CHAPTER I

INTRODUCTION

Paracetamol has become so popular as a substitute for aspirin because it does not cause the side effects exhibited by some patients following ingestion of salicylates. Paracetamol does not produce the gastric irritation, erosion or bleeding that sometimes occurs after salicylate ingestion. It does not depress the prothrombin time and it seems to have less overall toxicity.

Paracetamol (1) is used mainly as an analgesic and anti-pyretic drug. It can be obtained alone or in combined form with other drugs as syrup, tablet, and injectable preparations. Upon ingestion, paracetamol is rapidly absorbed into the blood stream and conjugated in the liver with glutathione and cysteine via the sulphydryl groups. In therapeutic dose, paracetamol do not cause hepatic damage since there is enough glutathione present to conjugate all of the toxic metabolites. However, when large doses of paracetamol are ingested, the stores of glutathione are rapidly depleted. The metabolites that are formed using an alternate pathway attach themselves to sulphydryl groups on the liver parenchymal cells in an irreversible manner which will causes damage of the liver tissue and necrosis leading to hepatotoxicity and coma.

The anti-dote substance that is now in general used for the treatment of paracetamol overdose is analogues of glutathione. They work mainly in preventing the alkylation of the liver cell proteins. But these analogues should not be administered after 12 hours post ingestion of paracetamol because the already damaged liver may not be able to metabolize them causing further liver

damage. Hence, the decision whether to administer glutathione analogues to patients who ingested large doses of paracetamol was based on the concentration of this drug in the plasma.

1. MEASUREMENT OF PARACETAMOL

Since paracetamol is being used now more frequently, there has been an increased interest in developing sensitive, specific, reproducibility and accurate methodology for estimation of paracetamol both in pharmaceutical preparations and in biological fluids. These methods may be divided into four main groups :-

1.1 Ultraviolet spectrophotometry

1.2 Colourimetry

1.3 Gas-liquid chromatography

1.4 High-performance liquid chromatography

1.1 ULTRAVIOLET SPECTROPHOTOMETRY

Paracetamol is assayed by UV-spectrophotometry by measuring the absorbance of the drug at a single specific wavelength or by scanning over the entire UV-spectrum. Dordini, et al. (2) calculated the paracetamol concentration by reading the absorbance of the ether extract at 250 nm. Although this method is quite rapid, it lacked sensitivity and was not specific. However, Spooner, et al. (3) later used the same method and found it worked quite satisfactory.

The technique of optical density scanning has been used by several people for the identification of paracetamol. An ether extract is scanned on an UV-spectrophotometer from 225-350 nm. Paracetamol gives a characteristic curve with a peak at 266-270 nm, a through at 230-235 nm; and a shoulder on the

peak at 290-300 nm (4). It is quite common to scan the extract to confirm the presence of paracetamol before readings at a specific wavelength are taken.

Routh, et al. (5) used the method of optical density scanning to identify paracetamol in the presence of salicylic and acetylsalicylic acid. In their method, the ether extract is treated with sodium bicarbonate. This solution is devided into two aliquots, and one is acidified and the other made alkaline. The two are scanned against each other with the acid portion serving as the blank. The paracetamol has a peak at 266 nm while salicylic acid has an isobestic point at this wavelength and, therefore, causes no interference. Knepil (6) introduced a slight change in the procedure. A protein precipitation preceded the ether extraction and the absorbance of the final extract peaked at 290 nm.

Shane and Kowblansky (7) presents a method for determination of four compounds commonly found in analgesic tablets containing aspirin, caffeine, paracetamol and salicylamide. The tablet is dissolved in 85 % methanol, filtered to remove the insoluble excipients. Then 0.5M sodium hydroxide was added to converted the aspirin to the disodium salt of salicylic acid and the solution was aliqouted into 2 beaker. One was added with phosphate buffer pH6 and act as the blank, the other was added with phosphate buffer pH10 as the sample. Consequently, the disodium salicylate converts to the monosodium salt (since the pKa_1 and pKa_2 of aspirin was 3.0 and 13.4, respec tively) and the absorbances of the two solution due to aspirin will exactly cancel each other. The caffeine (pKa = 13.39) is in the same form at both pH's. Therefore, when using the pH 6 buffer solution as the reference, and the pH 10 as the sample solution, the spectra will automatically subtract in the

spectrophotometer, giving new characteristic spectra for paracetamol which the absorbance can be measured at 263.5 nm (figure 1).

