KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCE

THE USE OF COMPOUNDS CHEMICALLY RELATED TO ANALYTE

AS SURROGATE REFERENCE STANDARDS IN QUANTITATIVE

HPLC

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DECLARATION

It is hereby declared that this thesis is the outcome of research work undertaken by the author. Any assistance obtained has been duly acknowledged. It is neither in part nor whole been presented for another degree elsewhere.

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DEDICATION

To my parents, Shuaibu King Mohammed and Suwaibatu Abubakar.

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ABSTRACT

The possibilities of using compounds chemically related to analyte as surrogate reference standards in quantitative high performance liquid chromatography (HPLC) have been explored. A quantitative high performance liquid chromatography method has been developed to determine a constant (K) with a surrogate reference that makes it possible to analyse paracetamol in a formulation in the absence of pure paracetamol as reference. Caffeine was used as internal standard while aspirin, phenacetin and benzoic acid were considered as surrogate references. The chromatographic apparatus consisted of Zobax C-18 column, UV detector at 257 nm and computer (Chromquest software) as a recorder. Elution was isocratic with a mobile phase consisting of methanol and 2.5% acetic acid 2:3. The retention times of the analyte were: 3.0 ± 0.01 mins (paracetamol), 4.7 ± 0.02 mins (caffeine), 8.1 ± 0.03 mins (aspirin), 11.1 ± 0.06 mins (phenacetin) and 11.7 ± 0.05 mins (benzoic acid). The constant K for the pure standards obtained were, aspirin: 18.23 ± 0.048, phenacetin: 1.15 ± 0.051 and benzoic acid: 11.66 ± 0.251. These constants were used in the analysis of nine samples of paracetamol tablets. The percentage content obtained for each sample was then compared with that obtained using the BP and the USP methods. The results showed that the surrogate reference standard can be used for the analysis of paracetamol without the use of pure paracetamol powder as reference.

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CHAPTER ONE

1.1 GENERAL INTRODUCTION

High performance liquid chromatography (HPLC) is the technique most commonly used for the quantification of drugs in formulations. Pharmacopoeial assay still rely quite heavily on direct UV spectroscopy, but in industry, detection by UV spectrophotometry is usually combined with a preliminary separation by HPLC (1).

HPLC is a precise, sensitive, and quantitative separation technique. Its popularity rests on its ability to separate a variety of analytes, including organics, ions, polymers, and biomolecules. It is the most popular analytical technique in the pharmaceutical industry and is used in all phases of drug discovery, development and quality control (2).

In recent years, HPLC is extensively used, because HPLC is not limited by sample volatility or thermal stability. HPLC is able to separate macromolecules and ionic species, labile natural products, polymeric material and a wide variety of other high molecular weight poly-functional group (3).

Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry, these fields currently comprise only about 50% of HPLC users. Currently HPLC is used by a variety of fields including cosmetics, energy, food, and environmental industries (4).

Increasingly, the determination of low concentrations of active ingredients either desired or undesired in complex mixtures, sold for human consumption, has become more necessary. Federal regulations have imposed strict limits on the type and concentrations of a host of substances sold as foods or drugs. Such requirements demand analytical techniques that are fast and reliable and combine the separation to alleviate interferences and analysis steps in a single operation (5).

In order to identify any compound by HPLC a detector must first be selected. Once the detector is selected and is set to optimal detection parameters, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. The first is the choice of column, another is the choice of mobile phase, and last is the choice of flow rate (6).

HPLC method development is mostly a process of finding the right mobile phase and adjusting its solvent strength because resolution in HPLC is controlled primarily by the mobile phase. Paradoxically, with all the HPLC hardware sophistication and computerization, most method development is still performed manually via trial and error, the total method development time may last only 15 minutes. In method validation, analytical performance data such as precision, accuracy, robustness, linearity, and sensitivity are gathered, and hundreds of assays are required (2).

Internal standards are frequently used in chromatographic analysis. The internal standard is a compound deliberately added to the analyzed mixture to aid in the quantification process. For example, in an HPLC experiment, a solution of known analyte and internal standard concentration is first run under a particular set of column conditions. The ratio of the detector signal for the analyte (A) and the internal standard (S) is then calculated to measure the relative sensitivity of the technique for the two materials $(A/S)_{known}$.

Next, the internal standard is added to the unknown solution and the concentration of the unknown is calculated. The ratio of the detector signal for the analyte and the internal standard in the unknown $(A/S)_{unknown}$ is in direct proportion to the ratio of their concentrations. Hence the concentration ratio of [A]/[S] in the unknown is simply the concentration ratio in the known solution multiplied by ($(A/S)_{unknown}$ divided by $(A/S)_{known}$).

Theoretically, internal standards added to the mixture to be analyzed are desirable if any loss of sample is likely to occur, for example, during handling. In this way, the ratio of concentrations of analyte and the internal standard remains constant regardless of the amount of solution lost (7).

Rather than generating calibration curves for each compound, modern chromatographic software packages automatically calculate response factors (RFs). The ratio of the peak area or height of the target compound in the sample to the peak area or height of the internal standard in the sample is determined and is compared to the equivalent ratio derived for the target compound and internal standard in each calibration standard. The value is termed the response factor (RF) or relative response factor (RRF):

$$\text{Response Factor} = \frac{(\text{Area of target compound}) \times (\text{Concentration of internal standard})}{(\text{Area of internal standard}) \times (\text{Concentration of target compound})}$$

An ideal internal standard concentration would yield an RF of 1.0 for each analyte, but this is not practical when there are multiple target analytes for each internal standard. Generally, the amount of internal standard to include should produce a response that is no more than 100 times that produced by the lowest concentration of the least responsive target compound associated with that internal standard. So the minimum RF should be no less than 0.01 for that target compound (2). If the compound exhibits linear response over the concentration range analyzed, the plot of RF (y-axis) versus concentration (x-axis) will be a relatively flat line, and the RFs can be used to calculate the concentrations of target compounds in samples:

$$\text{Target Compound Concentration} = \frac{(\text{area target compound}) \times (\text{concentration internal standard})}{(\text{Area of internal standard}) \times (\text{RF})}$$

Although the internal standard technique compensates for changes in injection volume, retention time, or detector response, it is advisable to perform a continuing calibration check at regular intervals (5).

The majority of applications of HPLC in pharmaceutical analysis are for the quantitative determinations of drugs in formulations. Usually these analyses do not require a large amount of time to be spent in selecting mobile phases, column and detectors since most quality control applications can be carried out with methanol:water (1:1) as mobile phase, an Octadecylsilane (ODS) column and a UV detector. The main potential interferants in analysis of formulation are preservatives, colourant, possible degradation product of the formulated drugs and formulations with more than one active ingredient. These may present more

analytical challenges since different ingredient have different chemical properties and may elute at different time (1).

HPLC assay of formulated drugs can often be carried out against a standard; this may be either an external or internal standard. The use of an internal standard is best since all loses due to extraction are compensated for. An ideal internal standard should be closely related to the analyte, chromatographically resolved from the analyte and any excipients present in the formulation extract. The best compounds to be used as internal standard should be compounds of similar structure compared to that of the analyte (1).

The HPLC is the modern instrument used in pharmaceutical industry and the Food and Drug Board Ghana (FDB) is encouraging the use of HPLC for the quality management of the drug products on the market. But the problem of how to come by pure standards for each one of these drugs is still a problem. This research therefore seeks to develop an alternative analytical procedure that would make it possible to use quantitative HPLC for assays without using reference powders of the target analytes, but chemically related compounds analytes as surrogate reference standard.

1.1.1 Justification

Quite a number of researches has been undertaken to determine constants which help chemists the world over in both chemical and drug assays. An example is the use of A (1%, 1cm) in the UV analysis of compounds where there are no pure standards for analysis. From the BP, diazepam tablet is analysed using A (1%, 1cm) as 450 at a maximum wavelength of 284nm. Another example is the use of internal standard in quantitative Nuclear Magnetic

Resonance (NMR), where drugs are quantified by measuring suitable proton signals against the intense singlet for the methyl groups in t-butanol used as the internal standard (1). The use of (NMR) in quantitative analysis of pharmaceuticals which utilized measurement of the area under selected signals both test and standard samples by means of electronic integrators has also been reported (46). The advantage of this method of quantitation is that a pure external standard for the drug is not required since the response is purely proportional to the number of protons present and this can be measured against a pure internal standard. Thus the purity or content of a substance can be determined without a pure standard of it being available (1). Paracetamol products are being used in this preliminary investigation because paracetamol is cheap and readily available both as a reference powder and as medicine. A lot of research has also been done on paracetamol and hence its data is readily available for comparison. Phenacetin, benzoic acid and aspirin are being investigated as the target surrogates because they are readily available and also show similar chemical properties as paracetamol.

1.1.2 Main Objective

This project is to investigate the use of compounds chemically related to the analyte as surrogates for pure reference samples in HPLC application.

1.1.3 Specific Objectives

The specific objectives of the research are:

1. To determine the retention times, limit of detection (LOD) and limit of quantitation (LOQ) of the analyte, an internal standard and all the target surrogate reference compounds by a suitable HPLC method.

2. To elute together the pure paracetamol sample, with an internal standard as well as a target surrogate reference to use the concentrations of analyte and chromatogram properties to determine a constant K, analogous to the response factor for the analytes.

3. To determine factors that affect the constants.

4. To use the determined constant to evaluate the content of paracetamol in selected samples.

5. To compare the results with that obtained by using British Pharmacopoeia (BP) and United State Pharmacopoeia (USP) methods.

1.1.4 Hypothesis of study

For the same compounds

$$\frac{A_{analyte}}{C_{analyte}} = \frac{A_{s\ tan\ dard}}{C_{s\ tan\ dard}}$$

For different compounds

$$\frac{A_{analyte}}{C_{analyte}} \alpha \frac{A_{s\ tan\ dard}}{C_{s\ tan\ dard}}$$

Therefore:

$$\frac{A_{analyte}}{C_{analyte}} = K\frac{A_{s\ tan\ dard}}{C_{s\ tan\ dard}}$$

Hence

$$K = \frac{A_{analyte} \times C_{s\ tan\ dard}}{C_{analyte} \times A_{s\ tan\ dard}}$$

Where K is a constant analogous to the response factor of the solute.

$A_{analyte}$ is the peak area ratio of the analyte.

$A_{standard}$ is the peak area ratio of the standard.

$C_{standard}$ is the concentration of the standard.

$C_{analyte}$ is the concentration of the analyte.

