and 2 M NaOH in a volume ratio (1:3) was prepared freshly. The sample solution (0.1 ml) was taken and 2 ml of mixture of hydroxylamine hydrochloride and NaOH was put into it. To this 2 ml of 2 M HCl and 1 ml of FeCl₃ solution were added. The change of colour was noted.

Spectral Identification: Indomethacin was identified by using UV and FTIR spectroscopy. The scanning in UV-Visible region was done in 0.0025% w/v solution of the sample drug in a mixture of methanol and 1 M HCl (9:1) and scanned in the region of 230 to 360 nm. FTIR Spectrum of indomethacin was recorded by FTIR spectrophotometer (Shimadzu FTIR Model 8300). The recorded spectrum is shown in Fig. 4.7. The spectrum was matched with those shown in Fig. 3.13 taken from official texts.

(b). Tests for Purity and Assay:
The sample was tested for the tests of purity as described in IP using pharmacopoeial procedures. The tests conducted were Heavy metals; Related substances; Sulphated ash and Loss on drying. Assay was also conducted by pharmacopoeial procedure. The results of pharmacopoeial analyses are reported in Table 4.8.
3.2 SELECTION AND OPTIMIZATION OF REACTIONS FOR SYNTHESIS OF MUTUAL PRODRUGS

Literature has revealed that the amide and azo prodrugs have enough potential to deliver the drug in the colon or distant ileum in the treatment of IBD. Since, the distal regions of the gastrointestinal tract is predominantly colonize by bacteria where the bacterial tot up in the colon is about 10^{11} per g, as compared with 10^4 per g in the small intestine. The enzymes produced by these colonic bacteria which are mostly anaerobic accomplished metabolism of endogenous and exogenous substances, for instance proteins and carbohydrates that escape digestion in the upper GIT and thus, are also responsible for the release of the most of drug in the colon.

3.2.1 General Methods of Synthesis of Mutual Prodrugs:

Most of the work on mutual prodrugs was subjected for IBD treatment, and it is believed that IBD is induced by the bacteria present in the colon and an infection always leads to inflammation. Therefore, antibacterial agents and NSAIDs are the first choice of drugs. As the drugs selected bare amino groups and carboxylic acid groups, respectively, thus, the basic reactions adopted were amidation and diazotization followed by coupling. Thus, the general methods of amidation are discussed here.

3.2.1.1 Amidation

(a). Direct conversion of carboxylic acid to an amide by reaction with an amine

Amides can be synthesized by the reaction of an acid with an ammonia or amine (1° and 2°) directly. The reaction is represented in Fig. 3.15

\[
\text{R} \text{O} \text{H} + \text{H}_2\text{N-R'} \xrightarrow{\triangle} \text{R} \text{O-N-H} \text{R'} + \text{H}_2\text{O}
\]

Fig. 3.15: Direct amidation reaction

The direct reaction is possibly expected to be not easy due to formation of highly unreactive carboxylate species by the deprotonation of carboxylic acid with the basic amine. However, when the ammonium carboxylate salt is heated to a temperature above 100 °C water is driven
off and an amide is formed. But this method cannot be used for present work due to following limitation. (Norman and Coxon, 1993; Vogel, 1971; Mann and Saunder, 1986)

- The reaction is difficult in nature due to formation of highly unreactive intermediate.
- Yield of product is low in this reaction.

**b). Amidation using Carbodiimides**

Carbodiimides have been evolved as novel methods for synthesis of peptides. Among these, Dicyclohexylcarbodiimide (DCC) was the first member of the family followed by several others. They are basically dehydrating and coupling agents. Now they are widely employed for the synthesis of esters and acid anhydrides also. The mechanism of formation of esters and amide using carbodiimides is shown in Fig. 3.16

![Mechanism of formation of amide and esters using carbodiimides](image)

Where $R = R_2 = $ Alkyl or Aryl Group $X = $ NH or O  
R_1 = $ Subtiuent on carbodiimide

3.16: Mechanism of formation of amide and esters using carbodiimides
Rebek and Feitler in 1974 postulated that an anhydride intermediate formation may be a common mechanism in route of the amide bond formation with an amine, particularly under anhy. conditions. When two molecules of the corresponding acid react in presence of one equivalent of DCC under milder conditions, symmetric anhydrides are formed (Mikolajczyk and Keilbasinski, 1981). The driving force of this reaction is the generation of isourea as byproduct. In the second step, the anhydride is then reacted with selected amine. Theoretically, no additional base is needed, as the addition produces carboxylate anion insitu. This efficient coupling scheme is well-suited for peptides formation only (Christian AGN). Apart from DCC, other carbodiimides like EDAC and CMC are also used to formed amide linkage between carboxylic acid and amine. These both are water-soluble derivative of carbodiimide. A number of chemical conjugates possibly produced by means of EDAC (Yamada et al. 1981; Chu and Ueno, 1977; Chase et al. 1983). Carbodiimide catalyzes the formation of amide bonds between carboxylic acids or phosphates and amines by activating carboxyl or phosphate to form highly reactive, O-acylisourea derivative. Nucleophilic attack on this adduct gives the acylated product (William and Ibrahim 1981). These reagents can be applied to form peptide bonds from acid and amines, and ester links from acid and alcohols or phenols (Tedder et al. 1972). Detailed protocols regarding nature and applications of carbodiimides are given by Hermanson (1996).