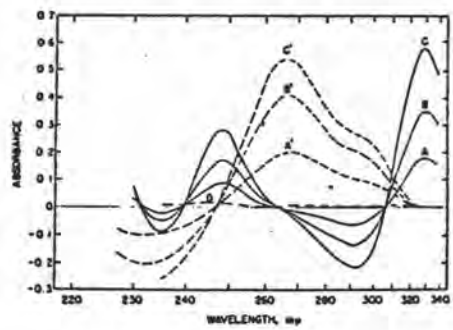


Figure 1 : Differential spectra of salicylamide(A+B+C), paracetamol (A'+B'+C'), and caffeine + aspirin (D).

El Sayed, et al. (8) proposed a method for determining paracetamol in different dosage forms without interference from absorbance due to excipients by using a differential spectrophotometric method or by Glenn's method of orthogonal function. That is the absorbance of the specimen in alkaline solution was measured at 267 nm using aqueous solution of the same concentration as blank. By Glenn's method, the calculation for quadratic coefficient was obtained from measuring over the wavelength range 240-250 nm at 2 nm intervals.

Korany, et al. (9) presented a method for determining paracetamol in the presence of their degradation product, p-aminophenol. The solution of paracetamol in 0.1N hydrochloric acid was analyzed by measuring their second derivative spectral response at 295 nm where the degradation product do not interfere (figure 2).

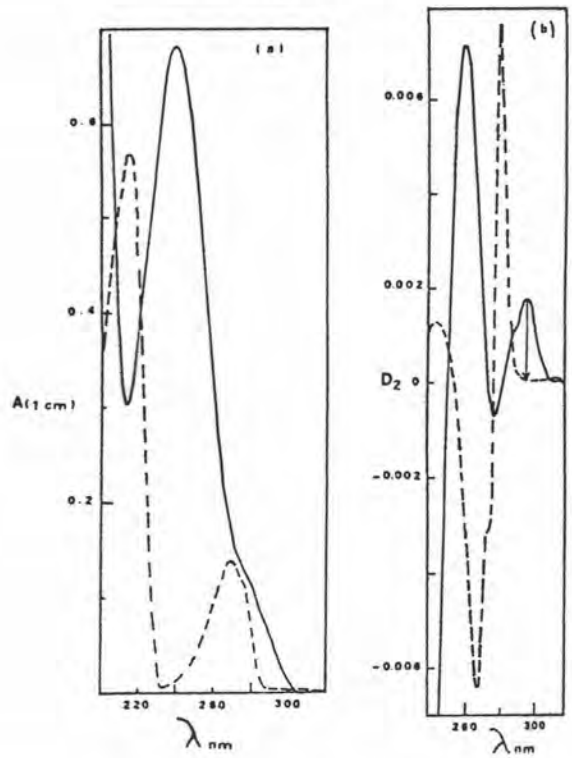


Figure 2 : Zero order absorption spectra (a) and second derivative spectra (b) of 1 mg%w/v paracetamol (--) and its degradation product (.....) in 0.1N HCl

1.2 COLOURIMETRY

When the significance of paracetamol was first recognized, and it was being measured, most of the methods used involved colourimetric techniques. These methods were overshadowed by the more sophisticated methods of UV-spectrophotometry, gas-liquid chromatography and high-performance liquid chromatography. Lately though increased interest has centred on colourimetric techniques due to improvements to old methods and descriptions.

The methods utilizing colourimetry can be devided into three main groups :-

1.2.1 Conversion to p-aminophenol

1.2.2 Dye reaction

1.2.3 Nitration

1.2.1 Conversion to p-aminophenol

The methods used here involve the hydrolysis of paracetamol to p-aminophenol. It then reacts with a colour reagent giving a relative stable product which can be measured spectrophotometrically.