Once K, $A_{analyte}$ and $C_{standard}$ are known for a particular system, $C_{analyte}$ can be calculated.

$$\text{Percentage content} = \frac{\text{Actual concentration}}{\text{Nominal concentration}} \times 100$$

1.2 LITERATURE REVIEW

The modern form of column liquid chromatography has been called high -performance, high-resolution, high-pressure and high-speed liquid chromatography. However, the abbreviation HPLC is now universally understood to describe the technique that separates mixtures on columns filled with small particles by elution with a liquid under high pressure. The essential equipment consists of an eluent reservoir, a high-pressure pump, an injector for introducing the sample, a stainless steel column containing the packing material, a detector, and a chart recorder. HPLC equipment can be obtained as a complete system or assembled from individual modules (8).

HPLC is a separation technique that can be used for the analysis of organic molecules and ions. It is based on mechanisms of adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. It involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases. HPLC has been used to assess the purity and/or determine

the content of many pharmaceutical substances (9). It has also been used to determine enantiomeric composition, using suitably modified mobile phases or chiral stationary phases (9).

1.2.1 Injectors

The primary goal for any injection is to introduce the sample into the column. The liquid samples are injected with small glass-syringes whose volume is between 1–10µL for capillary columns and 50µL for packed columns. Gaseous samples are normally injected with gas-tight glass-syringes with volumes between 1 and 10mL (10). The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. In modern HPLC systems, the sample injection is typically automated (11).

1.2.2 Chromatographic column

Columns are usually made of polished stainless steel, of about 50 to 300mm long, with internal diameter of between 2 and 5mm and an outside diameter of 6.3mm. They are commonly filled with a stationary phase with a particle size of 5 - 10µm (9). The outlet is terminated by a stainless steel mesh disk to retain the packing material. The mesh disk is held in position by the end-fitting with the end of the tubing to the detector flush against the center. This tubing should have an internal diameter of 0.25mm or less and be as short as possible to minimize peak broadening (8). Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally, the temperature of the mobile phase

and the column should be kept constant during an analysis. Most separations are performed at ambient temperature, but columns may be heated to give better efficiency. Normally, columns should not be heated above 60 °C because of the potential for stationary phase degradation or changes occurring to the composition of the mobile phase (9). The most efficient columns produce the sharpest peaks, which give better separation by minimizing band spreading (12).

There are two main classes of column, normal and reversed phase columns. The normal phase columns are most usually packed with silica gel and are used in partition or adsorption chromatography. Reversed phase columns are packed with a chemically bonded octadecylsilyl coated silica; such columns are referred to as C-18 and are very non-polar. This is the most popular column used in HPLC (13).

There are various columns that are secondary to the separating column or stationary phase. They are: Guard, Derivatizing, Capillary, Fast, and Preparatory Columns (14).

1.2.3 Mobile phases

Mobile phase selection is the most important parameter in HPLC hence proper selection of the mobile phase is an important step in the development of separation methods. The main requirement for the mobile phase is that it has to dissolve the analytes up to the concentration suitable for the detection. Type of mobile phase used may have a big effect on the retention (15).

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample. A sample solution is

injected into the mobile phase of an assay through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase (16).

The choice of mobile phases is based on the desired retention behaviour and the physicochemical properties of the analyte. For normal-phase HPLC, using unmodified stationary phases, lipophilic solvents should be employed. The presence of water in the mobile phase must be avoided as this will reduce the efficiency of the stationary phase. In reverse-phase HPLC, aqueous mobile phases, with and without organic modifiers, are used.

The mobile phase should be filtered through suitable membrane-type filters with a porosity of 0.45μm to remove mechanical particles. Adjustment of the pH, if necessary, should be made using the aqueous component of the mobile phase and not the mixture. Buffers of high molarity should be avoided in the preparation of mobile phases. If buffers are used, the system should be rinsed with an adequate mixture of water and the organic modifier of the mobile phase to prevent crystallization of salts. Mobile phases may contain other components, e.g. a counter-ion for ion-pair chromatography or a chiral selector for chiral chromatography using an achiral stationary phase (9).

1.2.4 Pumping system

Pump refers to the device that forces the mobile phase through a liquid chromatography column at pressures up to 10,000 psi and varies in pressure capacity (17). Their performance is measured on their ability to yield a consistent and reproducible flow rate.

Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns that are < 2 micrometres. These "Ultra Performance Liquid Chromatography" systems (UPLCs) can work at up to 15,000 lbf/in² (~100 MPa or about 1000 atmospheres) (18). HPLC pumping systems are required to deliver metered amounts of mobile phase at a constant flow rate.

Pumping systems that deliver solvent from one or more reservoirs are available (9). The flow rate through the column is generally between 1and 3 ml/min, with pressures ranging from 500 to 4000Ib/ in2 (3.4-27.6MPa). The pumps must show minimum fluctuations within these ranges to achieve maximum stability of the detector response and reproducible retention data. The simplest pump consists of the direct application of gas pressure on to the surface of the eluent contained in a pressure bottle or holding coil. Such devices are relatively cheap but have the disadvantage that the pressure limit is low typically 1500Ib/in2, 10.3MPa. The gas dissolved in the eluent may reappear as bubbles in the detector flow-cell, and a change of eluent requires the apparatus to be dismantled, washed and refilled. Commercial HPLC pumps use pistons or diaphragms to displace eluent, hence avoiding problem of dissolved gas (8).

Modern computer- or microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying (gradient elution) composition, according to a defined programme. In the case of gradient elution, solvent mixing can be achieved on either the low or high-pressure side of the pump(s). Depending on the flow rate and composition of the mobile phase, operating pressures of up to 42000kPa (6000 psi) can be generated during routine analysis (9).

1.2.5 Detectors

A detector is a component in the chromatographic system which senses the presence of a compound passing through it, and provides an electronic signal to a recorder or computer data station. The output is usually peaks known as the chromatogram (12).

It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (19). There are many types of detectors that can be used with HPLC. Some of the more common detectors include: Refractive Index (RI), Ultra-Violet (UV), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS) (6).

There are four types of detectors that have found widespread of application; an important factor in the choice of a detector is the amount of peak broadening which occurs as the eluted compounds pass through. This is largely controlled by the volume and geometry of the

detector flow-cell but is also influenced by the connecting tubing between the column and the detector (8).

1.2.6 Stationary phases

Separation of pharmaceuticals is usually achieved by partition of compounds in the test solution between the mobile and the stationary phases. HPLC systems consisting of polar stationary phases and nonpolar mobile phases are described as normal-phase chromatography; those with nonpolar stationary phases and polar mobile phases are called reversed-phase chromatography.

Many types of stationary phases are used including unmodified silica, HPLC alumina, or porous graphite. These are used in normal-phase chromatography, where separation is based on differences in adsorption. A variety of chemically modified supports prepared from polymers, silica, or porous graphite are used in reverse-phase HPLC, where separation is based principally on partition of the molecules between the mobile phase and the stationary phase. Resins or polymers with acid or basic groups are used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase. Porous silica or polymers are used in size-exclusion chromatography, where separation is based on the relative molecular mass of the molecules.

Most separations are based on partition mechanisms using chemically modified silica as the stationary phase and polar solvents as the mobile phase (reverse-phase HPLC). The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to

produce covalently bonded silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system (9).

1.2.7 Qualitative and Quantitative analysis

Analytical instruments such as gas chromatography-mass spectroscopy (GC-MS), atomic absorption spectroscopy (AAS), or HPLC can provide a lot of information about the contents of a sample. They can tell us what is in a mixture and how much is there (20). Determining the identities of components is referred to as qualitative analysis. In qualitative analysis, comparison of retention times of samples with those of authentic samples can be employed in the identification of a compound.

Determining the amounts of these compounds is called quantitative analysis. In quantitative analysis, the HPLC methods are generally more rapid compared to other classical and chromatographic methods (37). All sample analysis instruments have some means of detecting the presence of analytes. Analytes enter a detector and generate an electronic signal called a response. The response can have other names depending on the instrument or the type of signal generated by its detector. Other names include absorbance, intensity, abundance, etc. The data system of the instrument has some way of storing and displaying that response. Usually, the response is displayed on a graph where the x-axis is retention time and the y-axis is a measure of the intensity of the response. In chromatography, this graph is called a chromatogram. During the course of running a sample, the graph is constantly updated to produce a line.

When the run begins, there are no analytes in the detector, the response is zero, and the line produced on the chromatogram is called the baseline. As the analyte enters the detector, the response intensity increases, usually very rapidly. The line on the graph shoots upward until the maximum response occurs. As the analyte is swept out of the detector, the line returns to the baseline until another analyte enters the detector. The chromatogram will show a peak. The size of the peak is proportional to the concentration of the analyte. If we measure the peak, we can evaluate the concentration of the analyte.

There are several measurements used to determine the size of the peak. They include height, width and area. However, height and width are affected by how fast the analyte moves through the detector. An analyte that is moved slowly will produce a short, broad peak. If we speed up the process, the analyte will produce a tall, thin peak. Therefore, the preferred measurement is the area of the peak. We can determine that area by treating the peak like a triangle, and using some geometry and algebra. However, most data systems can determine the area more precisely for us using complex computer algorithms.

Before an analyte can be quantified, the relationship between its peak area and concentration should be known. The simplest method is to determine a response factor. The response factor (RF) is the proportionality constant for the analyte. Each analyte will have a unique RF under given instrumental conditions. Equation 1 shows us that RF is simply the concentration (C) divided by the area (A).

$$RF = C/A$$

If a sample of a known concentration is prepared and analysed, the peak area can be measured and the RF determined. This process is referred to as a calibration. Once the RF for the target analyte is known, the concentration of that analyte in the sample can determined. Concentration is simply

$$C = RF \times A$$

This method works fairly well provided the concentration in the calibrated standard is close to the concentration in the sample (20).

Drugs that previously required chemical derivatization for GC analysis can now be chromatographed by HPLC without any prior treatment. Also pharmaceuticals in complex matrices may not require a preliminary extraction procedure but be dissolved in a suitable solvent and infected directly into the system. HPLC presents the option of selecting solvents of different polarities and dielectric properties for use as the mobile or carrier phase to effect a greater margin of specificity and resolution in difficult separation problems. The capability of recovering the solute in the eluate on a preparative scale using greatly simplified fraction collectors for purposes of characterization can also be achieved. The major disadvantage is that no universally applicable detector system is yet available. The choice of HPLC for a particular analytical problem depends on the physical and chemical properties of the compounds to be analysed, the nature of sample and detection level required.

