CERTIFICATE

This is to certify that the dissertation entitled

" Determination of Paracetamol and Caffeine in Dosage Forms by High Performance Liquid Chromatography with Photo Diode Array Detection"

is the bonafide record of research work done by

Ismazarni bt Ismail

During the period January to March 2006
Under my supervision

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ACKNOWLEDGEMENT

IN THE NAME OF ALLAH, THE MOST GRACIOUS, THE MOST MERCIFUL, ALHAMDULILLAH, this dissertation is finally completed.

I greatly acknowledge the guidance and consistant support of my Supervisor, Associate Prof Dr Sayed Waliullah Shah during the course of this project. I am highly thankful to the Department of Pharmacology, School of Medical Science for their permission to use the HPLC instrument, also to the En Rosliza who helped me a lot in order to ensure this project is success and thanks to Pn Ruzilawati, and En. Nik Zahari and the Head Department of Pharmacology, Dr Mohd Suhaimi, thanks a lot.

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LIST OF SYMBOLS, ABBREVIATION

	DESCRIPTION
AA	Acetic acid glasial
ACN	Acetonitrile
CAF	Caffeine
CTM	Chlorpheniramine maleate
DBA	Di –*n*- butylamine
GGL	Glycerylguaiacolate
HPLC	High Performance Liquid Chromatography
HSA	Hexanesulphonic acid sodium salt
PCM	Paracetamol
PDA	Photo diode array
PPA	Phenylpropanolamine HCl

ABSRACT

A simple, accurate, and reproducible high performance liquid chromatographic method for the separation and simultaneous determination of paracetamol and caffeine in dosage forms is described. The HPLC system consisted of Water Alliance Series 2475 with photo diode array detection. The separations were carried out at room temperature on a C-8 (5 μm, 250 x 4.6 mm i.d) Luna 5μ C-8 (2) Phenomenax Prodigy) analytical column. A mixture of 0.01 M KH_2PO_4, methanol, acetonitrile, and isopropyl alcohol (420 : 20 : 30 : 30 v/v/v/v) was used as mobile phase at a flow rate of 0.5 ml/min. Mobile phase solvents were degassed for 30 min. using ultrasonic bath and filtered through a 0.45 μm Milipore filter. The wavelength was in range of 210 to 400 nm. The validation parameters : linearity (r = 0.9998, 0.996 respectively for caffeine and paracetamol). The calculations of the concentration of paracetamol and caffeine were based on peak areas using calibration plots. The retention time of the separation of the mixture of caffeine and paracetamol were recorded as 11.695 min and 9.684 min respectively. This method is successfully applied to the analysis of commercial pharmaceutical preparations, yielding better resolution, sensitivity, and ease of operation as compared to the existing methods.

INTRODUCTION.

Modern high performance chromatography began with the publication of Martin and James's paper on gas chromatography (James et.al.,1952). Their publication on the use of the gas as a mobile phase in the separation of volatile fatty acids initiated the research that has resulted widely used of chromatography.

There are various techniques which are included in general scope of liquid chromatography, commonly used are separation techniques. Tswett, a Russian botanists ' Father of Chromatography' introduced the simple column chromatography, and still used in large scale preparative work (Harvey, 2000).His work on 1906, result in significant historical interest and become fitting introduction to a discussion on the concepts of chromatography (Miller, 1988).

HPLC is a fast technique that with high precision and specificity, separates mixtures into individual ingredients. It can be used as a routine procedure, it has several advantages, it can be completely automated, sample clean up, and preparation are simple and the reproducibility of the packing material means that the analytical conditions remain the same for a new column (Nollet,1992).

According to Rony, separation is the hypothetical condition where there is complete isolation, by *m* separate macroscopic regions, of each of the *m* chemical components which comprise a mixture. In other words, the goal of any separation process is to isolate the *m* chemical components, in their pure forms,

into *m* separate vessels, such as glass vials or polyethylene bottles (Miller, 1988).

Different kinds of HPLC exist and many kinds of column packings and solvents are available. Retention behavior and resolution are affected by column characteristics (C- loading, chain length, porosity, etc.) and by elution scheme characteristics (mobile phase, pH, organic modifier, etc.). The samples can be separated on the basis of solubility and polarity of sample component (Nollet, 1992).