But this method cannot be used for present work due to following limitation.

- DCC is often tricky to take out from a bottle as its vapours are extremely harmful to the eyes.
- It would be difficult task to remove isourea byproduct (dicyclohexyl urea) of a reaction initiated by DCC as it is water-soluble and should be eliminated by washing through organic solvent.
- The main limitation using DCC as coupling agent in the formation of amide using carboxylic acid is that only half of the acid is effectively coupled, while the rest half is dissipated. This could be a setback if the acid is expensive.
- When EDAC is used as cross linker in the formation of amide, the major competing reaction is the hydrolysis by water in aqueous solution, as it cleave off the activated ester intermediate, forming an isourea and regenerating the carboxylate group.

(c). Amidation using Acid Chloride
Compounds containing carboxyl moiety can be triggered as acetyl halides, anhydrides, acyl azides, esters, acyl imidazoles etc. These reactive carboxyl derivatives are then coupled with an amine in different ways.

- An intermediate acylating agent so formed is then separated and subjected to aminolysis
- An acylating agent is generated from an acid by the addition of coupling agent in situ, is then treated with selected amine

As discussed in previous section that amides are normally not synthesized directly and by carboxylic acids due to several limitations. So, it is more convenient to use conventional methods in which acids are usually converted to functional derivatives - acyl chloride or acid anhydride before going reacting to amines (Norman and Coxon, 1993; Vogel, 1971). Here, we discuss amidation using acyl chlorides as it is most convenient method of synthesis of amides. Generally, amidation via acid chloride involved following steps, and the scheme is represented in Fig. 3.17

  - Synthesis of acid chloride.
  - Acylation of ammonia or amines (1° or 2°) with acid chloride using Schotten - Baumann reaction.

**Fig. 3.17: Scheme of synthesis of amides via acid chloride**

*General methods for preparation of acyl chloride:* Acid chlorides can be generated from their corresponding acids by reacting with any one of the phosphorus pentachloride (PCl₅), phosphorus oxytrichloride (POCl₃), thionyl chloride (SOCl₂) or oxalyl chloride (COCl₂). The phosphorous pentachloride is preferred in chlorinating aromatic acids having electron leaving moieties and which do not react readily with SOCl₂. The use of oxalyl chloride is going together with the stoichiometric production of two gas molecules, CO is one of them.
(Bruckner, 2002). So, the resulting chemical exposures must always be taken into contemplation before set up reaction (Urben, 1999). For synthesis of aliphatic acid chlorides, thionyl chloride is the most preferred reagent provided that product has a boiling point, which permits separation of excess of reagent by fractional distillation. The reactions are often catalyzed by the adding of one or two drop of dimethylformamide (DMF).

1. Heating carboxylic acid with phosphorous trichloride (PCl₃).

\[ 3\text{RCOOH} + \text{PCl}_3 \rightarrow 3\text{RCOCl} + \text{H}_3\text{PO}_3 \]

2. By heating carboxylic acid with phosphorous pentachloride (PCl₅).

\[ \text{RCOOH} + \text{PCl}_5 \rightarrow \text{COCl} + \text{POCl}_3 + \text{HCl} \]

3. By treating carboxylic acid with oxalyl chloride (COCl₂).

\[ \text{RCOOH} + \text{Cl}^+ + \text{O} \rightarrow \text{RCOCI} + \text{HCl} + \text{CO} + \text{CO} \]

4. Reaction of carboxylic acid with thionyl chloride (SOCl₂)

\[ 3\text{RCOOH} + \text{SOCl}_2 \rightarrow 3\text{RCOCl} + \text{HCl} + \text{SO}_2 \]

In the case of reactions involving phosphorus trichloride, phosphorus pentachloride, and phosphorus oxytrichloride, hydrogen chloride gas is generated violently. Therefore, thionyl chloride and oxalyl chloride are used generally for the synthesis of amides because of the possibility of controlled reaction. Mechanism of reaction involved in the synthesis of acyl chloride using thionyl chloride and oxalyl chloride is shown in Fig. 3.18 and 3.19, respectively.
3.18: Mechanism for acyl chloride synthesis using thionyl chloride

Fig. 3.19: Mechanism for acyl chloride formation using oxalyl chloride

d. Amidation using Acid Anhydride

Similarly, the carboxylic acids can be converted to acid anhydride before reacting to ammonia or amines ((1° or 2°) (Norman and Coxon, 1993; Vogel, 1971 and Mann and Saunders, 1986). Anhydride can be prepared by several methods. The most convenient method of synthesis of anhydride is by using dehydrating agent. This method involved the fact that N, N'- Dicyclohexylcarbodiimide (DCC), hich is an excellent dehydrating agent, reacts first with acid moiety to form an intermediate. Since the compound remains intact during this initial step. The intermediate is then attacked by another molecule of RCOO⁻ to give anhydride (RCO)₂O, which is the actual species that reacts with an amine to form amides. (March 1988), scheme is depicted in Fig. 3.20 and 3.21.
3.2.2 Diazocoupling Reaction

Most common method used for the formation of mutual azo prodrugs is diazotization followed by coupling, scheme is shown in Fig. 3.22. Diazotization involves reaction of primary amines with nitrous acid in acidic solution to give diazonium ions. Aromatic
Diazonium ions are moderately stable in aqueous solution at low temperature when present with anions of low nucleophilic power as compared to aliphatic diazonium ions which are too unstable. The mechanism of formation of diazonium ion may be summarized in Fig. 3.23 (Norman and Coxon, 1993).