HPLC has found extensive usage throughout the whole spectrum of pharmaceutical analysis, from quality control of raw materials and qualitative and quantitative analyses of formulated products to the separation and estimation of the concentration of drugs and their degradation

products in body fluids and tissues. The application of HPLC to pharmacokinetics and bioavailability studies of drug products has been made possible by the development of sensitive detectors to monitor column effluent and thereby separate and quantify drugs and their metabolites (37). Ferguson used quantitative HPLC analysis in the separation and determination of caffeine in an analgesic and amitriptyline hydrochloride and perphenazine in phychotherapeutic medication. In another study, quantitative and qualitative HPLC analysis of thermogenic weight loss products was done by Schaneberg and Khan (2004), an HPLC qualitative and quantitative method of seven analytes (caffeine, ephedrine, forskolin, icariin, pseudoephedrine, synephrine, and yohimbine) in thermogenic weight loss preparations available on the market was also developed. After 45 min, the seven analytes were separated and detected in acetonitrile: water (80: 20) extract (40).

1.2.8 Internal standard

An internal standard is a known amount of a compound, different from analyte that is added to the unknown. Signal from analyte is compared with signal from the internal standard to find out how much analyte is present. Internal standards are especially useful for analyses in which the quantity of sample analysed or the instrument response varies slightly from run to run for reasons that are difficult to control. For example, gas or liquid flow rates that vary by a few percent in a chromatography experiment could change the detector response. A calibration curve is only accurate for the set of conditions under which it is obtained. However, the relative response of the detector to the analyte and standard is usually constant over a wide range of conditions. If signal from the standard increases by 8.4% because of a change in solvent flow rate, signal from the analyte usually increases by 8.4% also. As long as the concentration of standard is known, the correct concentration of analyte can be

derived. Internal standards are widely used in chromatography because the small quantity of sample solution injected into the chromatograph is not very reproducible in some experiments. Internal standards are also desirable when sample loss can occur during sample preparation steps prior to analysis. If a known quantity of standard is added to the unknown prior to any manipulations, the ratio of standard to analyte remains constant because the same fraction of each is lost in any operation (21).

1.2.8.1 Choice of Internal Standard

Analysts are often reluctant to use internal standards in HPLC as selection of an appropriate standard can cause a significant increase in method development time. This increase results from the additional injections required to establish the elution position of each potential internal standard relative to the other peak(s) in the chromatogram. The migration position of peaks can be more easily predicted in free-solution capillary electrophoresis, as the migration time is related to the size and charge of the compound.

Therefore, to have an internal standard that migrates before a basic analyte of interest at low pH requires one with a smaller molecular weight or higher number of positive charges. The separation of anions at high pH would require an internal standard with larger molecular weight or fewer charges than the analyte to produce a peak that migrates before the solute peak. It is more difficult to predict an internal standard but solubility data can be useful, as a lower water-soluble compound will generally elute later than a more soluble one.

The nature and concentration of the exact substance selected depends on several factors. The main requirement is that the substance gives good peak shape and is resolved from the

analytes of interest and any other peaks in the separation. Other requirements for an appropriate internal standard include stability in solution, commercial availability in a high pure form, ready solubility in the diluent required, possession of acceptably high UV activity at desired wavelength, cheap and is non-toxic.

1.2.8.1.1 Stability

The internal standard should be sufficiently stable in the sample dissolving solvent to prevent the formation of degradation products, which would interfere with the integration results. It should also be chemically stable in the solid state to allow suitable storage (22).

1.2.8.1.2 Internal standard solubility

The internal standards should be freely soluble in the sample solvent. If low conductivity sample diluents are used then 'stacking' may result in a 10-fold increase in on-column concentration, which may cause problems of precipitation and/or peak tailing. This problem can also occur for the analyte and should be checked (22).

1.2.8.1.3 Commercial availability

The material selected should be cheap and readily available in a high-purity form from commercial suppliers so that the method can be readily reproduced elsewhere (22).

1.2.8.1.4 Toxicity

The toxicity of the internal standard should be minimal to reduce any handling precautions that may be required. The compound should have a good UV response at the detection wavelength so that a high signal can be obtained to reduce any integration-related variability generated with small peaks. Preferably, the internal standard should have a migration

position near to the peak of interest so that if there is a drift in migration times throughout an injection sequence the migration times for both the solute and internal standard peaks will be similar (22).

The best internal standard is an isotopically labeled version of the molecule you want to quantify. An isotopically labeled internal standard will have a similar extraction recovery, ionization response in ESI mass spectrometry, and a similar chromatographic retention time. It may be difficult to justify such a standard since a special synthesis of an isotopically labeled standard can be expensive and time consuming. Often, medicinal chemists have a library of compound analogue that can be used as internal standards. These analogues were made in the evolution of the compound to be tested and will be similar to the compound to be quantified and more importantly will be slightly different by parent mass. The use of de-methylated (-14) or hydroxylated (+16) analogues as internal standards are to be avoided since these are the most common mass shifts observed in naturally occuring metabolites of the parent compound. A common internal standard is a chlorinated version of the parent molecule. A chlorinated version of the parent molecule will commonly have a similar chromatographic retention time which is an important characteristic of an internal standard. It has been found that one of the most important characteristics of an internal standard is that it co-elutes with the compound to be quantified (23).

1.2.8.2 How to use an internal standard

First of all an internal standard should be added at the beginning of the sample work-up, typically before the plasma crash or solid phase extraction. The internal standard should be added at the same level in every sample including the standards. An internal standard should

give a reliable HPLC response. Care should be taken that the amount of the internal standard is well above the limit of quantitation but not so high as to suppress the ionization of the analyte. The amount of internal standard added is important but can be accomplished by making trial analyses of an early, middle and late time point with perhaps one or two standard points. This information will be very valuable when building an appropriate standard curve and in knowing how much internal standard to add (24).

1.2.9 Paracetamol

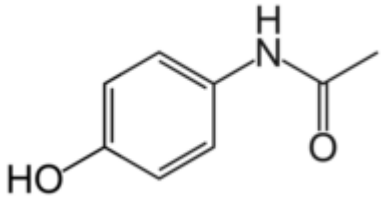


Fig 1.1. Chemical structure of paracetamol (N-(4-hydroxyphenyl) acetamide)

Paracetamol is a white, odourless crystalline powder with a bitter taste, soluble in 70 parts of water, 7 parts of alcohol (95%), 13 parts of acetone, 40 parts of glycerol, 9 parts of propylene glycol, 50 parts of chloroform, or 10 parts of methyl alcohol. It is also soluble in solutions of alkali hydroxides. It is insoluble in benzene and ether. A saturated aqueous solution has a pH of about 6 and is stable but stability decreases in acid or alkaline conditions, the paracetamol being slowly broken down into acetic acid and p-aminophenol.

Initially, paracetamol was found in the urine of patients who had taken phenacetin and in 1889, it was demonstrated that paracetamol was a urinary metabolite of acetanilide. Paracetamol, known as acetaminophen in the United States, is a painkiller that is popular throughout the world because it is remarkably safe and it does not irritate the stomach. It was

first discovered to have both analgesic and antipyretic properties in the late nineteenth century (25).

The legal category of paracetamol ranges from prescription only, through pharmacy only to general sales list depending on the quantity and strength of paracetamol supplied in a container or packet. High risk of liver damage can occur if an overdose of paracetamol is taken. The hazards of paracetamol overdose are greater in persistent heavy drinkers and in people with alcoholic liver disease (26).

1.2.10 Caffeine

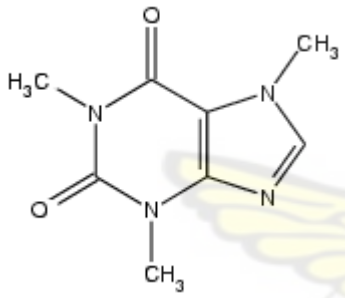


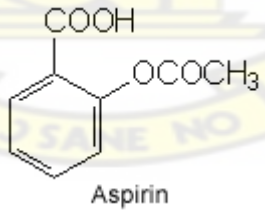
Fig. 1.2 Chemical structure of caffeine (1,3,7-trimethylpurine-2,6dione)

The Caffeine is an alkaloid of the methylxanthine family, which also includes similar compounds such as theophylline and theobromine. In its pure state, it is an intensely bitter white powder. Its chemical formula is $C_8H_{10}N_4O_2$. Pure caffeine occurs as odourless, white, fleecy masses, glistening needles or powder. It is soluble in water (1 gm in 46 mls water) pH 6.9 (1% aqueous solution). The Chinese first discovered the effects of caffeine in the form of medicinal tea about five thousand years ago, for the purpose of staying awake. The active ingredient in coffee and tea was not identified until the nineteenth century and it was not until 1820 that caffeine was truly discovered (27).

Chemically speaking, pure caffeine is a plant-based alkaloid which stimulates the central nervous system of any creature that ingests it. It is also known as guaranine, mateine or theine depending on the source plant. It is considered a psychoactive drug. However, it has not been designated a controlled substance, so its use in teas, coffees and sodas is not illegal (28).

Caffeine is a chemical found in many different plants from all over the world and it is a stimulant. Of all caffeine consumed in the world, 54 % is in coffee, 43 % is in tea and the other 3 % is in other sources, such as cola drinks and medicines. Most of the caffeine in many young people's diets is in cola drinks. It is difficult to say exactly how much is too much caffeine, because individuals will have different reactions and responses to particular foods or substances. Some people seem to be able to tolerate much more caffeine than others. However, 250mg (approximately) will probably cause some mild effects. Most researchers say that 600mg per day is 'safe', however many people have problems from this, and would feel better if they had much less caffeine (29).

1.2.11 Aspirin



Aspirin

Fig. 1.3 Chemical structure of aspirin (Acetylsalicylic acid)

Aspirin is an odourless, colourless or a white crystalline powder with a molecular weight of about 180.15g/mol. It also has melting and boiling points of 135^{o}C and 140^{o}C respectively.

Aspirin, one of the first drugs to come into common usage, is still widely used in the world. Aspirin is prepared by chemical synthesis from salicylic acid, by acetylation with acetic anhydride. Aspirin is analgesic, anti-inflammatory, and antipyretic and is an inhibitor of platelet aggregation. It inhibits fatty acid cyclo-oxygenase by acetylation of the active site of enzyme and the pharmacological effects of aspirin are due to the inhibition of the formation of cyclo-oxygenase products including prostglandins, thromboxanes and prostacyclin (30).

The problem with aspirin is that it upsets the user's stomach fairly badly. In fact, some people had bleeding in their digestive tracts from the high doses of aspirin needed to control pain and swelling (31).

1.2.12 Phenacetin

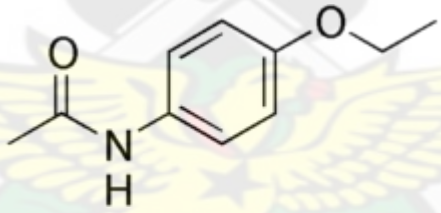


Fig. 1.4. Chemical structure of phenacetin N-(4-ethoxyphenyl)acetamide

Phenacetin, introduced in 1887, is used principally as an analgesic. Typical doses of 300 mg to 500 mg a day result in an analgesic effect. Its analgesic effects are due to its actions on the sensory tracts of the spinal cord. It also is an antipyretic, acting on the brain to decrease the temperature set point. It is also used to treat rheumatoid arthritis, intercostal neuralgia, and some forms of ataxia. In addition, phenacetin has a depressant action on the heart, where it acts as a negative inotrope (32).