Brodie and Axelrod (10) published a method in which the paracetamol is extracted into an iso-amyl alcohol or ether mixture. The extract is made alkaline and then it is hydrolyzed in acid to form p-aminophenol. The p-aminophenol is diazotized and coupled with alpha-napthol giving a red-violet colours which is measured at 510 nm.

The method developed by Lester and Greenberg (11) involved the use of a protein free filtrate. The filtrate is extract into dichloroethane and then made alkaline, followed by acid hydrolysis. After cooling, the colour is developed using alpha-napthol and sodium hydroxide, then extract into butanol at the absorbance read at 635 nm. Gwilt, et al. (12) combined both of the above methods when they used an extraction with ether in a Soxhlet thimble and followed by colour development according to Lester and Greenberg.

Davis, et al. (13) utilized a slightly different method in that after hydrolysis with hydrochloric acid, the p-aminophenol is reacted with

hypochlorite to form p-quinone chlorimide, which undergoes a reaction with phenol and borate buffer (pH 9.9) to give an indophenol blue chromogen that is measured at 625 nm. Murfin and Wragg (14) used sodium arsenite to remove the excess of hypochlorite. Frings and Saloom (15) used ammonium hydroxide instead of borate buffer and measured the indophenol dye attained at 620 nm. Love (16) used perchloric acid for the hydrolysis as well as for a protein precipitation. The p-aminophenol is reacted with 1% O-cresol and ammonia and measured at 615 nm.

Sanghavi and Vishwasrao (17) described a method which is applicable to pharmaceutical preparations based on formation after hydrolysis of a complex with p-dimethylaminobenzaldehyde, which has maximum absorption at 444 nm.

All the above methods are quite similar in that they involve hydrolyzing the paracetamol to p-aminophenol and then developing colour. It is important that the pH does not rise above neutrally when using methods that measure p-aminophenol because it is unstable in alkaline conditions.

1.2.2 Dye Reaction

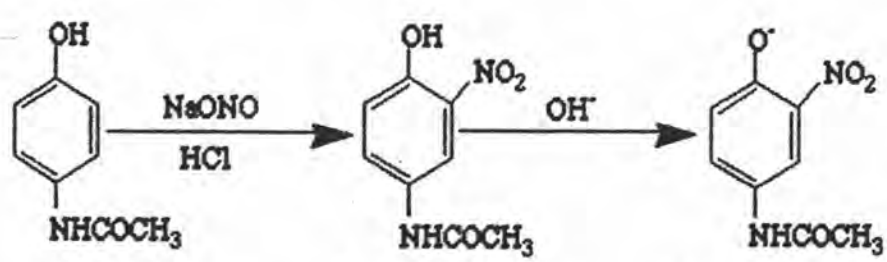
A method where the decrease in the intensity of a substance is measured was developed by Routh, et al. (5) for the measurement of paracetamol. A violet coloured dye (2,2-diphenyl-1-picrylhydrazine) is used in the procedure. This dye reacts with secondary and tertiary aromatic amines and some of their derivatives form a yellow coloured diphenylpicryl-hydrazine and the resulting decrease in intensity of colour is measured at 527 nm. The

paracetamol (which reacts with the dye) is separated from other compounds in the serum which might interfere by adding sodium sulfate and sodium sulfite and the extracting into dichloroethane containing 3% iso-amyl alcohol. A serum blank is used to eliminate the presence of any naturally occurring amines.

Meola (18) used a method involving the extraction of paracetamol into a mixed solvent. The extract is combined with Folin-Ciocalteu reagent at pH 11.0 to give an indophenol dye which is measured at 660 nm.

1.2.3 Nitration

Paracetamol is reacted with nitrous acid to give a yellow colour which becomes a more intense orange-red colour when the solution is made alkaline. This method was first used by Chafetz, et al. (19) to measure paracetamol in tablets. The specimen is treated with 6N hydrochloric acid and 10% sodium nitrite. Fifteen percent of sulfamic acid is added to destroy any excess nitrous acid present. The specimen is then made alkaline using 10% sodium hydroxide and the absorbance of the resulting specimen is measured at 430 nm using water as a blank (Scheme 1).