**3.22: General scheme of mutual azo prodrug formation**

**3.23: Mechanism of formation of Diazonium ion**

The reactions of diazonium ions with aromatic nuclei are known as coupling reaction. Diazonium ions are weakly electrophilic and react only with those aromatic compounds which are very powerfully activated towards electrophiles (such as phenols, amines and heterocyclic systems such as pyrrole), scheme is depicted in Fig. 3.24. Azo mutual prodrugs of the NSAIDs with peptide, including an antibiotic peptide temporin analog are synthesized by Kennedy et al. 2011. Similarly, azo mutual prodrugs of 5-ASA with essential amino acids were prepared following above reaction method by Dhaneshwar et al. in 2009.
3.2.3 Selected Methods of Synthesis of Mutual Prodrugs

The proposed mutual prodrugs are the amides of NSAIDs with the antibacterial. Several mutual amides prodrugs have been reported by various authors. Prodrugs of 4-ASA with phenylalanine and tryptophan have been prepared by Dhaneshwar et al. in 2009. NSAIDs viz. ibuprofen, flurbiprofen and diclofenac were conjugated with sulfonamides via amide bond is also reported (Makhija et al. 2013). Mutual amide prodrugs of ibuprofen with various sulfa drugs have also been reported (Nazeruddin and suryawanshi, 2010). As we discussed in the previous section that carbodiimide chemistry has several limitations and amide formation via acid anhydride involves half wasted of carboxylic acid. Therefore, Schotten-Boumann reaction for amide prodrugs formation via acid chloride was tried in the present research work as it is most convenient method which gave satisfactory results. For this purpose we used thionyl chloride for the preparation of acyl chloride which is then reacted with amine in presence of pyridine by refluxing for several hours. In place of pyridine, the typical acylation can also be carried out in presence of other non-nucleophilic tertiary organic bases like DMAP (dimethylaminopyridine), TEA, 4-picoline, N-methylmorpholine and sodium 2-ethylhexanoate (Rajput and Gore 2011). We selected this conventional method for amidation as it results in appreciable yield of prodrugs. Furthermore, the entrapment was avoided by using calculated amount of drugs. The reaction is schematically represented in Fig. 3.25.
For the synthesis of azo prodrugs of sulfonamides with NSAIDs, the most familiar method of diazotization followed by coupling reaction was used. General scheme of synthesis of azo prodrugs is shown in Fig. 3.26.

**Fig. 3.26: Scheme of synthesis of azo prodrugs**

Several mutual azo prodrugs have been reported on time to time. Recently, a mutual azo prodrug of 5-ASA and 2-phenylbenzoxazole-2-yl-5-acetic acid has been synthesized (Jilani et al. 2013). Many mutual azo prodrugs of anti-inflammatory drugs with essential amino acids have been synthesized (Nagpal et al. 2006; Dhane 2007, 2007 and 2009). Synthesis of mutual prodrugs of NSAIDs with sulfonamide conjugates using amino acids as spacers have been synthesized (Abdulhadi and Qasir 2013).

The aimed mutual prodrugs are cataloged in Table 3.1.

**Table 3.1: List of Aimed Mutual Prodrugs**

<table>
<thead>
<tr>
<th>Product Code</th>
<th>Drug Moiety I (Anti-inflammatory Drugs)</th>
<th>Drug Moiety II (Antibacterial Drugs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>Aspirin</td>
<td>Norfoxicin</td>
</tr>
<tr>
<td>AI</td>
<td>Aspirin</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td></td>
<td>IN</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>---</td>
<td>----</td>
<td>--------------</td>
</tr>
<tr>
<td>IT</td>
<td>Indomethacin</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>SDS</td>
<td>Salicylic acid</td>
<td>Sulphadiazine</td>
</tr>
<tr>
<td>SMS</td>
<td>Salicylic acid</td>
<td>Sulphamethoxazole</td>
</tr>
</tbody>
</table>

**3.2.4 Experimental Procedure of Synthesis of Mutual Amide Prodrugs:**
Schotten-Boumann reaction was used to prepare amide conjugates of aspirin and indomethacin by reacting their acid chloride with norfloxacin and trimethoprim in pyridine (Norman and Coxon, 1993; Vogel, 2010; Rawat et al. 2007). The formation of acyl chloride of aspirin and indomethacin followed by coupling with the given antibacterial drugs norfloxacin and trimethoprim are schematically depicted in Fig. 3.27 and 3.28, respectively.
3.2.4.1 Synthesis of mutual prodrug of Aspirin and Norfloxacin [[Acetylsalicylamido-norfloxacin (AN)]]