Phenacetin, and products containing phenacetin have been shown in an animal model to be carcinogenic. In humans, many case reports have implicated products containing phenacetin in urothelial neoplasms, especially transitional cell carcinoma of the renal pelvis. In one prospective series, phenacetin was associated with an increased risk of death due to urologic or renal diseases, death due to cancers, and death due to cardiovascular diseases. In addition, people that are glucose-6-phosphate dehydrogenase deficient may experience acute hemolysis while taking this drug (33).

1.2.13 Benzoic Acid

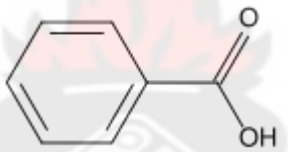


Fig. 1.5 Chemical structure of Benzoic acid (benzene carboxylic acid)

Benzoic acid, the simplest aromatic carboxylic acid containing carboxyl group bonded directly to benzene ring, is a white, crystalline organic compound; melting at 122 ^{o}C, boiling at 249 C; slightly soluble in water, soluble in ethanol, very slightly soluble in benzene and acetone. Its aqua solution is weakly acidic. The name derived from gum benzoin, which was for a long time the only source for benzoic acid. This weak acid and its salts are used as a food preservative. Benzoic acid is an important precursor for the synthesis of many other organic substances. Benzoic acid was discovered in the 16th century, Justus von Liebig and Friedrich Wöhler determined the structure of benzoic acid in 1832 while in 1875 Salkowski discovered the antifungal abilities of benzoic acid, which were used for a long time in the preservation of benzoate containing fruits (34).

Use of benzoic acid in the production of glycol benzoates for the application of plasticizer in adhesive formulations is increasing. It is also used in the manufacture of resins and drilling mud additive for crude oil recovery applications and as a rubber polymerization activators and retardants. Benzoic acid is converted to its salts and esters for the use of preservative application in foods, drugs and personal products. Sodium benzoate, sodium salt of benzoic acid, is used preferably as one of the principal anti-microbial preservatives used in foods and beverages but concentrations exceeding 0.1% is poisonous, as it is about 200 times more soluble than benzoic acid. Sodium Benzoate is also used in medications, anti-fermentation additives and tabletting lubricant for pharmaceuticals (35).

Benzoic acid is present as part of hippuric acid (N-Benzoylglycine) in urine of mammals, especially herbivores. Humans produce about 0.44 g/L hippuric acid per day in their urine, and if the person is exposed to toluene or benzoic acid it can rise above that level. For humans the intercellular perfusions of cell (IPC) suggest a provisional tolerable intake of 5 mg/kg body weight per day and a lethal dose for humans is 500 mg/kg (46).

CHAPTER TWO

2.1 EXPERIMENTAL METHODS, MATERIALS AND REAGENTS.

2.1.1 Materials/ Reagents

Pure paracetamol (BDH), phenacetin (BDH), aspirin (BDH) , benzoic acid (BDH), methanol (BDH), glacial acetic acid (BDH), acetic anhydride (BDH), toluene (BDH), sodium hydroxide(BDH), hydrochloric acid (BDH), ethanol (BDH), sulphuric acid (BDH), ammonium cerium (IV) sulphate and perchloric acid were provided by the Department of Pharmaceutical Chemistry, KNUST Kumasi, Ghana. Paracetamol tablets manufactured by Letap Pharmaceuticals, Kinapharma Ltd, Enerst Chemist, M&G Pharmaceuticals, Phyto Riker Ltd, Dannex, Eskay Therapeutics, Ayrton Drugs and G&R Pharmaceuticals were bought from retail pharmacies at Adum, Kumasi.

2.1.2 Instrumentation

The component of the liquid chromatograph include**;** Zobax ODS column (4.6mm x 25cm), a pump (spectra system P4000), an auto sampler (spectra system AS3000) a detector (spectra system UV1000 and integrator (Dell Pentium IV with chromoquest software).

Table 2.1. Profile of Paracetamol samples (500mg)

Paracetamol sample	Manufacturing Company	Batch number	Expiry date
LP	Letap Pharmaceutical Ltd	6002116	01/08
KP	Kinapharma Ltd	035	08/09
EC	Ernest Chemist Ltd	**	**
MG	M & G Pharmaceuticals Ltd	PA393H	09/08
PR	Phyto Riker, Ltd.	F03013	02/07
DN	Dannex	**	**
ET	Eskay Therapeutics Ltd	75	07/08
AD	Ayrton Drugs	11	01/09
GR	G & R Pharmaceuticals Ltd	13	01/08

** Information was not available

2.1.3 Preparation of solutions

2.1.3.1. Preparation of 0.5M Sulphamic acid

Sulphamic acid (4.4527g) was weighed into a beaker. Distilled water (40ml) was added and stirred with a clean rod to dissolve. It was transferred quantitatively into a clean dried 100 ml volumetric flask using a funnel. The beaker was rinsed with small amount of distilled water till the solution was made to volume. The funnel was then removed and the flask was stoppered, the solution was then labeled and dated.

2.1.3.2. Preparation of 0.5 M sodium hydroxide

Sodium hydroxide (10.160 g) was weighed directly in a beaker, fast and carefully because it easily reacts with moisture in the atmosphere. Distilled water (200 ml) was poured into the beaker to dissolve it, the reaction is exothermic. The solution was stirred with a clean dried glass rod to ensure total dissolution. The solution was made to cool and quantitatively transferred into the 500ml volumetric flask using a cleaned and dried funnel. This was done

to avoid expansion and contraction of the flask which can change the volume of the calibrated volumetric flask. The beaker was rinsed with distilled water and transferred into the flask. This was repeated two times to make sure everything was transferred into the flask. The funnel was also rinsed into the flask, and then it was removed. The solution was stoppered and shaken to ensure proper mixing. The solution was then topped to the mark carefully with a pipette. The solution was stoppered, gently shaken, labeled and dated. Aliquots were taken for the titrations.

2.1.3.3. Preparation of 0.5 M hydrochloric acid

Small quantity of the stock HCl was carefully poured into a small clean and dried beaker to rinse it. Another quantity was carefully poured into the beaker again which was around the volume needed to avoid the contamination of the stock by pouring the rest into it. A dried clean 250ml volumetric flask was filled to about half its capacity with distilled water. The HCl (10ml) was pipetted from the beaker carefully using pipette filler. The acid was carefully released into the flask containing the distilled water by the wall of the flask. The solution was stoppered and shaken to ensure proper mixing. The solution was topped to the mark using a pipette. The solution was stoppered, shaken, labeled and dated. Aliquots of the solution were taken for the titrations.

2.1.3.4. Preparation of 0.1M Perchloric Acid

Glacial acetic acid (900 ml) was measured into a 1L volumetric flask. Perchloric acid (10.2 ml of 60%) was slowly added with continuous and efficient mixing. The perchloric acid was well diluted with the glacial acetic acid before the addition of 30 ml acetic anhydride. This was done to prevent the danger of forming acetyl perchlorate. The addition was made slowly

with continuous and efficient mixing. The volume was adjusted to 1 L with glacial acetic acid. The solution was allowed to stand for 24 hrs before it was used. The acetic anhydride used was to react with any molecules of water in the perchloric acid and glacial acetic acid to make the solution virtually anhydrous.

2.1.3.5. Preparation of solution of surrogate reference standards

Exactly 0.1000 g of the pure paracetamol, caffeine, aspirin, benzoic acid and phenacetin powders were weighed accurately into separate 100 ml clean volumetric flask. To each of the flask, a small amount of the diluent was added and shaken manually to aid dissolution. It was topped to the mark using the diluent and then filtered with a Whatmann's filter paper. The resultant solution was stored in a volumetric flask and labeled.

2.1.3.6. Assay preparation (paracetamol tablet sample solution)

A quantity of the powder equivalent to 0.1g of paracetamol was weighed into a 100 ml volumetric flask. A small amount of the diluent was added to the powder and shaken to dissolve. The volume was made to the mark by using the diluent and then filtered. One milliliter (1 ml) of the resultant solution was pipetted into another clean 100 ml volumetric flask and made to the mark using the diluent.

Table 2.2 Weight of sample equivalent to 0.1 g of pure paracetamol

Sample	Weight equivalent
LP	0.1180
KP	0.1100
EC	0.1123
MG	0.1153
PR	0.1108
DN	0.1082
ET	0.1225
AD	0.1222
GR	0.1194

2.1.4 Standardisation of solutions

2.1.4.1. Standardisation of 0.1M ammonium cerium (IV) sulphate

An amount of ammonium cerium (IV) sulphate (65.0002g) was dissolved in a mixture of 30 ml H_2SO_4 and 500 ml of water. This was allowed to cool and diluted to 1000 ml with water. To 25 ml of the resulting solution, 2.0000g of potassium iodide and 150 ml of water was added. This was immediately titrated with 0.1 M sodium thiosulphate vs using starch as indicator. Each ml of sodium thiosulphate is equivalent to 63.26 mg.

2.1.4.2. Standardisation of (0.1 M) perchloric acid

Potassium hydrogen phthalate was used in the standardization of the perchloric acid. Potassium hydrogen phthalate (0.5064 g) was weighed into a cornical flask. Glacial acetic acid (25 ml) was pipetted into the cornical flask. The solution was warmed to dissolve the

salt. The solution was allowed to cool and two drops of crystal violet were added to it. The resulting solution was then titrated potentiometrically with the 0.1M perchloric acid.

2.1.5 Assay of pure standards

2.1.5.1 Aspirin

One gram (1.0000 g) of Aspirin was dissolved in 10 ml of ethanol (96%), 50 ml of 0.5 M sodium hydroxide Vs was added. The flask was stoppered and allowed to stand for 1 hour. Dilute phenolphthalein solution (0.2 ml) was added to it and titrated with previously standardised 0.5 M HCl. The operation was repeated without the substance being examined. The difference between the titrations represents the amount of sodium hydroxide required. Each ml of 0.5 M sodium hydroxide Vs is equivalent to 0.0450 g of $C_9H_8O_4$ (42).

2.1.5.2 Benzoic acid

Benzoic acid (2.5000g) was dissolved in 15 ml of warm alcohol (95%) previously neutralized with phenolphthalein solution, 20 ml of water was added and titrated with 0.5 M sodium hydroxide using phenolphthalein solution as indicator. Each ml of 0.5 M sodium hydroxide is equivalent to 0.061006 g of $C_7H_6O_2$ (41).