In HPLC, there are two active phases, the stationary phase and mobile phase; changing one or both of the phases may have a large impact on the retention behavior (Zeeuw et.al.,1989). A mixture of the analytes is introduced into the mobile phase and is carried through the system by it. A separation is effected as various components emerge from the bed at different times, which are called *retention times.*

Chromatographic parameters is very important in the of HPLC. The column capacity ratio, *k'*, or simply the *k'* value of a solute is the usual method of indicating solute retention. As shown in the equation 1, *k'* values are obtained from the elution chromatogram by

$$K' = \frac{t_r - t_o}{t_o} \qquad (1)$$

where t_r is the retention time of the given peak and t_o is the retention time of the unretained or solvent peak.

The k' value in HPLC is related to the R_f value in TLC by

$$K' = 1/R_f - 1 \qquad (2)$$

The efficiency of a chromatographic column is measured by the number of theoretical plates, *N*, to which the column is equivalent. This parameter is calculated from equation 3

$$N = 16\,(t_r/W)^2 \qquad (3)$$

Where t_r is the retention time of the peak and *W* is the base width of the peak measured in the same units (mm, and s, etc.) and is obtained by extrapolation of tangents at the points of inflection to the baseline. The plate number of a column is a measure of the amount spreading of a solute band as it travels down the column, and the efficient systems are characterized by high values of *N*.

The height equivalent to theoretical plate (H.E.T.P) or simply the plate height, H of a column is given by

$$H = L/N \qquad (4)$$ (Pride & Gilbert, 1979)

Applications of HPLC are widespread. HPLC is routinely used for both qualitative and quantitative analyses of environmental, pharmaceutical, industrial, forensic, clinical and consumer product samples (Harvey, 2000), widely used because of the precision in HPLC is often better due to routine use of loop injectors, and the range of compounds that can be analyzed is larger.

Application of HPLC in pharmaceutical preparation are proved to be more specific.

Chemical name of paracetamol is N-(4- Hydroxyphenyl)acetamide 4'-Hydroxyacetanilide. The other names are acetaminophen, Tempra, Tylenol. Molecular formula is $C_8H_9NO_2$, with MW 151.2 and. Paracetamol appears as a white crystalline powder with slightly bitter taste.It is soluble in boiling water and freely soluble in alcohol.It has a pKa of 9.51 (Xu & Trissel,2003).

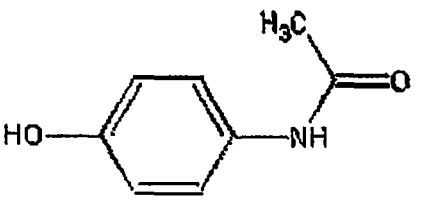

Figure 1 : Chemical structure of Paracetamol

The chemical name for caffeine is 3,7 – Dihydro – 1,3,7 – trimethyl – 14 – purine –2,6 – dione 1,3,7 – Trimethylxanthine. The other name is cafedrine. There are two form that are available, caffeine and caffeine citrate. Caffeine with molecular formula $C_8H_{10}N_4O_2$, molecular weight 194.2, and caffeine citrate with molecular formula $C_8H_{10}N_4O_2.C_6H_8O_7$, molecular weight 386.3. Caffeine and caffeine citrate occur as white powders. Caffeine is sparingly soluble in H_2O and alcohol. Caffeine citrate is freely soluble in H_2O and soluble in alcohol.

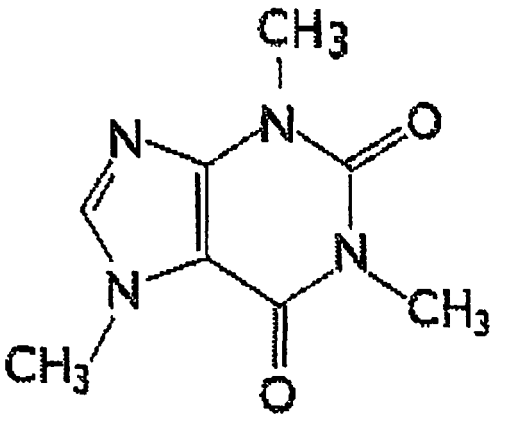

Figure 2 : Chemical structure of Caffeine.