(a). Preparation of acyl chloride of aspirin [2-acetoxybenzoyl chloride]:
Firstly, benzene was dried with flattened pieces of sodium. Aspirin (0.05 mol, 9 g) was dissolved in 60 ml of sodium-dried benzene taken in a 100 ml volumetric flask, and then freshly distilled SOCl₂ (0.05 mol+20% excess, 4.5 ml) was added slowly in small portions with the addition of 1 or 2 drop of DMF as catalyst. The reaction mixture was refluxed for 3 h at 70-80 °C, until the evolution of HCl and SO₂ ceased. The excess of SO₂ and benzene were distilled off under reduced pressure, when the volume reduced to about 20 ml the contents of the flask were poured into a china dish and dried in an oven at 100° C. The product yield was
noted (it was found 9.23 g, 93 %) and taken for coupling with norfloxacin and trimethoprim by dividing into two equal parts (4.61 g).

(b). Coupling of 2-acetoxybenzoyl chloride with norfloxacin:
Norfloxacin (0.02 mol, 6.38 g) was first dissolved in 100 ml acetone by stirring continuously on magnetic stirrer for about 1 h and then taken in a R.B.F containing 2-acetoxybenzoyl chloride (0.02 mol, 3.98g) and 20 ml of pyridine. The mixture was refluxed for 1 h at 100º C on water bath. After cooling, it was kept aside. After 24 h the mixture was added into crushed ice and the resultant compound was obtained as ppt., which was then filtered and rinsed with water and drained well. The crude white to light yellow solid was then recrystallized from rectified alcohol. The yield of the recrystallized product was 5.68 g (59 %).

3.2.4.2 Synthesis of mutual prodrug of Aspirin and Trimethoprim [Acetylsalicylamido- trimethoprim (AT)]
This step involves coupling of the 2-acetoxybenzoyl chloride with trimethoprim. 2-acetoxybenzoyl chloride as prepared by the procedure described in the previous section was taken as such for this step. First trimethoprim (0.02 mol, 5.80 g) was placed directly in round bottom flask containing about 20 ml of pyridine and 2-acetoxybenzoyl chloride (0.02 mol, 3.98g). This mixture was refluxed for 1 h at 100 ºC on water bath. After cooling the mixture, it was kept aside. After 24 h it was transferred into crushed ice with vigourous shaking and the resultant prodrug was obtained as a precipitate, which was sifted and then rinsed with water. The compound (AT) so obtained was purified by recrystallization from rectified alcohol. The yield of the prodrug was 5.79 g (64 %).
3.28: Scheme of synthesis of IN and IT

3.2.4.3 Synthesis of mutual prodrug of Indomethacin and Norfloxacin
[Indomethacinamido-norfloxacin (IN)]
(a). Preparation of acyl chloride of Indomethacin [2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetyl chloride]

Indomethacin (0.03 mol, 10.73 g) was first dissolved in 150 ml of CHCl₃ taken in a R.B.F and freshly distilled SOCl₂ (0.03 mol+20% excess, 2.7 ml) was added slowly to the above solution. Now add about 1 or 2 drop of DMF as catalyst in the reaction mixture. The mixture was then refluxed for 3 h at 70-80 ºC, until the evolution of HCl and SO₂ ceased. The excess of SOCl₂ and benzene were distilled off under reduced pressure. When the volume of the content reduced, it was transferred to a china dish and dried at 100º C in an oven. The yield of the white crude product so obtained was 8.91 g (79 %). It was then taken for the next step of coupling.

(b). Coupling of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetyl chloride with norfloxacin:

Accurately weigh amount of norflloxacin (0.01 mol, 3.19 g) was taken in about 5 ml of 5% ice cooled solution of NaOH and 60 ml acetone was added to it. The mixture was kept at magnetic stirrer to dissolve it completely for 1 h. The above solution was then placed in a R.B.F containing 3.76 g (0.01mol) acetyl chloride of indomethacin and about 10 ml pyridine. The solution was then refluxed for 1 h at 100 ºC on water bath. After cooling, it was kept aside for 24 h. The mixture was then transferred into crushed ice to give ppt., which was filtered off and rinsed several times with water. The crude white to light yellow colour solid was then recrystallized from rectified alcohol. The yield of the recrystallized product was 3.76 g (57 %).

3.2.4.4 Synthesis of mutual prodrug of Indomethacin and Trimethoprim [Indomethacinamido-trimethoprim (IT)]

Acyl chloride of indomethacin was prepared by the same method given previously was coupled with trimethoprim for the synthesis of IT. For this, weigh amount of trimethoprim (2.90 g, 0.01 mol) was taken in a R.B.F containing about 10 ml of pyridine and 3.76 g (0.01 mol) acyl chloride of indomethacin. The mixture was refluxed for 1 h at 100º C on water bath. After cooling, the mixture was kept aside for 24 h and then transferred into crushed ice. The product was gained as precipitate, which was sifted and rinsed with water and recrystallized from rectified alcohol. The yield of the crystalline white substance was 62 % (3.90 g).
3.2.5 Experimental Procedure of Synthesis of Mutual Azo Prodrugs: [Norman and Coxon 1993]

Diazotization reaction followed by coupling process was used for the preparation of azo conjugates of sulphadiazine and sulphamethoxazole with salicylic acid. The reaction involves first the formation of diazonium salt of sulfa drugs which later on coupled with the salicylic acid to give mutual azo prodrugs. The scheme of syn is represented in Fig. 3.29.