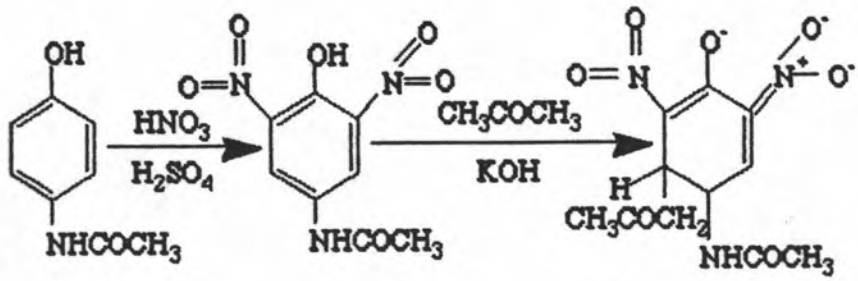


λ_{max} 235, 285 nm λ_{max} 370 nm λ_{max} 430 nm

Scheme 1

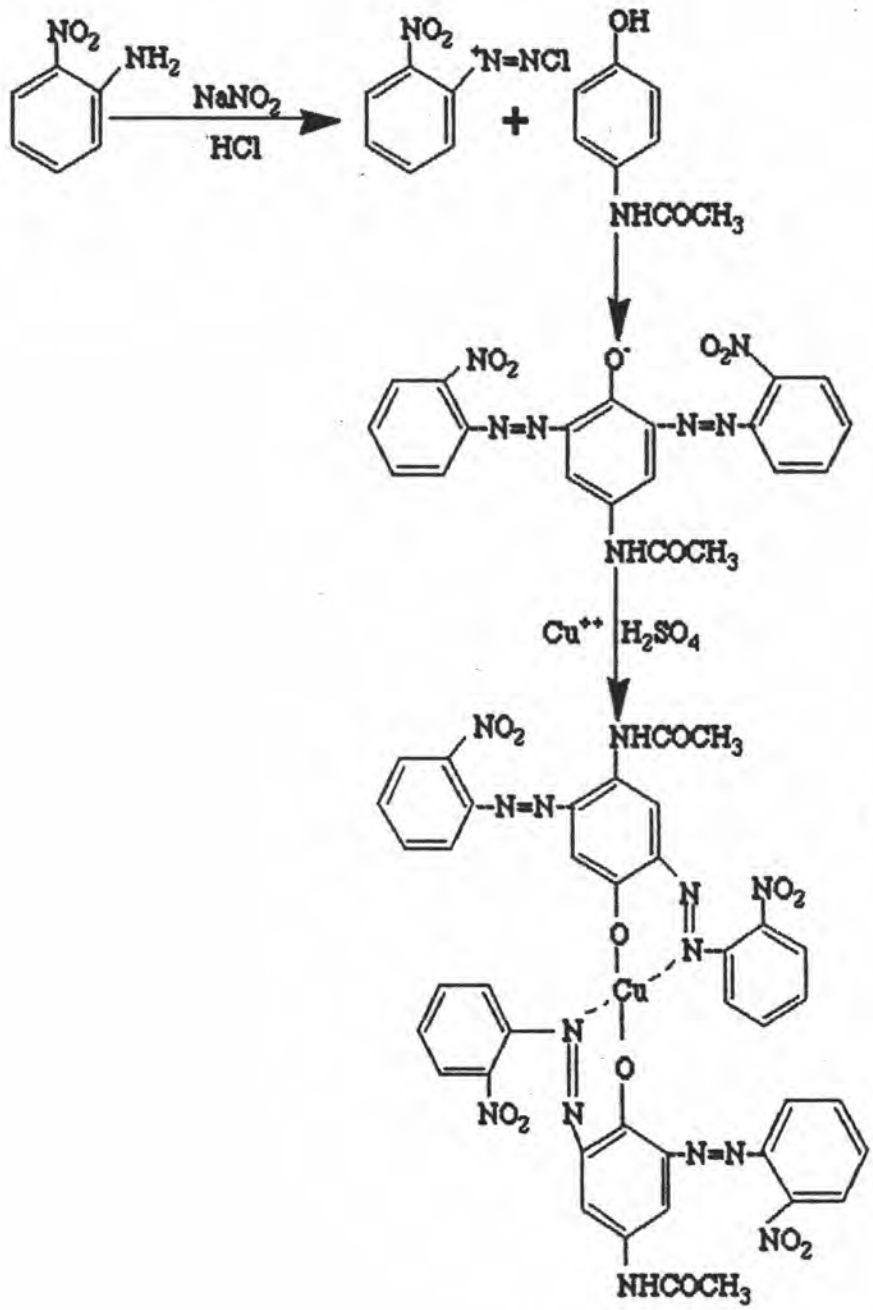
Daly, et al. (20) later developed an automated system for the analysis of the paracetamol in elixirs using the same reaction. Independently and later, Inamdar and Kaji (21) reported use of the chromophore formed by the reaction of nitrous acid with paracetamol for dosage form assay; however, they measured the yellow colour in a solution instead of the orange-red colour of the phenolate ion, and they attributed the chromophore to nitroso derivatives. Glynn and Kendal (22) used this method for the analysis of paracetamol in blood. The process is preceded by a protein precipitation step with 10% trichloroacetic acid and the supernatant is then treated with nitrous acid. In Belal, et al. (23) method, this nitroso derivative was used to form chelates with cobalt (III) and copper (II) ions. The absorbance measured at 400 nm and 535 nm for cobalt (III) and copper (II), respectively.