2.1.5.3 Caffeine

Caffeine (0.1700 g) was dissolved in 5 ml of anhydrous glacial acetic acid with the aid of heat. It was allowed to cool, and 10 ml of acetic anhydride and 20 ml of toluene were added to it. A non-aqaueous titration was carried out. Each ml of 0.1 M perchloric acid Vs is equivalent to 0.01942 g of $C_8H_{10}N_4O_2$ (42).

2.1.5.4 Paracetamol

Paracetamol (0.3000 g) was dissolve in a mixture of 10 ml of water and 30 ml of 1 M sulphuric acid. It was boiled under reflux for one hour, cooled and diluted to 100 ml with water. To 20 ml of the solution, 40 ml of water, 40 g of ice, 15 ml of 2 M hydrochloric acid, and 0.1 ml of ferroin sulphate solution were added and titrated with 0.1 M ammonium cerium (IV) sulphate Vs until a yellow colour was obtained. The procedure was repeated without the substance being examined. Each ml of 0.1 M ammonium cerium (IV) sulphate Vs is equivalent to 0.007560 g of $C_8H_9NO_2$ (43).

2.1.5.5 Phenacetin

Phenacetin (0.3000g) was dissolved in a mixture of 10ml of water and 30ml of 1M sulphuric acid. It was boiled under reflux for one hour, cooled and diluted to 100 ml with water. To 20 ml of the solution, 40 ml of water, 40 g of ice, 15 ml of 2 M hydrochloric acid and 0.1 ml of ferroin sulphate were added and titrated with 0.1 M ammonium cerium (IV) sulphate Vs until a yellow colour was obtained. The procedure was repeated without the substance being examined. Each ml of 0.1 M ammonium cerium (IV) sulphate Vs is equivalent to 0.008761 g of $C_{10}H_{13}NO_2$ (43).

2.1.6 HPLC Method development

2.1.6.1 Development of mobile phase and diluent

From the literature searched on the physico-chemical properties such as solubility, stability in solution of paracetamol and the other selected surrogate references advantage was taken of these properties to try a number of mobile phase combinations which involved methanol and water. The ionic strength and pH of the mobile phase was modified with glacial acetic acid.

All the selected surrogate references dissolved in methanol and water in the ratio 1:1. This was chosen as the diluent. Another mobile phase tried was methanol, water and acetic acid mixture in the ratio 28: 69:3, this gave poor retention times and tailing peaks. Methanol and 2.5 % glacial acetic acid in the ratio of 2:3 was used as the mobile phase for the analysis. Since the volume of mixtures do not usually equal the sum of the separate volumes making up the mixture as a result of differences in density and volume, the mobile phase and diluent used were separately measured. These were mixed together filtered with filter paper. The resultant solution was stored in a conical flask and labeled.

2.1.6.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

A stock solution of 0.1% w/v of all the surrogate standards were prepared and serially diluted to different concentrations. Ten micro-litres (10 µl) of the resultant solution was injected onto the column and eluted isocratically. Chromatograms were recorded and peak areas measured electronically. This was to help determine the LODs and LOQs so as to choose a good concentration for analytical work, using the formula given below

LOD = 3 x s/n

LOQ = 10 x s/n

Where s is the standard deviation of concentrations

n is the number of sample

2.1.6.3 Analytical performance parameters (reproducibility, accuracy and precision)

Five milliliters (5 ml) of the 0.005% w/v pure paracetamol solution (analyte) was pipetted into a 10 ml clean volumetric flask. An aliquot of the 0.2% w/v caffeine solution (0.20 ml) (internal standard) was added. It was mixed and made to the mark using the diluent. Ten

micro-litres (10 µl) of the resultant solution was injected onto the column and eluted isocratically. Chromatograms were recorded and peak areas measured electronically. There were ten replicate determinations for each concentration. The whole process was repeated using paracetamol tablets so as to determine its percentage content.

2.1.6.4 Determination of K using the surrogate reference standards.

Five milliliters (5 ml) of the 0.004% w/v paracetamol solution (analyte) was pipetted into a 10 ml clean volumetric flask. An aliquot of the 0.2% w/v caffeine solution (0.20 ml) (internal standard) and 1 ml of the 0.1% w/v aspirin solution (as standard) were added. It was mixed and made to the mark using the diluent. Ten micro-litres (10 µl) of the resultant solution was injected onto the column and eluted isocratically. Chromatograms were recorded and peak areas measured electronically. The constant K was determined. The concentration of the paracetamol was varied to determine whether these variations would affect the constant K. There was ten replicate determinations for each concentration. The whole process was repeated using benzoic acid (0.6 ml of 0.06% w/v) and phenacetin (5ml of 0.005% w/v) sequentially in place of aspirin. Effort of variations in concentration of paracetamol on K was investigated in each case.

2.1.6.5 Analysis of paracetamol tablets using the surrogate reference standards

Five milliliters (5 ml) of the paracetamol tablet sample solution (analyte) was pipetted into a clean 10 ml volumetric flask. An aliquot (0.2 ml) of the caffeine solution (internal standard) and 1ml of aspirin (as standard) were added. It was mixed and made to the mark using the diluent. Ten microlitres (10 ul) of the resultant solution was injected onto the column and

eluted; the major peaks, corresponding to paracetamol, caffeine and aspirin were measured and recorded. There were ten replicate determinations for each concentration. The whole process was repeated using benzoic acid (0.6 ml of 0.06% w/v) and phenacetin (5ml of 0.005% w/v) sequentially in place of aspirin. Effort of variations in concentration of paracetamol on K was investigated in each case. The concentration of the analyte was determined by making use of the constant earlier on elucidated.

2.1.7 Standard method (USP method)

2.1.7.1 Standard preparation

An accurately weighed quantity (0.1002 g) of USP acetaminophen RS was dissolved in the mobile phase to obtain a solution having a known concentration of about 0.01 w/v.

2.1.7.2 Preparation of mobile phase

Methanol and water were measured separately in the ratio of 1:1, mixed together and filtered before it was used for analysis.

2.1.7.3 Assay preparation

Twenty tablets were weighed and finely powdered. An accurately weighed portion of the powder equivalent to about 100 mg of acetaminophen was transferred into a 200 ml volumetric flask; and 100 ml of mobile phase was added. It was mechanically shacken (Orbital shaker, Stuart SO1, speed 200) for 10 minutes and diluted with mobile phase to volume and mixed. Five milliliters (5 ml) of the solution was transferred into a 250 ml volumetric flask and diluted with mobile phase to volume and mixed. A portion of the solution was filtered through a finer porosity filter discarding the first 10ml of the filtrate. The clear filtrate was used as the assay preparation.

2.1.7.4 Procedure for preparation

Equal volumes (10 ul) of the standard preparation and assay preparation were separately injected into the chromatograph. The chromatograms were recorded and the response for the major peaks was measured. The quantity in mg of acetaminophen in the portion of tablets taken was calculated by the formula 10,000 C (ru/rs) in which C is the concentration in mg/ml of USP acetaminophen RS in the standard preparation and ru and rs are the acetaminophen peak response obtained from the assay preparation and the standard preparation respectively (44). This was repeated three times.

2.1.8 Standard method (BP)

Twenty tablets each of the nine paracetamol brands were weighed and powdered. A quantity of the powder containing 0.15 g was added to 50 ml of 0.1 M sodium hydroxide. It was diluted to 100 ml with water, hand-shaken for 15 minutes and sufficient distilled water was added to produce 200 ml of solution. The resulting solution was filtered and 10 ml of the filtrate was diluted to 100 ml with water. Ten milliliters (10 ml) of the resulting solution was added to 10 ml of 0.1 M sodium hydroxide and diluted to 100 ml with water. The absorbance of the resulting solution was then measured at 257 nm using a double beam UV spectrophotometer 5000i (curry) with a glass cuvette of path length 1 cm. A 0.1 M solution of sodium hydroxide was prepared and used as the reference solution (45). 715, which is the A(1 %, 1 cm) for paracetamol was used for the calculation. This was repeated three times.

Table 2.3 Weight of sample equivalent to 0.15 g of pure paracetamol

Sample	Weight equivalent
LP	0.1782
KP	0.1650
EC	0.1689
MG	0.1729
PR	0.1663
DN	0.1623
ET	0.1838
AD	0.1840
GR	0.1716

CHAPTER THREE

3.1 RESULTS AND CALCULATIONS

Table 3.1 melting range of the surrogate reference powders

Surrogate reference powders	Melting range ^{o}C	BP range ^{o}C
Aspirin	140-142	about143
Benzoic acid	122- 124	121-124
Caffeine	233- 235	234-239
Paracetamol	168-170	168-172
Phenacetin	133-136	134-136

Table 3.2 Percentage deviation of tablets; sample *AD*

Number	individual weight(g)	Deviation	*% deviation
1	0.6150	-0.0017	-0.2772
2	0.6064	0.0069	1.1251
3	0.6067	0.0066	1.0761
4	0.6012	0.0121	1.9729
5	0.6391	-0.0258	-4.2067
6	0.5925	0.0208	3.3915
7	0.6132	1×10^{-04}	0.0163
8	0.6439	-0.0306	-4.9894
9	0.6228	-0.0095	-1.5490
10	0.6226	-0.0093	-1.5164
11	0.6120	0.0013	0.2120
12	0.5777	0.0356	5.8047
13	0.6201	-0.0068	-1.1088
14	0.6172	-0.0039	-0.6359
15	0.5803	0.0330	5.3807
16	0.6249	-0.0116	-1.8914
17	0.6157	-0.0024	-0.3913
18	0.5656	0.0477	7.7776
19	0.6205	-0.0072	-1.1740
20	0.6289	-0.0156	-2.5436

* % Deviation = $\frac{\text{Deviation}}{\text{Average}} \times 100$

Average wt = 0.6133g

Table 3.3 Standardization of 0.5 M NaOH with sulphamic acid.

Burette readings/ml	1st determination	2nd determination	3rd determination
Final reading	21.00	21.00	21.00
Initial reading	0.00	0.00	0.00
Titre volume	21.00	21.00	21.00

Mean titre = 21.00 ± 0.000 ml

Table 3.4 Assay of Aspirin powder.