One of the earliest papers on the analysis of analgesics by HPLC was that of Henry and Schmit where various combinations from aspirin, caffeine, paracetamol, phenacetin, and salicylamide in six types of tablets were analyzed on a 1m X 2.1mm column of Zipax SAX with an aqueous mobile phase buffered at pH 9.2 (Pryde & Gilbert, 1979).

In order to achieve effective separations, the selection of mobile phase influence the selectivity. This is due to the elution order of solutes in HPLC is governed by polarity. Retention times are controlled by selecting mobile phase, with a less polar mobile phase leading to longer retention time. Less polar mobile phase leading to longer retention times and result in a good separation and more opportunity to an acceptable separation (Harvey, 2000). We can determine the polarity due to the polarity index where the larger values of polarity index (*P'*) correspond to more polar solvents. The selection of methanol, acetonitrile for example as the mobile phase that have more *P'* result in long retention time. The

polarity index measures the intermolecular attraction between a solute and a solvent (Miller, 1988). The mobile phase is chosen for the following reasons:

1. proper strength or polarity
2. low viscocity
3. compatibility with detector, and
4. volatility if the analytes are to be recovered by evaporation of the mobile phase

The chromatographic process is controlled by equilibrium, and as a consequence its retention parameters are related to thermodynamic partition coefficients. Chromatography is dynamic, and concentration gradients exist in an analyte zone as it passes through the chromatographic bed or column. Those gradients result in diffusion, a kinetic process (Miller, 1988).

Due to the configurations of the stationary phase, it can be either solid or liquid. The stationary phase may be partially soluble in mobile phase, causing it to 'bleed' from the column over time. To prevent this loss of stationary phase, it is covalently bound to the silica particles (Harvey, 2000). When the types of stationary phase is nonpolar and mobile phase is polar, the types of chromatography used is reversed phase chromatography which is a commonly encountered form of HPLC where the stationary phase use an organochlorosilane for which the R group is an *n* – octyl (C_8) or *n* – octyldecyl (C_{18}) hydrocarbon chain. Most reverse phase separations are carried out using a buffered aqueous solution as a polar mobile phase (Harvey, 2000).