![Scheme of synthesis of SDS and SMS](image)

3.2.5.1 Synthesis of SDS (Salicylazosulfadiazine)

(a). Preparation of diazonium salt of Sulphadiazine:

Sulphadiazine (0.15 mmol, 3.75 g) was first dissolved in 50 ml of 3N HCl solution by stirring. The solution was cooled in cryostatic bath and the temperature was maintained at 0 to 5 ºC throughout. To the above mixture, 0.15 mmol or 1.03 g of sodium nitrite was mixed with vigorous stirring. Yellow colour solution was obtained. The hydrolysis of diazonium salt was avoided by maintaining the reaction at 0-5 ºC.

(b). Coupling of diazonium salt of Sulphadiazine with salicylic acid:

Weigh amount of salicylic acid (2.07 g, 0.15 mmol) was liquefied in 50 ml NaOH aq. solution (20 %). The above solution was set in an ice tub at below 5 ºC temperature for about 2 min. It was now poured to the diazonium salt solution with rapid and vigorous stirring, the dark red color solution was obtained. The reaction mixture was set aside for 5 min. After 5 min, about 50 ml of 3N HCl was mixed rapidly with vigorous stirring to the above solution. The red colour soon disappears and the product was separated from the solution as brownish yellow solid. The resulting solid was now passed through a filter and rinsed with water 2-3 times and dried under vacuum. For purification of the product, the residue was dissolved in pyridine and loaded on a silica gel open column and eluted with ethyl methyl ketone/acetone/water (16:16:1) at flow rate 1.2 ml/min, the second of the three bands to elute is synthesized prodrug which is collected and solvent remove by evaporation (Stone and Gorby 1974). The yield of the product is 4.73 g (79 %)

3.2.5.2 Synthesis of SMS (Salicylazosulfamethoxazole)

(a). Preparation of diazonium salt of Sulphamethoxazole:

It was prepared by the same process as described above for the synthesis of diazonium salt of sulphadiazine. Accurately weight 3.79 g or 0.15 mmol of sulphamethoxazole was first
dissolved in 50 ml of 3N HCl solution by stirring and the solution was kept cooled in cryostatic bath at 0º to 5º C. To the above mixture, 1.0 g (0.15 mmol) sodium nitrite was added with vigorous stirring. It gave a light yellow coloured solution.

(b). Coupling of diazonium salt of Sulphadiazine with Salicylic acid: Meanwhile, salicylic acid (2.07 g, 0.15 mmol) was dissolved in 50 ml NaOH aqueous solution (20 %) and the mixture was set aside below 5 ºC in an ice tub for 2 min. The above solution was now put in to the diazonium salt of sulphamethoxazole with rapid and vigorous stirring; the dark red solution was obtained. The reaction was set aside for 5 min; then 50 ml of 3N HCl was rapidly added to it with vigorous stirring. The red colour disappears and the product was separated from the solution as light brownish yellow solid. The resulting product was filtered and rinsed with water and dried under vaccum and then purified by using column by the same method mentioned in the previous section. The yield of the prodrug so obtained was 4.46 g (74%).

The amount of the drug moiety I and drug moiety II used for the synthesis of different mutual prodrugs and their yield data are given in Table 3.2

<table>
<thead>
<tr>
<th>Product code</th>
<th>Drug Moiety I</th>
<th>Drug Moiety II</th>
<th>Prodrug Obtained (g)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>Aspirin</td>
<td>Norfloxacin</td>
<td>5.68</td>
<td>59</td>
</tr>
<tr>
<td>AT</td>
<td>Aspirin</td>
<td>Trimethoprim</td>
<td>5.79</td>
<td>64</td>
</tr>
<tr>
<td>IN</td>
<td>Indomethacin</td>
<td>Norfloxacin</td>
<td>3.76</td>
<td>57</td>
</tr>
<tr>
<td>IT</td>
<td>Indomethacin</td>
<td>Trimethoprim</td>
<td>3.90</td>
<td>62</td>
</tr>
<tr>
<td>SDS</td>
<td>Salicylic acid</td>
<td>Sulfadiazine</td>
<td>4.73</td>
<td>79</td>
</tr>
<tr>
<td>SMS</td>
<td>Salicylic acid</td>
<td>Sulfamethoxazole</td>
<td>4.46</td>
<td>74</td>
</tr>
</tbody>
</table>

3.3 CHARACTERIZATION OF THE SYNTHESIZED MUTUAL PRODRUGS
Characterization is an important factor for the confirmation of the formation of desired compounds. It provides support to what we have done in the synthesis section.
Characterization of the synthesized conjugates involves both course of characterization, viz., chromatographic and spectroscopic method along with physicochemical characterization like melting point, elemental composition determination etc. These studies are conclusive enough to characterize them.