Burette readings/ml	1st determination (1.0028 g)	2nd determination (1.0020 g)	3rd determination (0.9999 g)
Final reading	26.20	25.40	25.40
Initial reading	0.00	0.00	0.00
Titre volume	26.20	25.40	25.40

Table 3.5 Blank titration (assay of aspirin)

Burette readings/ml	1st determination	2nd determination	3rd determination
Final reading	48.60	48.60	48.60
Initial reading	0.00	0.00	0.00
Titre volume	48.60	48.60	48.60

Table 3.6 Assay of benzoic acid powder

Burette readings/ml	1st determination	2nd determination	3rd determination
Final reading	43.80	43.50	43.30
Initial reading	0.00	0.00	0.00
Titre volume	43.80	43.50	43.30

Table 3.7 Assay of paracetamol powder

Burette readings/ml	1st determination	2nd determination	3rd determination
Final reading	7.90	7.90	7.90
Initial reading	0.00	0.00	0.00
Titre volume	7.90	7.90	7.90

Average titre = $\frac{7.90 + 7.90 + 7.90}{3}$

= 7.90 ml

Table 3.8 Assay of phenacetin powder

Burette readings/ml	1st determination	2nd determination	3rd determination
Final reading	6.80	6.80	6.80
Initial reading	0.00	0.00	0.00
Titre volume	6.80	6.80	6.80

Average titre = 6.80 ± 0.000 ml

Table 3.9 Assay of caffeine

Burette readings/ml	1st determination (0.1758 g)	2nd determination (0.1762 g)
Final reading	9.20	9.35
Initial reading	0.00	0.00
Titre volume	9.20	9.35

3.2 Calculation of percentage purity of aspirin

Factor for sulphamic acid (H_2SO_3H) = $\frac{\text{actual wt}}{\text{Nominal wt}}$

= $\frac{4.4527g}{4.4545g}$ = 0.9996

Factor for NaOH = $\frac{\text{factor of } H_2SO_3H \text{ x volume of } H_2SO_3H}{\text{Volume of NaOH}}$

$= \frac{0.9996 \text{ x } 20.00 \text{ ml}}{21.00 \text{ ml}}$

$= 0.952$

Factor for HCl = $\frac{\text{factor of NaOH x volume of NaOH}}{\text{Volume of HCl}}$

$= \frac{0.952 \text{ x } 20.00 \text{ ml}}{20.10 \text{ ml}}$

$= 0.9473$

3.2.1 Sample A

Weight of sample A = 1.0028 g

Volume of HCl equivalent to NaOH that reacted with Aspirin:

= (48.60 – 26.20) ml

= 22.40 ml

Actual volume of HCl = equivalent volume x factor of HCl

= 22.40 ml x 0.9473

= 21.21 ml

But 1ml of NaOH ≡ 0.04504 g of aspirin (42)

Thus 21.21ml = 21.21 x 0.04504 g = 0.9553 g

Percentage purity of Aspirin = $\frac{\text{Actual wt x}}{\text{Nominal wt}}$ 100%

$= \frac{0.9553 \text{ g}}{1.00280 \text{ g}} \text{ x } 100$

= 95.26 %

3.2.2 Sample B

Weight of sample B = 1.0020 g

Volume of HCl equivalent to NaOH that reacted with Aspirin:

= (48.60 – 25.40) ml

= 23.2 ml

Actual volume of HCl = 23.2 ml x 0.9473 = 21.98 ml

But 1ml of NaOH ≡ 0.04504 g of aspirin

Thus 21.98 ml ≡ 21.98 x 0.04504 = 0.9900 g

Percentage purity Aspirin = $\frac{0.9900\ g}{1.0020\ g} \times 100$

=98.80 %

3.2.3 Sample C

Weight of sample C = 0.9999 g

Volume of HCl equivalent to NaOH that reacted with Aspirin:

= (48.60 – 25.40) ml = 23.2ml

Actual volume of HCl = 23.2 x 0.9473 = 21.98 ml

But 1 ml of NaOH ≡ 0.04504 g of aspirin

Thus 21.98 ml ≡ 21.98 x 0.04504 g = 0.9900 g

Percentage purity of Aspirin = $\frac{0.9900}{0.9999} \times 100$

= 99.01 %

Average percentage purity = $\frac{95.26 + 98.80 + 99.01}{3}$

= 97.69 ± 2.11 %

3.3 Calculation of percentage purity benzoic Acid

Factor for sulphamic acid = 0.9996

Factor for NaOH = $\frac{\text{factor of } H_2SO_3H \times \text{volume of } H_2SO_3H}{\text{Volume of NaOH}}$

= $\frac{0.9996 \times 20.00\ ml}{21.00\ ml}$

= 0.952

3.3.1 Sample A

Weight of sample A = 2.5104 g

Volume of NaOH that reacted with Benzoic Acid = 43.80 ml

Actual volume of NaOH = equivalent volume x factor of NaOH

= 43.80 ml x 0.952

= 41.6976 ml

But 1 ml of NaOH ≡ 0.06106 g benzoic acid (41)

Thus 41.6976 ml ≡ 41.6976 x 0.06106 g

= 2.5461 g

Percentage purity of Benzoic Acid = $\frac{\text{Actual wt x } 100}{\text{Nominal wt}}$

= $\frac{2.5461 \text{ g x } 100}{2.5104 \text{ g}}$

= 101.42 %

3.3.2 Sample B

Weight of sample B = 2.5028 g

Volume of NaOH that reacted with Benzoic Acid = 43.50 ml

Actual volume of NaOH = equivalent volume x factor of NaOH

= 43.50 ml x 0.952

= 41.412 ml

But 1 ml of NaOH ≡ 0.06106 g benzoic acid

Thus 41.412 ml ≡ 41.412 x 0.06106 g

= 2.5286 g

Percentage purity of Benzoic Acid = $\frac{2.5286 \text{ g x } 100}{2.5028 \text{ g}}$

= 101.03%

3.3.3 Sample C

Weight of sample C = 2.5009 g

Volume of NaOH that reacted with Benzoic Acid = 43.30 ml

Actual volume of NaOH = equivalent volume x factor of NaOH

= 43.30 ml x 0.952

= 41.2216 ml

But 1 ml of NaOH ≡ 0.06106 g benzoic acid

Thus 41.6976 ml ≡ 41.2216 x 0.06106 g

= 2.5170 g

Percentage purity of Benzoic Acid == $\frac{2.5170 \text{ g}}{2.5009 \text{ g}} \times 100$

= 100.64 %

Average Percentage purity of Benzoic Acid = $\frac{101.42 + 101.03 + 100.64}{3}$

= 101.03 % ± 0.39

3.4 Calculation of percentage purity paracetamol powder

Factor for ammonium cerium (IV) sulphate ($2(NH_4)_2SO_4Ce(SO_4)_2.2H_2O$) = 1.0056

Weight of sample = 0.3019 g

Volume of $2(NH_4)_2SO_4Ce(SO_4)_2.2H_2O$ that reacted with paracetamol = 7.90 ml

But 20ml of $2(NH_4)_2SO_4Ce(SO_4)_2.2H_2O$ ≡ 7.90 ml

Therefore 100 ml = $\frac{100}{20} \times 7.90$

= 39.5 ml

Actual volume of $2(NH_4)_2SO_4.Ce(SO_4)_2.2H_2O$ =

Equivalent volume x factor of $2(NH_4)_2SO_4Ce(SO_4)_2.2H_2O$

= 39.5 ml x 1.0056

= 39.7212 ml

But 1 ml of $2(NH_4)_2SO_4Ce(SO_4)_2.2H_2O$ ≡ 0.007560 g of paracetamol (43)

Thus 39.7212 ml ≡ 39.7212 ml x 0.007560 g = 0.3003 g

Percentage purity of paracetamol = $\frac{\text{actual wt.}}{\text{nominal wt.}} \times 100$

$= \frac{0.3003\ \text{g}}{0.3019\ \text{g}} \times 100$

$= 99.47\ \%$

3.5 Calculation of percentage purity phenacetin powder

Weight of sample (phenacetin) = 0.3002 g

Volume of $2(NH_4)_2SO_4\ Ce(SO_4)_2.2H_2O$ that reacted with phenacetin = 6.80 ml

But 20 ml of $2(NH_4)_2SO_4.\ Ce(SO_4)_2.2H_2O$ = 6.80 ml

Therefore 100 ml = $\frac{100}{20} \times 6.80$

= 34.00 ml

Actual volume of $2(NH_4)_2SO_4.Ce(SO_4)_2.2H_2O$ =

Equivalent volume x factor of $2(NH_4)_2SO_4.Ce(SO_4)_2.2H_2O$

= 34.00 ml x 1.0056

= 34.1904 ml

But 1 ml of $2(NH_4)_2SO_4.Ce(SO_4)_2.2H_2O$ ≡ 0.008761 g of phenacetin

Thus 34.1904 ml ≡ 34.1904 ml x 0.008761 g

= 0.2995 g

Percentage purity of phenacetin = $\frac{\text{actual wt.}}{\text{nominal wt.}} \times 100$

$= \frac{0.2995\ \text{g}}{0.3002} \times 100$

$= 99.77\ \%$

3.6 Calculation of percentage purity caffeine

Factor for Potassium hydrogen phthalate (KHP) = $\frac{\text{actual wt}}{\text{Nominal wt.}}$

$= \frac{0.5064 \text{ g}}{0.5000 \text{ g}}$

$= 1.0128$

Factor for perchloric acid ($HClO^{4-}$); $F_1V_1 = F_2V_2$

Therefore $F_2 = \frac{F_1V_1}{V_2}$

Where F_1 is the factor for KHP

F_2 is the factor for $HClO_4^-$

V_1 is the pipetted volume

V_2 is the titre volume

Therefore $F_2 = \frac{1.0128 \times 25 \text{ ml}}{25.15 \text{ ml}}$

$= 1.0068$

3.6.1 Sample A

Sample A mass = 0.1758 g

Titre value = 9.20 ml

Actual titre value = titre value x factor for $HClO_4^-$

= 9.20 x 1.0068

= 9.26 ml

1 ml $HClO_4^-$ = 0.01942 g of caffeine (42)

Therefore ml = $\frac{9.26 \text{ ml}}{1 \text{ ml}} \times 0.01942 \text{ g}$

= 0.1798 g

% content = $\frac{\text{actual wt}}{\text{nominal wt}} \times 100$

$= \frac{0.1798 \text{ g}}{0.1758 \text{ g}} \times 100 = 102.29\ \%$

3.6.2 Sample B

Sample B mass = 0.1762 g

Titre value = 9.35 ml

Actual titre value = titre value x factor for $HClO_4^-$

= 9.35 x 1.0068

= 9.41 ml

1 ml $HClO_4^-$ ≡ 0.01942 g of caffeine

Therefore 9.41 ml ≡ $\frac{9.41 \text{ml}}{1 \text{ ml}}$ x 0.01942 g = 0.1828g

% content = $\frac{\text{actual wt}}{\text{nominal wt}}$ x 100

= $\frac{0.1828 \text{ g}}{0.1762 \text{ g}}$ x 100

= 103.75 %

Average % content = $\frac{102.29 + 103.75}{2}$ = 103.02 % ± 1.03

Table 3.10 Comparison of percentages purities of sample to BP reference range

Pure sample	Calculated percentages (%)	BP percentage (%)	Comment
Aspirin	97.69	99.5 – 101.0	Failed
Benzoic acid	101.03	Not less than 95	Passed
Caffeine	103.02	98.5 – 101.5	Failed
Paracetamol	99.47	99.5 – 101.0	Passed
Phenacetin	99.77	99.5 – 101.0	Passed

Table 3.11 Limit of detection and quantification of the pure standards.