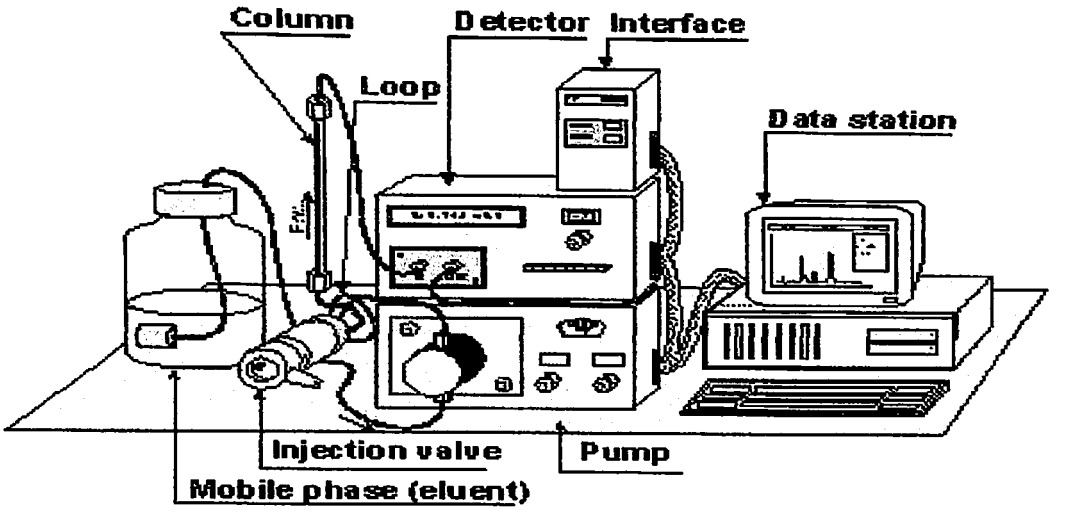

Figure 3 : The schematic diagram of HPLC

From the schematic diagram above, a brief description on the operation of HPLC is given below. Eluent from the solvent reservoir is filtered, pressurized and pumped through the chromatographic column. A mixture of solutes injected at the top of the column is separated into components on traveling down the column and the individual solutes are monitored by the detector and recorded as peaks on a chart recorder. The main components of a high performance liquid chromatograph are a high pressure pump, a column/ injector system, and a detector, and minor components may be required (Pryde & Gilbert, 1979)

In order to achieve good and effective method development, typical experimental factors that need to be investigated are :

1. HPLC conditions: % organic, pH, flow rate, temperature, wavelength, column age
2. Sample preparation: % organic, pH, shaking/ sonication, sample size, sample age

3. Calculation/ standardization: integration, wavelength, standard concentration, response correction.

HPLC has already made a significant impact in pharmaceutical and clinical sciences and is an ideal complementary technique. Preparative HPLC is also beginning to make an impact and is success due to lack of problem of volatilization and thermal instability.

REVIEW OF LITERATURE

The commonly used methods of quantitative determination of analgesic compounds utilize spectroscopic measurements (UV-visible, fluorescence/ chromatographic assays (GLC, HPLC, and TLC), microbiological procedures and radioimmunoassay techniques (Pryde & Gilbert, 1979).

Various analytical methods such as fluorimetry, voltammetry, FTIR, spectrophotometry, flow injection analysis, chemiluminescence, electrochemical analysis, mass spectrometry, gas chromatography, capillary electrophoresis, and liquid chromatography have been employed for the determination of paracetamol. All these methods were used for the determination of paracetamol either alone or in combination with other drugs (Rao & Narasaju, 2006). The analysis of paracetamol and caffeine that will be discussed include spectroscopic measurements, flow injection analysis and chromatographic assays.

When the caffeine and paracetamol are mixed together, there are some possible interactions and caffeine – paracetamol interactions have been studied in 2000, where a demonstration of the possible intermolecular interaction between caffeine and paracetamol is presented. The influence of intermolecular interaction on the solubility, transmembrane permeation and phase partitioning of paracetamol were examined under different aqueous conditions. The results showed a general increase in the solubility of paracetamol in the presence of

caffeine. The effect of the interaction on the transmembrane transport of paracetamol was clearly discernible under the alkaline conditions and in distilled water. By means of the permeation rate data, it was possible to establish that the interaction was based on a 1:1 stoichiometry (Vincent & Anselm, 2001).

The methods of analysis of paracetamol and caffeine in pharmaceutical preparations include the spectroscopic measurements. Spectroscopic (UV / Visible) methods for the analysis of drugs are based on the measurement of absorbance at a characteristic wavelength. In the assay of formulated products, the drug may have to be extracted from UV-visible absorbing excipients prior to spectroscopic analysis (Pryde & Gilbert, 1979). Two spectroscopic methods and HPLC were proposed for the simultaneous analysis of caffeine and paracetamol in a tablet formulation. The ratio spectra derivative method is based on the use of analytical signals obtained by measuring at 267.9 and 291.0 nm for caffeine and 237.0 and 251.8 nm for paracetamol in the first derivative of the ratio spectra. The results were compared and it was found that HPLC method could be utilized for more specific than spectrophotometric methods (Dinc,1999).

In Vierordt's method, (1 %, 1 cm) values of paracetamol and caffeine were determined at 242.9 and 273.0 nm in zero – order spectra (Dinc, 1999). Medina et al (1999) proposed a simple and rapid analytical procedure for the simultaneous determination of caffeine, paracetamol, and acetylsalicylic acid in pharmaceutical preparations by partial least – squares (PLS) treatment of a flow