El Kheir, Belal, et al. (24) developed a method for the assayed of paracetamol in the presence of oxyphenbutazone and salicylamide by prepared and used the polynitro derivatives as intermediates in the spectrophotometric determination, through interaction with alkaline ketone reagents to form Meisenheimer-type complexes. The paracetamol was treated with nitric acid and sulfuric acid to form dinitro derivative. Afterwhich the derivative was reacted with acetone and potassium hydroxide and the absorbance of the resulting colour was measured against a reagent blank at 355 nm (Scheme 2).



Scheme 2

Belal, et al. (25) proposed a method for determination of paracetamol based on its coupling with diazotized o-nitroaniline and subsequently chelated with copper (II) ion. Copper (II) chelation with the coupled compound makes the method highly sensitive for pharmaceutical preparations. The copper chelate chloroform extract was diluted in sodium diethyldithiocarbamate solution and the absorbance was measureed at 442 nm against a blank (Scheme 3).



Scheme 3

Hasssan, et al. (26) used a method involving the coupling of phenolic group of the paracetamol with diazosulfanilic acid or diazo-p-nitroaniline. Sulfanilic acid or p-nitroaniline was diazotized in sodium nitrite and phosphoric acid (instead of hydrochloric acid as in Chafetz's method) and

the excess nitrous acid was eliminated by adding methanol. Afterwhich it was coupling with paracetomol in alkaline solution. The absorbance was measured at 480 nm for diazosulfanilic acid and diazo-p-nitroaniline respectively

1.3 GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography is a process by which a mixture of compounds are separated into constituent parts by moving a mobile phase over a stationary phase. The mobile phase is an inert gas which carries the components through a column. The stationary phase is a non-volatile liquid which is coated on an inert solid support. The effluent leaving the column carries the separated constituents to the detector which transmits the results to a recorder. The constituent is identified by its retention time when compared to that of known standards.

This technique has been used by several people for the identification of paracetamol. Prescott (27) extracted paracetamol into ethyl acetate and made trimethylsilyl derivatives using N-trimethylsilylimidazole. N-trimethylsilylimidazole is an agent that selectively silylates the hydroxyl groups which make the method specific for paracetamol. P-chloracetanilide is use as the internal standard. The column is packed with 10% OV-17 on 80/100 mesh Gas Chrom Q and maintain at 220°c. Thomas and Coldwell (28) used Regisil (bis-[trimethylsilyl] trifluoroacetamide) as silylating reagent which permits the use of less carefully controlled conditions, more powerful silylating reagent, more volatile and causes less detector contamination than N-trimethyl-silylimidazole or N,O-bis (trimethylsilyl) acetamide. The column was 3% OV-1 on Gas Chrom Q and maintained at 160°c.