Pure powders	Limit of detection (w/v)	Limit of quantification (w/v)
Aspirin	0.007	0.02
Benzoic acid	0.001	0.004
Caffeine	0.0006	0.002
Paracetamol	0.0004	0.001
Phenacetin	0.0001	0.0004

3.7 Calculation of limit of detection (LOD) and limit of quantitation (LOQ)

LOD = 3 x s/n

LOQ = 10 x s/n

Where s is the standard deviation of concentrations

n is the number of sample

eg. aspirin the concentrations (%w/v) were: 0.00005, 0.0005, 0.001, 0.005, 0.01 – 0.06

n =10 and s = 0.022203

Therefore LOD = $3 \times \frac{0.022203}{10} = 0.006661$

LOQ = $10 \times \frac{0.022203}{10} = 0.022203$

3.8 Chromatographic conditions

Wave length: 257 nm

Flow rate: 1ml/min

Column: Zobax C-18 column

Detector: UV detector

Table 3.12 Mean Retention time of the pure samples (n=10).

Pure sample	Mean Retention time (minutes)
Aspirin	8.05 ± 0.028
Benzoic acid	11.73 ± 0.054
Caffeine	4.72 ± 0.019
Paracetamol	3.02 ± 0.012
Phenacetin	11.11 ± 0.060

Table 3.13 Analytical performance parameters (assay of paracetamol tablet)

Replicates	Aspirin	Benzoic acid	Phenacetin
1	94.0	110.0	97.5
2	95.7	114.8	96.3
3	96.0	110.8	101.7
4	95.5	110.3	98.0
5	95.1	114.6	98.8
6	93.4	114.0	97.9
7	103.6	114.5	97.1
8	96.6	113.2	97.2
9	93.9	115.6	98.8
10	94.4	110.2	98.8
Mean	95.8 ± 2.93	112.8 ± 2.21	98.2 ± 1.48
*RSD	3.05	1.96	1.51
**SEM	0.93	0.70	0.47

* Relative Standard Deviation
** Standard Error of Mean

Table 3.14 Constant (K) values of pure paracetamol using aspirin as standard at varying concentration.

Concentration mg/ml	Mean K
0.0003	18.26 ± 0.917
0.0004	18.22 ± 0.862
0.0005	18.19 ± 0.732
0.0006	18.19 ± 1.286
0.0007	18.30 ± 1.065

Table 3.15 Constant (K) values of pure paracetamol using benzoic acid as standard at varying concentration.

Concentration mg/ml	Mean K
0.0002	11.75 ± 0.239
0.0003	11.73 ± 0.533
0.0004	11.25 ± 0.543
0.0005	11.93 ± 0.406
0.0006	11.66 ± 0.411

Table 3.16 Constant (K) values of pure paracetamol using phenacetin as standard at varying concentration.

Concentration mg/ml	Mean K
0.0002	1.24 ± 0.065
0.0003	1.15 ± 0.038
0.0004	1.14 ± 0.043
0.0005	1.12 ± 0.046
0.0006	1.16 ± 0.055