through multisensor based on integration of the retention and UV detection of the analytes an a solid support. Diode array spectrophotometry has been used to obtain spectra (240 – 350 nm) of the analytes retained on C_{18} bonded phase beads packed in a flow cell. Limitations to analyze mixtures of organic compounds improved by the use of solid support in combination with chemometric techniques, provides a high selectivity and increase in sensitivity. Dinc et al, proposed two methods used to determine paracetamol, caffeine and propyphenazonin ternary mixtures and tablets. Derivative ratio spectra zero crossing procedure was based on the simultaneous use of the first derivative of ratio spectra and measurements of derivative ratio analytical signals corresponding to the zero crossing points of wavelengths. By using propyphenazon as a divisor, the amounts of paracetamol and caffeine in the ternary mixture were determined by measuring the first derivative ratio amplitudes at 242.8 nm (zero crossing point for caffeine) and 251.2 and 273.8 nm (zero crossing point for paracetamol) respectively (Dinc et al, 2001). Dou et. al (2005) proposed diffuse reflectance near infrared (NIR) spectroscopy that are widely used for rapid and non destructive analysis of solid samples. A method for simultaneous analysis of the two components of compound paracetamol and diphenhydramine hydrochloride powdered drug developed by using artificial neural network (ANN) on near infrared (NIR) spectroscopy. The determination of active compounds content of compound paracetamol and diphenyhydramine hydrochloride drug of the state drug standard made by using UV – spectrophotometer (paracetamol in 240 and caffeine in 272 nm). Dinc et. al

(2005) accomplished quantitative multiresolution of tablets and ternary mixtures of paracetamol (PAR), acetylsalicylic acid (ASP), and caffeine (CAF) having strongly overlapping spectra by two graphical transform methods as ratio spectra first derivative zero crossing and ratio spectra continuous wavelet transform zero crossing (ratio spectra CWT – zero crossing) method. In this study, ratio spectra derivative – zero crossing and ratio spectra CWT- zero crossing methods are based on the use of transformed signals of the ratio spectra and their calibration graphs were obtained by measuring the $\delta A / \delta\lambda$ and CWT amplitudes of the ratio spectra corresponding to zero crossing points. The obtained result statistically showed good agreement.

Quantitative analysis by chromatography assay using planar chromatography have been done by Franeta et. al, (2001). The analyzed analgoantipyretics in dosage form using a pre – coated HPTLC silica gel plates (10 x 20 cm^2)by development in the mobile phase dichloromethane – ethyl acetate – cyclohexane – isopropanol – 0.1 M HCl – formic acid (9:8:3:1.5:0.2:0.2 v/v/v/v/v/v). Migration distances were 68.6 $\pm$ 0.2 mm, 54.1 $\pm$ 0.1 mm, 36.4$\pm$0.14 mm and 85.9$\pm$0.11 mm for acetylsalicylic acid, paracetamol, caffeine, and phenobarbirone, respectively with low RSD values (0.13—0.39 %) showing a satisfactory reproductivity in the chromatographic system). TLC scanner was used for direct evaluation of the chromatograms in the reflectance / absorbance mode (Franeta et. al, 2001). Satinsky et. al, reported a sequential injection chromatographic determination of paracetamol, caffeine and acetylsalicylic acid

in pharmaceutical tablets based on a novel reversed phase sequential injection chromatography (SIC) technique with UV detection. A Chromolith® Flash RP – 18e, 25-4.6 mm column (Merck, Germany) and a FIAlab®3000 system (USA) with an 8 port selection valve and a 5 ml syringe were used for sequential injection chromatographic separations. The mobile phase used was acetonitrile 0.01 M , phosphate buffer (10 : 90 v/v) pH 4.05, flow rate 0.6 ml/min. UV detection at 210 and 230 nm. Validation parameters showed good results, with linearity ($r > 0.999$) for all compound (Satinsky et al, 2004).

This paper also briefly discuss about the theory of HPLC and method development that are suitable for qualitative and quantitative analyses. In this project, isocratic elution is used, where a separation uses a single mobile phase of fixed composition. Here, the mobile phase, potassium phosphate, methanol, acetonitrile,and isopropanol are fixed to 420/20/30/30. However, isocratic is often difficult to find a single mobile phase composition that is suitable for all solutes (Harvey, 2000). The one of the difference between the gradient and isocratic elution is the composition of the mobile phase varies during the run. Gradient elution is a means to overcome the general elution problem which is the problem of dealing with a complex mixture of solutes, where some of the solution may elute quickly while others have high k' values (Pryde & Gilbert, 1979). By using the gradient elution, as the separation progresses, the mobile phase's composition is made less polar (Harvey, 2000) . Isocratic is simplest types of HPLC and is the only types possible with the certain pump.