Prescott (29) later modified this method in which the silylation was replaced by alkylation and the new internal standard used was N-butyryl-p-aminophenol. The column was packed with 3% HI-EFF 8 BP on Gas Chrom Q at 220°c.

Street (30) extracted plasma plus the internal standard (N-butyryl-p-aminophenol) with ether and then into a 5% sodium carbonate solution. Benzoyl derivatives are prepared by adding benzoyl chloride and these are extracted with ether, evaporated and the residue taken up in acetone and injected into the column.

The above mention utilized the preparation of a derivative which is necessary to avoid tailing of the peak. Several methods involving no derivative preparation have been report in the literature. Grove (31) used an ether extraction on plasma saturated with ammonium sulfate. The extract is evaporated and the residue is redissolved in ether and injected into the column. Some tailing of the paracetamol peak resulted. There was also a large peak which could be eliminate by protein precipitation.

Hackett and Dusci (32) used a completely different extraction procedure. Plasma is mixed with a borate/celite mixture and this preparation is transferred to a column and the paracetamol is eluted out using ether. The internal standard, diphenyl pyraline, is added and the mixture was evaporated. To the remaining residue, pyridine and acetic anhydride are added and this mixture is injected into the gas chromatograph. Huggett, et al. (33) used N-methylimidazole instead of using pyridine which was said to be much more reactive than pyridine.

Thoma, et al. (34) used methylene chloride in the extraction procedure for the reasons of safety and performance, while recovery may be superior using ether, so also is the recovery of extraneous material. The use of methylene chloride instead of ether in the method of Prescott and Evans with the dual protein precipitation and salting out effect of ammonium sulfate resulted in remarkably clean extracts and recovery sufficient for high sensitivity. The internal standard in this assay was 2-acetamidophenol which is structural similar to paracetamol to minimize differences in derivatization. Stationary phase was SP 2250-DA (a special deactivated version of OV-17).

Chan and McCann (35) synthesized the pentafluorobenzyl derivative of paracetamol and used N-butyryl-p-aminophenol as internal standard. Penta-fluorobenzylbromide and anhydrous potassium carbonate were added and refluxed. Afterwhich, it was extracted into n-hexane and injected onto the GLC column. The column used was packed with 3% SP 2100 coated on Supelcoport 100-120 mesh which was silanised in situ with hexamethyl disilane before use.

Evans and Harbison (36) described a simultaneous method for analysing paracetamol and phenacetin using flame ionization detector and p-bromoaceta-nilide as internal standard. After the extraction procedure, the sample was reacted with trimethylanilinium hydroxide in methanol. The product obtained was less polar and does not make excessive detector contamination.

Kaa's method (37) used 2-acetaminophenol as an internal standard and the reagent used were toluene and trifluoroacetic anhydride. Alvan, et al. (38) used 3-acetaminophenol instead of 2-acetaminophenol. But 2-acetamino-

phenol give better separated chromatographic peak and the other drugs does not coincide with the internal standard peak.

Dechtiaruk, et al. (39) described a GLC procedure for paracetamol which the drug is chromatographed as the O-heptyl-N-methyl derivative. This derivative is prepared by a sequential alkylation procedure in which the phenolic hydroxyl group of the parent compound is alkylated off-column with heptyl iodide and the amide group is derivatized on-column by reacting with trimethylanilinium hydroxide.

1.4 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) is a liquid chromatographic technique in which the test solution is pumped through the column at the pressure up to and sometimes exceeding 4000 psi. inlet pressure.

The stationary phase may be solid or liquid, but must have a large surface area. It is liquid chromatography which affords high resolution, high speed, high efficiency, and high sensitivity at the same time. The use of pressure to the eluent through the column is the main feature that distinguishes this particular techniques from other types of chromatography. This technique is still relatively new and is used mainly in the measurement of paracetamol in biological fluids in research situations. The process involves extraction of plasma /internal standard mixture. The extract is then dissolved in a solvent and an aliquot of this is injected into a column.