3.3.1 Physicochemical Characterization of Synthesized Compounds

For the synthesized compounds determination of physical constants was carried out in preliminary stage.

3.3.1.1 Solubility:
Solubility can be expressed quantitatively as the concentration of the solute in a saturated solution at a certain temperature. I.P. (2007) expresses solubility at the temperature ranging from 20–30 °C. Solubility may be avowed in units of Concentration, Molality, Mole ratio, Mole fraction, and other units. The solubility of various synthesized prodrugs was studied in various solvents.

*Experimental procedure:* Approximately 10 mg of compd. was liquefied in 5 ml of each solvent at 37± 1 °C in different glass test tubes. Test tubes were shaken gently and solubilities were examined. When any insoluble fraction was observed, the known amt. of solvent was further included to determine the solubility of the given compd. (Gennaro A R, 2008).

3.3.1.2 Melting Point Determination:
The melting points of the intermediates formed during course of reaction and the synthesized conjugates were determined by open capillary tube using Toshniwal Melting Point Apparatus and errors are uncorrected.

3.3.1.3 Elemental analysis:
Elemental analysis of intermediates and synthesized prodrugs was performed on Thermo scientific (FLASH 2000) Elemental Analyzer, SAIF, Panjab University, Chandigarh. The results are given in the form of percentage of each element analyzed. They were in an acceptable error range.

3.3.1.4 Thin Layer Chromatography:
The purity of the synthesized derivatives was ensured by subjecting to thin layer chromatography. It was carried out on silica gel precoated plates of Merck with CHCl₃: CH₃OH: glacial CH₃COOH (4:4:2) as solvent system used for prodrugs AN, AT, IN, IT and acetonitrile: methanol: water (4:2:1) was used for prodrugs SDS and SMS, iodine vapours
and UV light were used as detecting agent for visualization. All synthesized derivatives gave brown spot. \( R_f \) values were calculated from the TLC plates.

### 3.3.2 Spectroscopic Methods for Characterization of Synthesized Derivatives

The synthesized compounds were analyzed and their structures were supported and corroborated by spectroscopic analyses viz., FTIR, NMR (\(^1\)H and \(^{13}\)C) and Mass spectroscopy.

#### 3.3.2.1 UV Spectroscopy:

The \( \lambda_{max} \) of the synthesized prodrugs AN and AT was determined in methanol; IN and IT was determined in chloroform; SDS and SMS was determined in acetonitrile on a Shimadzu 1700 UV double beam spectrophotometer.

#### 3.3.2.2 Infrared Spectroscopy:

The infrared spectra of the prodrugs were recorded (scanning range between 4000 cm\(^{-1}\) to 250 cm\(^{-1}\) and resolution of 1 cm\(^{-1}\)) on Shimadzu FTIR in KBr pellets (anhy.) at the University of Rajasthan, Jaipur and on Perkin Elmer-Spectrum RX-IFTIR in KBr pellets at SAIF, Panjab University, Chandigarh.

#### 3.3.2.3 \(^1\)H NMR and \(^{13}\)C NMR Spectroscopy:

The \(^1\)H NMR and \(^{13}\)C NMR spectral characterization of the synthesized compounds were done and their spectrum was recorded in deuterated Dimethylsulfoxide (DMSO-d\(_6\)) as solvent using Bruker Avance II 400 NMR spectrometer, SAIF, Panjab University.

#### 3.3.2.4 Mass Spectroscopy:

The molecular weights of synthesized compounds were determined from their Mass spectrum traced at Jeol SX-102(FAB) Mass spectrometer, SAIF, Panjab University, Chandigarh.

### 3.4 IN VITRO RELEASE STUDIES OF SYNTHESIZED MUTUAL PRODRUGS FOR COLONIC RELEASE

#### 3.4.1 General Consideration

A harnessing of curiosity of researches is never ends as every step give rise to another step to be completed in order to get fruitful outcome. After successful characterization of the new synthesized derivatives, the next step is to see whether these derivatives serve the purpose for what they had been synthesized. The purpose of this research was to deliver the drugs in the colon to reduce inflammation and to get rid from bacterial infection by the safer and time-
tested drugs. To achieve success many hypothesis are to be tried. As all the synthesized compounds are of higher molecular weight and thus according to new evolve Biopharmaceutical classification system are hydrophobic in nature. Two synthesized compounds (IN and IT) also obey Lipinski golden rule of five suggests that these prodrugs would not be absorbed in the GIT. Therefore, attempts to study the absorption and other parameters relating to absorption were not made. In this section we are focused on the fate of the newly generated xenobiotics in the GIT environment. Life is too precious and to sacrifice the animals for proving theories must come after getting firm confirmation of simulated studies. Hence, the confirmation by studies in different simulated fluids is provided in this section. In order to provide the requisite, the studies are carried out as following:

- Establishment of methods for release of drugs in different fluids used for in vitro studies viz. simulated gastric fluid, simulated jejunal fluid, simulated intestinal fluid, and simulated colonic fluid.
- In vitro release studies of drugs from mutual prodrugs in simulated gastro intestinal environment.