Focusing on the HPLC method on analysis of paracetamol and caffeine, it has indeed become a widely used technique in the field of pharmaceutical and drug analysis (Pryde & Gilbert, 1973). Gupta (1980) developed a simultaneous quantitation of acetaminophen, aspirin, caffeine, codeine phosphate, and salicylamide by high pressure liquid chromatography. The method is based on reversed phase high pressure liquid chromatography with a mobile phase buffered with phosphate (pH 2.3). The procedure not only separated these six active ingredients but also salicylic acid, the major decomposition product of aspirin. Lowering the pH, increased the retention time of some weak acids and decreased that of some weak bases. Krieger (1984) developed a simple, rapid LC method for the separation and determination of acetaminophen in analgesic preparations containing up to 6 additional active components. The method uses a C-18 reverse phase column, methanol – 0.75% acetic acid (1+3 v/v) mobile phase, and photometric detection in the ultraviolet region. Acetaminophen was effectively separated from chlorpheniramine maleate, phenylpherine hydrochloride, caffeine, salicylamide, aspirin, and phenacetin, as well as from salicylic acid, a degradation product of aspirin. Willems et. al,(1985) determined some anticonvulsants, antiarryhthmics, benzodiazepines, xanthines, paracetamol and chloramphenicol by reversed phase HPLC. Using a uniform working procedure with five different mobile phases and one HPLC system. Changing from one eluent to another is simple and a stable base line is achieved within half an hour. Indrayanto et. al, (1995) developed a simultaneous assay of phenylpropanolamine hydrochloride, caffeine, paracetamol, glycerylguaiacolate

and chlorpheniramine maleate in Silabat ™ tablet using HPLC with diode array detection. A modified mobile phase of previous work consisting a mixture of acetonitrile ion pair solution (15 : 85 v/v ;pH 3.3) was used for initial separation. The ion pair solution contained an aqueous solution of HSA (5 mM), DBA (10 mM), AA (0.8% v/v) and PA (0.12 % v/v). The flow rate was 1 ml/min. The eluent was monitored by PDA detector in the range of 210 – 400 nm. Quantitations were performed at 260 nm for (PPA), 298 nm (CAF), 310 nm (PCM), 284 nm (GGL), and 265 nm (CTM). Pietra et. al,(1996) developed a method of HPLC analyses of pharmaceutical dosage forms containing analgesics and related compounds (acetylsalicylic acid, paracetamol, propyphenazone, caffeine and chlorpheniramine) using C-18 and cyano columns under reversed phase conditions. The performance was enhanced by introducing postcolumn on line photochemical derivatization in combination with diode array detection. The column effluents were subjected on line to UV irradiation (254 nm). LC method for the analysis of acetylsalicylic acid, caffeine, and codeine phosphate in pharmaceutical preparations have been reported by Kartal et. al, (2001) and separation was performed by using a mμ Bofidapack C-8 column by isocratic elution with flow rate 1.0 mL/min. The mobile phase composition was 125/125/250/0.5 (v/v) isopropyl alcohol, acetonitrile, water and omicron – phosphoric acid. The samples were detected at 215 nm using photo diode array detector.

Martos et. al, (2001) in their paper described a rapid reversed phase liquid chromatographic method with UV detection, for the simultaneous