Gotelli, et al. (40) used a procedure that determined paracetamol using acetoacetanilide as the internal standard. Silica beads chemically bonded with octadecyl trichlorosilane are used in the column. The detector is set at 254

nm and an acetonitrile phosphate buffer (pH4.4) mixture is used to elute the column at a flow rate of 3 ml/min. The specimen is extract into ethyl acetate and allowed to dry. The dried residue is dissolved in methanol and 10-20 µl are injected into the column. This method showed little interference from other drugs, but theophylline would produce the greatest interference.

Mrocheck, et al. (41) used a system to investigate paracetamol metabolism in man. It involves anion exchange chromatography with detection and quantitation of paracetamol using both UV and cerate oxidimetric detectors. It is fairly lengthy in that the cycle time is approximately 24 hours per sample.

Hovitz and Jaflow (42) used a reversed phase chromatography. The column used contained octadecylsilane-coated silica with the column being eluted with acetonitrile in potassium phosphate solution. N-propionyl-p-amino-phenol is used as the internal standard. Hovitz and Jaflow prefered this method since it is fast enough to be used for emergency determinations of paracetamol as well as being sensitive enough to measure serum levels after the patient has been given therapeutic doses.

Blair and Rumack (43) devised a micro method which by passed the extraction stage. An equal amount of serum and internal standard (N-butyryl-p-aminophenol) are mixed and injected onto the column. A cation exchange resin is used and the detector is set at 254 nm. The main problem with this method is that a disposable pre-column has to be used to prevent protein deposition and eventual column damage.

Wallo and D'adamo (44) reported a reverse phase HPLC method for simultaneous quantitative analysis of paracetamol with the presence of hydrocodone bitartrate in a tablet formulation. The preparation was based on

an octadecylsilane column with phosphate buffered (pH4.5) methanol-water (1:3) moblie phase. Measurement was with a UV-spectrophotometer set at 283 nm, compared to external standard.

Thomis, et al. (45) described a method enables the quantitation of the components and the main impurities of tablets containing aspirin, paracetamol and vitamin C. A C_8 reverse-phase column was used; the mobile phase was methanol-0.2M phosphate buffer (pH3.5)-water (20:10:70).

Carnevale (46) demonstrated the separation and simultaneous determination of cough mixture using chemically bonded octadecylsilane stationary phase with a mobile phase of methanol-water-acetic acid (45:55:2) containing the ion-pairing agent octanesulfamic acid.

Krieger (47) proposed the HPLC method for separation and determination of paracetamol in analgesic preparations containing up to six additional active components. The method uses a C_{18} reverse phase column, methanol-0.75% acetic acid (1+3) mobile phase, and photometric detection in the UV region (254 or 280 nm depending on composition of dosage form)

Stewart, et al. (48) used a reverse-phase HPLC for determining paracetamol-containing muscle relaxant mixture. The compounds were chromatographed on an octadecylsilane column using methanol-water (50:50) solvent system.

2 STATEMENTS OF PROBLEM

Paracetamol is a highly polar compound which shows marked "tailing" (49) and "broadening" (50) on gas-liquid chromatography. In small amounts, it was said that there is significant absorption losses (51) due to an irreversible