3.4.2 Prodrugs Analysis in Simulated Gastro-intestinal Environment

The residence period of the food contents during gastric transit in different parts of the organs of GIT was studied by various workers and it was revealed that food remains in stomach for about 2 hours. From the stomach the contents reach to jejunum where it remains for about 2 hours, wherefrom it travels to ileum and then to colon. So, it takes nearly about 8 hours to reach to colon. However, the given time period is an average time for healthy individuals. On the basis of this fact, the release studies in simulated gastro-intestinal fluids were carried out using following simulated fluids:

1). Simulated Gastric Fluid (SGF, pH 1.2)
2). Simulated Jejunal Fluid (SJF, pH 4.5)
3). Simulated Intestinal Fluid (SIF, pH 7.4)
4). Simulated Colonic Fluid (SCF, pH 7.0)

Therefore, the analytical methods have to be developed for the prodrugs in these fluids before carrying out release studies. All the selected drugs and their respective synthesized prodrugs show absorption of light of good magnitude in UV region and as the \( \lambda_{\text{max}} \) of the parent drugs were found to be substantially different from their corresponding synthesized compounds, thus we used UV Spectrophotometric methods of analysis of the drugs.
3.4.2.1 Preparation of Simulated Gastro-intestinal Fluids

For colon release studies different types of simulated gastro-intestinal fluids have been tried by various authors. The main difference observed in these papers was the embodiment of enzymes in the simulated gastrointestinal contents. *In vitro* colon release studies without using any enzymes was performed by Ashford *et al.* 1993 and Rama Prasad *et al.* 1998 etc., while a number of researchers has incorporated gastro-intestinal enzymes as well (Gliko-Kabir *et al.* 2000, Raju *et al.* 2011, Akhgari *et al.* 2012 etc.) Generally, the equipment used for such analysis was Type I dissolution test apparatus as described in I.P. however, the use of Type III has been specifically recommended for guar based colonic delivery formulations because of their adhesive properties by Wong *et al.* in 1997. Unconventional method for dissolution test for hydrophilic matrix tablets has also developed (Durig and Reza, 2000). Gauri *et al.* 2011 used Type I described in U.S.P. XXIV for the evaluation of CTDDS for metronidazole and it is most commonly recommended for *in vitro* release studies now a days. (Dhaneshwar *et al.* 2009)

In the present work, SGF (simulated gastric fluid) and SIF (simulated intestinal fluid) were prepared by the methods depicted in USP (2004). SCF (simulated colonic fluid) was made by using the formula as was used by Gliko-Kabir *et al.* 2000.

**Procedure:**

- **Simulated Gastric Fluid (SGF) preparation:** Sodium chloride (2 g) was dissolved in 50 ml water and then 7 ml of Conc. hydrochloric acid was mixed. To the mixture 3.2 g of pepsin was added and stirred well. Finally volume of the solution was made up to 1000 ml and pH is adjusted to 1.2 ± 0.05 using 5 % w/v solution of NaOH.

- **Preparation of Simulated Intestinal Fluid (SIF):** Potassium dihydrogen phosphate (6.8 g) was first dissolved in 250 ml distilled water and then 190 ml of 0.2 N sodium hydroxide and 400 ml water were added. Add 10 g of pancreatin to the above solution and mixed well. The pH of resulting solution was adjusted to 7.5 ± 0.1 with 0.2 N NaOH and volume was made up to 1000 ml with distilled water.

- **Preparation of Simulated Jejunum Fluid (SJF):** SJF was prepared by mixing 61 parts of SIF and 39 parts of SGF by volume and the pH is adjusted to 4.5 with 0.2 M sodium hydroxide or 0.2 M hydrochloric acid.

- **Preparation of simulated colonic fluid (SCF):** First Sorenson’s buffer was prepared by dissolving 500 mg Na₂HPO₄ and 301 mg KH₂PO₄ in 1000 ml distilled water and
adjusted pH to 7.0. SCF was prepared by dissolving azoreductase enzyme (20 U) obtained from *Escherichia coli*, amidase obtained from *Proteus vulgaris* (20 U) in Sorenson’s buffer (pH 7.0).

### 3.4.2.2 UV Spectrophotometric Determination of Prodrugs in SGF, SJF, SIF and SCF

All the prodrugs synthesized have chromophores in UV region; therefore, they were scanned after dissolving in SGF, SIF, SJF and SCF and selected a proper wavelength of UV light for the determinations. After selecting the wavelength of UV light for the determinations, the calibration curves were prepared for all the synthesized drugs in SGF, SIF, SJF and SCF.

**Procedure:**

- **Scanning of \( \lambda_{max} \):** The weigh amount of prodrug (10 mg) was transferred to 100 ml volumetric flask and volume was made up to 100 ml with PBS (pH 7.4) to obtain a stock solution of 100 \( \mu \)g/ml. From this 1 ml was withdrawn each time and taken in four 10 ml volumetric flask. Volume was made up to 10 ml separately with SGF, SJF, SIF and SCF, respectively. These solutions were scanned between 220-380 nm on Shimadzu 1700 UV double beam spectrophotometer.