determination of acetylsalicylic acid, caffeine, codeine, paracetamol, pyridoxine, and thiamine in pharmaceutical preparations. A reversed phase C-18 Nucleosil column was used. The mobile phase consisted of 2 successive eluents: water (5 min) and acetonitrile – water (75+25 v/v;9 min), both adjusted to pH 2.1 with phosphoric acid. Salicylic acid, caffeine, paracetamol, pyridoxine and thiamine were detected at 285 nm, whereas codeine was detected at 240 nm. A rapid, precise, and specific high performance liquid chromatographic has been reported for the simultaneous determination of paracetamol, phenylepherine HCl, and chlorpheniramine maleate in combined pharmaceutical dosage forms. It involves the use of μ Bondapak CN RP analytical column (10 μm, 3.9 x 150 mm) at 22°C as the stationary phase with the mixture of acetonitrile and phosphate buffer (pH 6.22, 78 : 22 v/v) as the mobile phase (Cenyuva & Ozden, 2002). Franeta et. al, (2002) proposed a HPLC method for simultaneous determination of acetylsalicylic acid, paracetamol, caffeine and phenobarbital in tablets, using chromatographic system consisting a Bio Rad 18 01 solvent pump, Rheodine 71 25 injector, and Bio Rad 1801 UV – Vis Detector. Separation was achieved using Bio SiL HL C-18, 5 μm, 250 x 4.6 mm column. Mixture of acetonitrile – water (25: 75 v/v) adjusted to pH 2.5 with phosphoric acid was used as a mobile phase at a flow rate of 2.0 ml/min. UV detection was at 207 nm range 0.01 AUFS.

Altun (2002) developed an accurate, simple, reproducible and sensitive method for the determination of paracetamol, caffeine, and dipyrone. Paracetamol, caffeine, and dipyrone were separated using a μ- Bondapak C-8 column by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase

composition was 0.01 M KH_2PO_4 – methanol – isopropanol – acetonitrile (420:20:30:30) (v/v/v/v) and spectrophotometric detection was carried out at 215 nm.

A simple and rapid gradient reversed – phase HPLC method for simultaneous separation and determination of paracetamol and its related compounds in bulk drugs and pharmaceutical formulations has been developed in nine process impurities and one degradation product of paracetamol have been separated on a symmetry C-18 column (4.6 x 250 mm i.d particle size 5 μm) with gradient elution using 0.01 M potassium dihydrogen phosphate buffer (pH 3.0) and acetonitrile as mobile phase and photo diode array detection at 215 nm (Rao & Narasaraju, 2006).

Aukunuru et. al, (1999) described the simultaneous determination of acetaminophen, salicylamide, phenyltoloxamine, and related products by HPLC. The stationary phase was a Phenomenax Prodigy C-8 column (150 x 4.6 mm, 5 μm particle siize). Mobile phase A was 0.1 M phosphate buffer adjusted to pH 2.7 with phosphoric acid. Mobile phase B was acetonitrile. The flow rate was 1 ml/min. UV detection was performed at 220 nm with injection volume 50 μl (Xu & Trissel, 2003). Wallo & D' Adamo (2000) developed an HPLC method for the simultaneous assay of hydrocodone bitartrate and acetaminophen in a tablet formulation. A Waters model ALC 204 liquid chromatograph with a Schoeffel SF 770 variable – wavelength UV detector. The stationary phase was a Waters μ Bondapak C-18 reversed phase column. The mobile phase consisted of 25 % methanol and 75 % of an aqueous solution containing 0.01 N monobasic

potassium phosphate and 0.05 N potassium nitrate, with flow rate about 1.1 ml/min. UV detection was performed at 283 nm and 2.0 AUFS. The injection volume was 13 μl (Xu & Trissel, 2003).

Use of HPLC in pharmaceutical preparation are done in antibiotics for the quantitative determination (Pryde & Gilbert, 1979). Besides, it developed to variable field quantitative determination of antibacterials like sulphonamides, antiseptic agents, in antidepressants like xanthines, theophylline, amphetamine, in central nervous system (CNS) depressants, like hypnotics and sedatives like anticonvulsants, tranquilizers phenothiazines, in analgesics and inflammatory drugs like analgesics, anti-inflammatory and so on (Pryde & Gilbert, 1979).

OBJECTIVE OF THE STUDY

1. To learn the operation of a high performance liquid chromatograph
2. To develop a suitable and effective method t in order to achieve best separation and quantification.
3. To construct a calibration plot of detector response versus concentration for checking the linearity of the response of HPLC
4. To separate and quantitate analgesics in dosage forms.