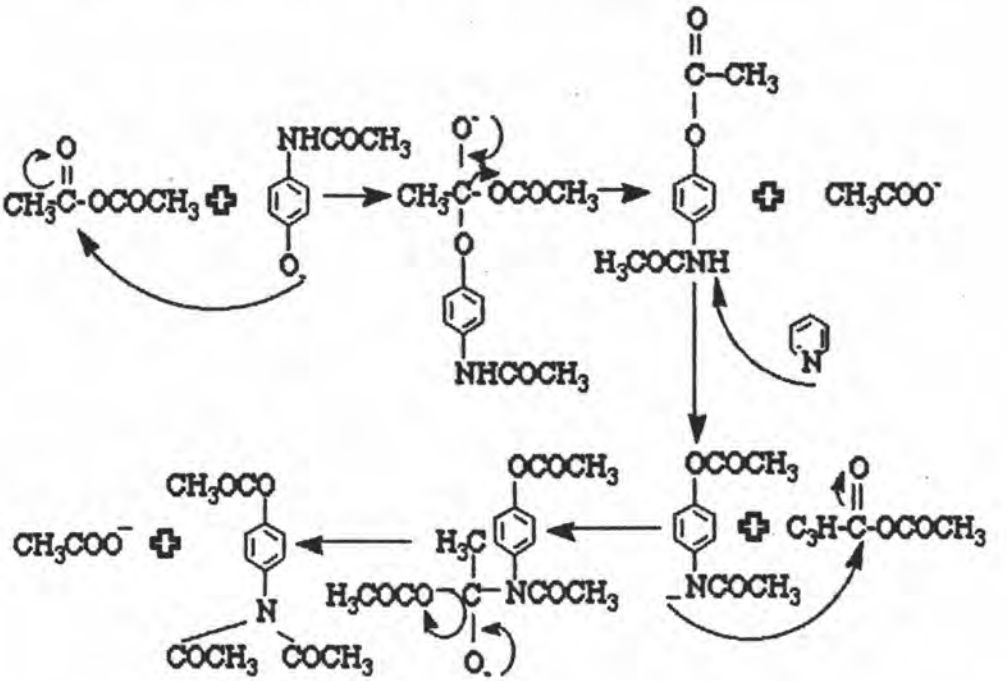
adsorption caused by interaction between polar functional groups of the compound and the polar groups of the liquid phase. Moreover, active hydrogen atoms of paracetamol can form intermolecular hydrogen bonds. Therefore, more energy is needed to separate the molecules in changing the substance from a liquid into a vapour causing a high boiling point.

These undesirable properties could be improved by forming a suitable derivative prior to the GLC analysis. Derivatization reaction (51) usually used is methylation, silylation and acetylation. Acetylation reaction is general in use, easy to perform, give quite good results and economically

Methylating and silylating reagents are expensive, cause rapid deterioration of the column and the product yield are susceptible to hydrolysis so in this experiment we used a low cost derivatizing reagents instead which is acetic anhydride that gives the product yield as good as of other reagents. Pyridine (52) is used as proton-accepting base (catalyst) and solvent.

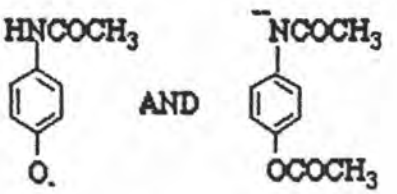
All of the measurement methods mentioned above have difficulties in extracting procedure, time-consuming, lack of sensitivity and cost high expense (53). In this experiment, a new GLC method for quantitative analysis of paracetamol in pharmaceutical preparations and physiological fluid has been developed and reported. Various conditions affecting the reaction were examined to obtain the optimum conditions for determining of paracetamol in various formulation. The results obtained were also compared to those obtained from the USP XXII method.

The acetylation reaction is as in Scheme 4 :-



Scheme 4

The reaction pathway (52) is normally nucleophillic addition/elimination via a tetrahedral intermediate, resulting in overall substitution of nucleophiles (Scheme 5) for leaving group -$OCOCH_3$. Drugs containing one or more of a range of different functional groups, usually possessing a replaceable hydrogen atom, can act as the nucleophile (Scheme 5)



Scheme 5

3 THE OUTLINE OF THIS THESIS IS BASED ON THE FOLLOWING STATEMENTS

1. The acetylation reaction of paracetamol with pyridine and acetic anhydride is studied. The resulting paracetamol derivatives is examined and identified by their melting points, thin-layer chromatography, infrared spectrophotometry, ultraviolet spectrophotometry, gas-liquid chromatography, and ^{1}H-nuclear magnetic resonance spectrometry.

2. Various conditions affecting the reaction are examined : temperature, time, varying concentration of paracetamol and amount and composition of acetylating reagents (pyridine and acetic anhydride). The optimum conditions for the assay are selected.

3. The acetylation reaction method is applied to determine the content of paracetamol in tablet, injection and syrup formulations which are available commercially. The results obtained are compared to those obtained from the official USP XXII method.

4. The accuracy and precision of the acetylation reaction method are measured by determining the percent recovery and the percent coefficient of variation, respectively, which are compared to those obtained from the official USP XXII method.