- **Calibration curve preparation of prodrug in SGF, SIF, SJF and SCF:** The prodrug (10 mg) was weight accurately taken in 100 ml volumetric flask and was liquefy in least amount of corresponding fluid. The volume was made up to the mark with sufficient quantity of corresponding fluid to give the concentration of 100 \( \mu \)g/ml of stock solution. Set of standard dilutions of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 \( \mu \)g/ml of drug was prepared by transferring 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml aliquots to a series of 10 ml volumetric flasks and volume was made with buffer to 10ml. Absorbance of each dilution were measured against buffer as blank using Shimadzu 1700 UV double beam spectrophotometer at the wavelength selected for corresponding synthesized prodrug.

### 3.4.3 Release Studies of Synthesized Mutual Prodrugs

Generally the diffusion method is used for such type of analysis and the equipment used is Type I dissolution test apparatus described in U.S.P. XXIV (Dhaneshwar *et al.*, 2009). Six station Dissolution Test Apparatus with paddle stirrer given in U.S.P. XXIV has been used for release study of enteric coated tablet of prednisolone by Raju *et al.* in 2011. U.S.P. dissolution apparatus II was also second-hand in the *in vitro* drug release studies of mutual
prodrugs of ibuprofen (Bhosale et al. 2004) and drug release behavior of polymeric prodrug of aspirin by Chandrasekar et al. in 2001. However, the use of Type III has been specifically recommended by Wong et al. in 1997 for guar based colonic delivery formulations. In the present research Type I dissolution test apparatus described in U.S.P. 2004 was used as most of the colon release experiments relating to mutual prodrugs were conducted in U.S.P. Type I dissolution test apparatus.

The aptitude of the prodrug to colon release of the drugs is investigated by incubating it under conditions mimicking GIT environment. The schedule of time for performing release studies is based on the average transit period in the healthy human as stated earlier. The total time period used for the study was 24 hours. For first two hours the dissolution was conducted in SGF. The fluid was then replaced with SJF retaining the solid contents for conducting the simulated release in jejunum for 2 hours. Further, the fluid was replaced second time retaining solid contents with SIF and the dissolution was carried out in SIF for next 2 h. Finally the fluid was replaced third time with SCF retaining the solid contents and the dissolution was carried out for further 16 hours.

Apart from the release of parent drugs, other components such as dissolved mutual prodrug, the intermediates may be released or dissolved that may contribute to the absorbance in UV spectrophotometry. For the analysis of such kind of the mixtures the methods based on HPLC resolution may be found more suitable. In the present study, analysis were performed spectrophotometrically for the estimation of prodrug remaining and the free drugs which were supposed to be released did not interfere with the absorption of prodrug because their $\lambda_{\text{max}}$ were found to be substantially different from the prodrug and furthermore aliquots were extracted by the suitable organic solvent in order to remove the interference by free drugs before going to spectrophotometrical assay.

Procedure:

- **Release studies of drug in SGF at pH 1.2**

  The mutual prodrugs were *in vitro* studied for the release of drugs in SGF using dissolution test apparatus I described in U.S.P. XXIV. Accurately weigh amount of mutual prodrug (10 mg) was kindly spread over the surface of 900 ml of SGF taken in basket and the contents were rotated at 100 rpm and were kept thermostatically controlled at 37 ± 0.5°C as specified in the I.P. (2007). Perfect sink condition was maintained during the dissolution of drug. The samples were withdrawn at intervals of 30 minutes, while first sample was withdrawn after an hour from the dissolution vessel and replaced with equal volume of SGF. The aliquots
were shaken or extracted with the same amount of CHCl₃ (ether in case of IN and IT) two times in order to remove the intervention by free drugs which were thought to be released by the prodrugs. The aliquots were now estimated spectrophotometrically at the wavelength corresponding to the prodrug for the amount of prodrug remaining. The concentration of the remaining prodrug was determined from calibration curve of the respective prodrug. The amount of prodrug in the samples was calculated and percentage of prodrug was determined. The cumulated percent release of free drugs from its prodrug was calculated from the above data.

In every case, free drugs which were thought to be released by the synthesized prodrugs did not interfere with absorption of prodrug because their λₑₘₐₓ were found to be substantially different from prodrug. The study in SGF was carried for a period of 2 hours.

- **Drug release studies in SJF (pH 4.5)**
  The studies for release of drugs in SJF were carried out by similar method as described above for SGF. The SGF was replaced with SJF retaining solid contents and the studies were done for a period of 2 h. Sampling was takes place at the interval of 30 minutes. Aliquots were analyzed by the same manner as discussed in release study in SGF.

- **In vitro release studies in SIF at pH 7.4**
  The studies for release of drugs in SIF were carried out by similar method as described above; the fluid was replaced second time with SIF retaining solid contents and the studies were carried out for a period of 2 h and sampling was done at the interval of 30 minutes. Same method of analysis as mentioned earlier was followed.

- **Drug release studies in SCF (pH 7.0)**
  The studies for release of drugs from synthesized conjugates in SCF were carried out by similar method as described earlier. The SIF was replaced with SCF retaining solid contents. The studies were performed for 18 h and sampling was done at the interval of 30 minutes. Same method of analysis was used as mentioned above and the amounts of prodrug remaining in aliquots were estimated directly on UV spectrophotometer.