MATERIALS AND METHODS

EQUIPMENT

Below is a list of the equipment that were used during the research work. All equipment were available at the Pharmacology Lab, School of Medical Sciences, Universiti Sains Malaysia and at the Lab Facility Unit (UKM – Unit Kemudahan Makmal)

1. Complete HPLC unit - Water Alliance Series 2475 (Multi λ Flourescence Detector) plus autosampler using 20 µl sample loop.
2. Empower software program.
3. PDA Detector Water 2996
4. Sonicator
5. 0.45 µm membrane filter
6. Phenomenax Prodigy analytical column C-8 (5 µm, 250 x 4.6 mm i.d) Luna 5µ C8 (2)
7. Glass vial

CHEMICALS :

1. Paracetamol (50.00 mg)
2. Caffeine (15.00 mg)
3. Methanol (20 ml)
4. HPLC Grade acetonitrile (30 ml)
5. Isopropanol (30 ml)
6. 0.01M K_2HPO_4
7. E. pure

1. MATERIALS

Commercial tablet formulation.

a. Paracetamol

Paracetamol commercial tablet formulation (Panadol® 500 mg produced by UPHA) was available in market. It was ground and weighed to 50 mg.

b. Caffeine

Caffeine commercial tablet formulation (Caffergot® Anti Migraine tablet produced by Novatis Pharma AG, Basle, Switzerland) contains 100 mg caffeine and 1 mg ergotamine per tablet.

2. METHODS

I. Sample preparation

During the sample preparation, the selection of the phase column is determined either using the normal phase or reverse phase column.This is important in order to choose suitable organic component as mobile phase. The sample, Panadol and Caffergot were finely powdered using the mortar and pestle and weighed 50 mg and 15 mg. The samples were then transferred into 10 ml volumetric flask.

II. Preparation of mobile phase

The mobile phase was prepared separately and the amount of the organic component selected by measuring the polarity index of the solvents and that is available in many text books that mainly focusing on the HPLC and also in the manual of the machine itself. The organic solvents that are used as the mobile phase were acetonitrile, isopropanol, methanol, and potassium dihydrogen phosphate (30/30/20/420). Potassium are available in powder, so make into solution by adding an E. pure and make into 420 ml. All the mobile phase components were then mixed together into 500 mL Duran bottle and filtered througha 0.45 μm milipore filter .

Figure 4 ; Filter (0.45 milipore filter)

After the filtration, the mobile phase was left until the bubble were removed. The mobile phase was then degassed before use in sonicator for about 30 minutes to remove dissolved air, otherwise gas bubbles can form in the detector cell.

III. Preparing stock solutions :

The stock solution was prepared by transfering both of the sample into separate 10 mL volumetric flasks. The volume was made to the mark with mobile phase.

IV. Preparing working solutions :

For the working solution, the concentration of 400, 200, 100, 50, and 25 ppm or μg per mL was prepared separately in the mobile phase, by diluting appropriate volume of stock solutions to 10 ml with the mobile phase.

The mixture of the sample solutions was prepared by taking aliquots from their working solutions.

Lastly, the blank solution was prepared using the mobile phase.

100 µl of all the sample is pipetted into the glass vial and the injection is automatically done by the system since this type of system use an autosampler. Triplicate 10 µl injections were made for each mixed standard solution and once for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph. The five concentration of each compound were subjected to regression analysis to calculate the correlation coefficients.

V. Operational of HPLC system :

The HPLC system was turned ON and allowed to warm up and get stabilized. The stabilization of the system took about half day. The mode of the instrument was set to isocratic and the flow rate of the mobile phase was adjusted to 0.5 mL /min. The detector wavelength of the diode array detector was adjusted in range of 210 to 400 nm. The C-8 column (5 µm, 250 x 4.6 mm i.d) was packed and terminated at top and bottom, and connected the detector to get the system stabilized.

After the system was stabilized (pressure < 2000 psi, temperature of the column at 22.9 ^{0}C), the sample was injected. The injections were made by a fixed volume injection loop. The retention time and peak area was recorded by system itself. The retention time for each working solution of paracetamol and caffeine, mixture of paracetamol and caffeine were