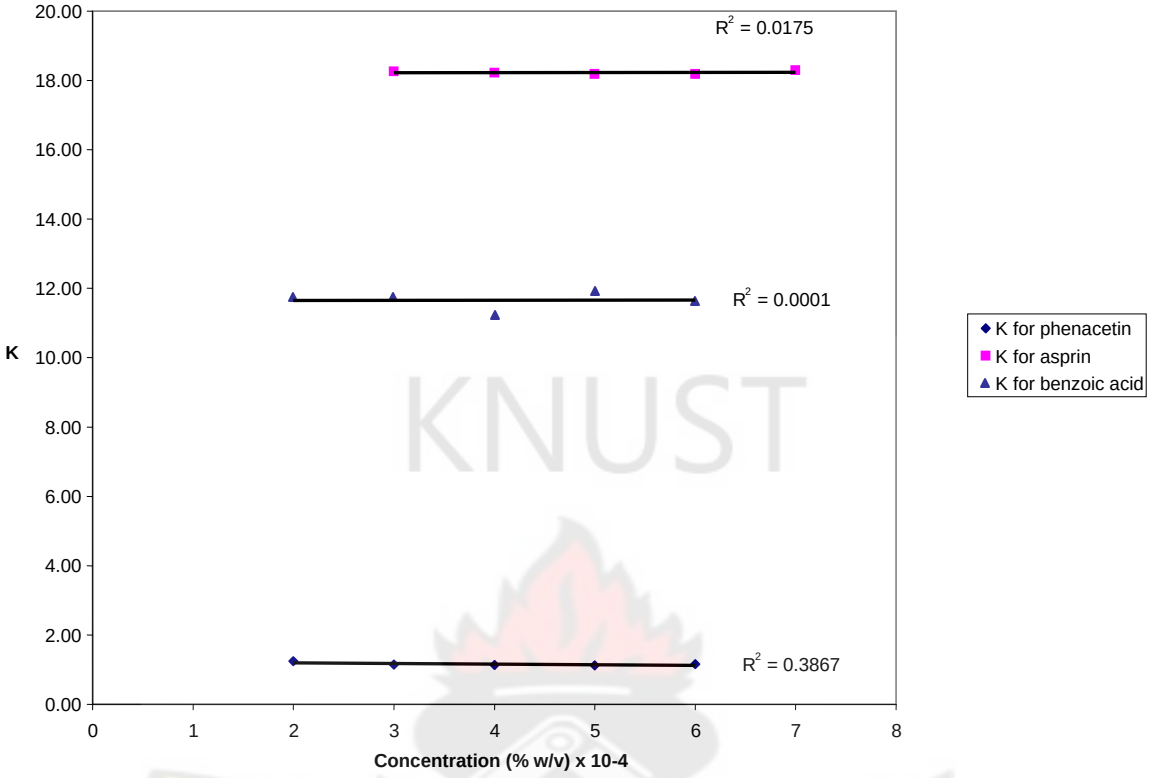


Fig. 3.1 The constant (K) curve for Phenacitin, Aspirin and Benzoic acid

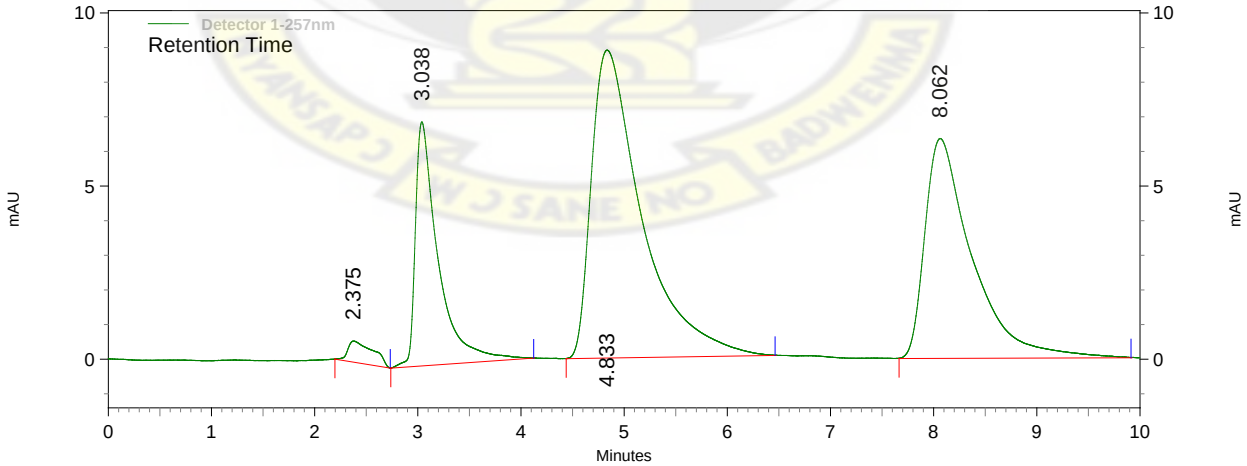


Fig 3.2 Standard Aspirin, Paracetamol and Caffeine

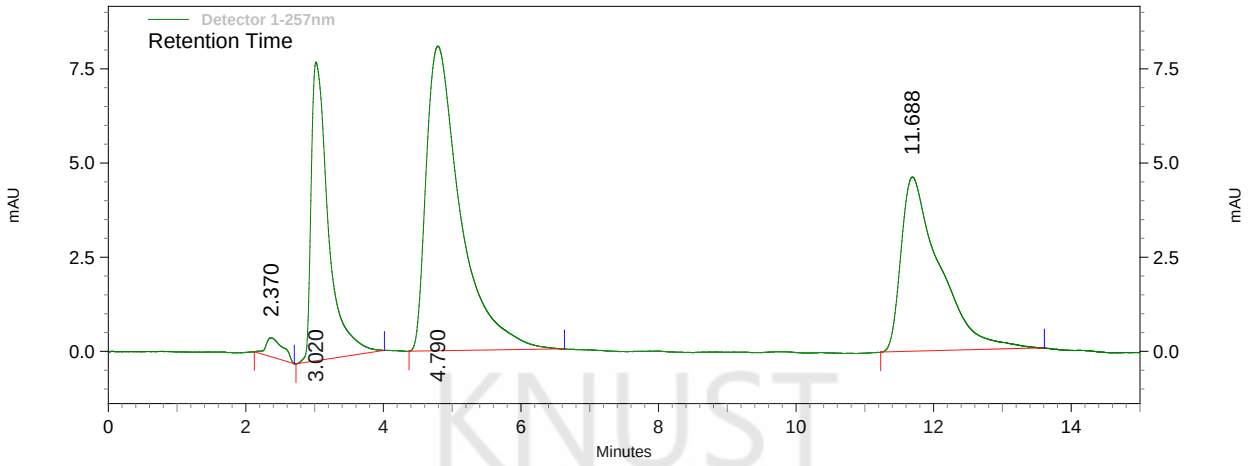


Fig 3.3 Standard Benzoic Acid, Paracetamol and Caffeine

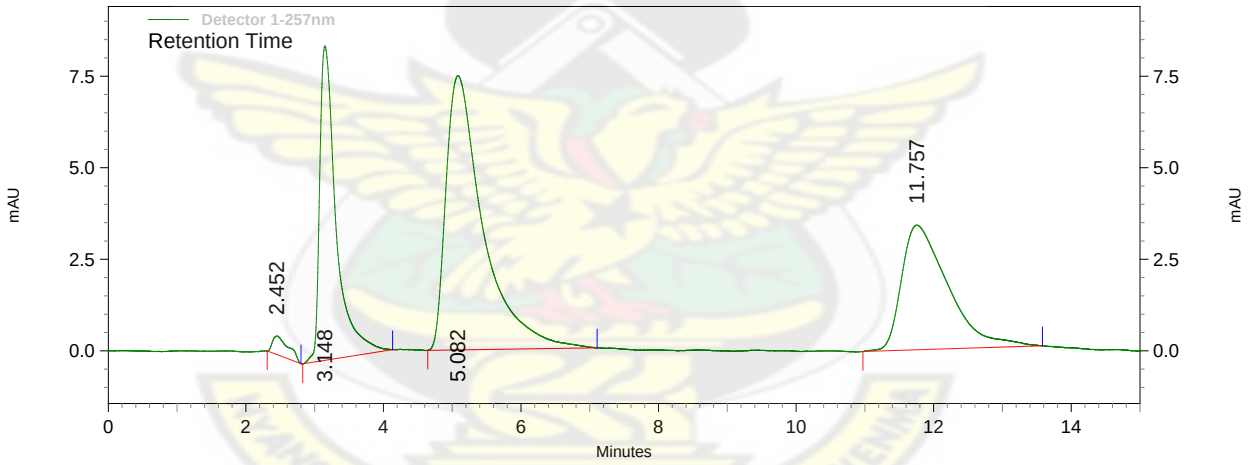


Fig 3.4 Standard Phenacetin, Paracetamol and Caffeine

Table 3.17 Assay of paracetamol samples using the USP method.

Samples	1st determination	2nd determination	3rd determination	Mean
AD	96.51	95.85	95.33	95.90 ± 0.593
DN	93.14	91.62	93.82	92.86 ± 1.131
EC	99.44	98.11	99.68	99.08 ± 0.847
ET	107.73	108.15	110.42	108.77 ± 1.453
GR	103.29	97.83	101.67	100.93 ± 2.805
KP	98.05	96.61	96.93	97.20 ± 0.756
LP	98.58	97.29	98.65	98.18 ± 0.764
MG	104.48	97.29	105.18	102.32 ± 4.364
PR	98.03	96.89	97.68	97.53 ± 0.587

Monograph requirement: 90-110%

Table 3.18 Assay of paracetamol samples using the BP method.

Sample	Mean Absorbance	Actual concentration %w/v	Nominal concentration %w/v	%content
AD	0.561	0.000785	0.00075	104.62
DN	0.526	0.000736	0.00075	98.09
EC	0.559	0.000782	0.00075	104.24
ET	0.550	0.000769	0.00075	102.56
GR	0.571	0.000799	0.00075	106.48
KP	0.563	0.000787	0.00075	104.99
LP	0.562	0.000786	0.00075	104.80
MG	0.572	0.000800	0.00075	106.67
PR	0.559	0.000782	0.00075	104.24

Monograph requirement: 95- 105%

Table 3.19 Assay of paracetamol tablet using the developed method.

Samples	Surrogate reference standards		
	Aspirin	Benzoic acid	Phenacetin
AD	103.90 ± 4.720	101.30± 4.609	97.13 ± 2.563
DN	101.92 ± 1.795	95.82 ± 1.917	90.64 ± 1.751
EC	104.83 ± 4.910	103.40 ± 3.619	99.70 ± 1.524
ET	106.77 ± 3.023	98.75 ± 1.802	94.40 ± 2.931
GR	111.26 ± 2.636	109.30 ± 6.931	98.60 ± 3.758
KP	106.33 ± 6.370	113.30 ± 4.940	97.70 ± 3.490
LP	106.14 ± 3.432	97.55 ± 4.540	95.63 ± 4.016
MG	98.85 ± 2.996	101.90 ± 1.987	99.25 ± 2.761
PR	122.95 ± 4.626	100.40 ± 2.339	102.00 ± 4.844

Table 3.20 Comparative assay data of the USP, BP and methods developed method.

Samples	Developed method			USP method	BP method
	Aspirin	Benzoic acid	Phenacetin		
AD	103.90	101.30	97.13	95.90	104.61
DN	101.92	95.82	90.64	92.86	98.09
EC	104.83	103.40	99.70	99.08	104.24
ET	106.77	98.75	94.40	108.76	102.56
GR	111.26	109.30	98.60	100.93	106.48
KP	106.33	113.30	97.70	97.20	104.99
LP	106.14	97.55	95.63	98.18	104.80
MG	98.85	101.90	99.25	102.31	106.67
PR	122.95	100.40	102.00	97.53	104.24

3.9 Statistical analysis

Table 3.21 Comparison of values at varying concentration of the pure paracetamol standard.

Standards used	p-values	Comments (p at 0.01)
Phenacetin	0.466	Not significant
Benzoic acid	0.999	Not significant
Aspirin	0.230	Not significant

Table 3.22 Comparison of the developed method and the standard USP method

Standards used	p-values	Comments (p at 0.01)
Phenacetin	0.022	Not significant
Benzoic acid	0.212	not significant
Aspirin	0.750	not significant

Table 3.23 Comparison of the developed method and the standard BP method

Standards used	p-values	Comments (p at 0.01)
Phenacetin	0.348	Not significant
Benzoic acid	0.314	not significant
Aspirin	0.248	not significant

CHAPTER FOUR

4.0 Discussion, Conclusion and Recommendations

4.1 Discussion

4.1.1 Quality Assurance

Quality assurance involves the sampling and testing of starting materials, intermediate, finished and packaging materials to ensure compliance with appropriate standards and specifications. The pharmaceutical industries have an obligation to design, test and produce dosage forms that provide the customer with products having attributes of quality, purity, uniformity of content, stability, safety and physiological availability. Quality must be safeguarded for the purpose of clinical trials just as much as for the manufacturer and sales of product of proven efficacy since in the absence of adequate safeguards, the trial may well be rendered uninformative or in an extreme case be completely vitiated if the product of doubtful composition or stability were used.(37)

The state of pure standards used in analytical work is a requirement of the pharmacopoeia since it affects the outcome. A state of absolute purity is virtually unattainable but may be approached as closely as desired, provided sufficient care is taken in the manufacturing process (37). The melting points of compounds are determined to help in the identification of the compounds and their levels of purity. The melting points of the surrogate reference standards were therefore determined to confirm their identities. Refer table 3.1. The purity levels of the chosen standards (paracetamol, caffeine, aspirin, benzoic acid and phenacetin) were compared with the standards in the BP. For paracetamol, phenacetin and benzoic acid their purities were found to be 99.47%, 99.77% and 101.03% respectively. Their purity levels

were within the BP range of 99.5-101.0 for paracetamol and phenacetin and not less than 95% for benzoic acid. The percentage purity of caffeine and aspirin, were 103.02% and 97.69% respectively. That for caffeine was not within the BP range of 98.5-101.5%, although it failed the purity test it was still used because it was used as an internal standard hence its effect would cancel out. Aspirin on the other hand also fell without the BP range of 99.5-101.0%. The purities of all the standards were taken into consideration during the analysis. Refer to Table 3.10

All tablets are subjected to a test for uniformity of weight to control the extent of deviation from the average. The permitted percentage deviation decreases with increase in size of the tablet. The uniformity of weight is a standard requirement in the pharmacopoeia. This is to ensure that tablets from a batch will have uniform weight (45). From the BP, it is expected that for every twenty tablets which are taken through the weight uniformity test, not more than two should deviate by ± 5% for uncoated tablet and none must deviate by more than twice that limit. Among the nine tablets from different manufacturers analysed, they all passed the uniformity of weight test, signifying a control over the dosage of active ingredients in the tablets from the manufacturers. Refer to Tables 3.2 and Appendix 1-8.

The determination of percentage active ingredient or content is a pharmacopoeia requirement. This is to ensure that the amount of active ingredient stated on the drug falls within acceptable range. Since individual tablets may vary in weight, twenty tablets are selected at random for the determination, where twenty tablets are not available, a smaller number not less than five tablets may be used. In spite of variations of the selected tablets for

analysis, the tablet granules from which the determination was made had reasonable composition so that determination of the active ingredient content of the powdered tablets is an accurate measure of the amount of active principal present (50). The BP and USP methods were used in the percentage content determinations of the nine samples. With the USP method all the samples fell within the range of 90-110% making them pass the percentage content. Refer table 3.16. All tablets with the exception of GR and MG samples fell within the BP range of 95-105%. Refer table 3.18.

4.1.2 Method Development

The High Performance Liquid Chromatography (HPLC) method was developed using an internal standard so as to minimize errors due to sample preparation, injection of sample, detector responds and power fluctuations. In this method development, surrogate reference standards were chosen based on their structures and solubility properties. The structures were taken into consideration because theoretically, compounds with similar chemical structures are expected to have similar elution properties (1). The solubility property of the compounds was important because HPLC deals solely with liquid, that is, every sample has to be in the liquid state before the analysis can be made possible. There was therefore the need to select a solvent in which the selected compounds could easily dissolve. Here a compound may have a similar structure as that of the analyte but may not be selected because it does not dissolve in the chosen solvent. The choice of different solvents for different compounds for analysis may lead to difficulties in the selection of a mobile phase. The first mobile phase tried was methanol, water and acetic acid mixture in the ratio 28: 69:3. The analyte was paracetamol while caffeine was the internal standard. Their retention times were 4 and 7 minutes

respectively. The retention times of the surrogate standards (aspirin, benzoic acid and phenacetin) were beyond 20 minutes with tailing peaks. This was not convenient because of long analysis time which would lead to poor quantitation as a result of poor resolution of peaks. The mobile phase was then changed to methanol and water mixture in the ratio1:1. Unfortunately poor resolution was obtained because all the drugs had almost the same retention time. The ideal mobile phase was found when methanol and 2.5 % glacial acetic acid were combined in the ratio of 40:60. Emphasis was placed on shortening run time, resolution of key components, optimizing selectivity, and understanding potential interferences by reaction components. The chosen method was highly selective for all the surrogate reference standards. Refer fig. 3.2-4

Retention times of the surrogate standards were determined and found to range between 3.025 – 11.729 minutes. This range is a reasonably good running time for analytical purposes. Refer to Table 3.12 and Fig 3.2-4.

Since the work was purely instrumental analysis, analytical performance factors such as reproducibility, LOD, LOQ, precision and accuracy were evaluated. This was required to demonstrate that the developed method was suitable for intended use. Ten injections of one sample solution were made to evaluate the performance of the analytical procedure. This type of precision study is known as instrument precision or injection repeatability (48). The instrument precision (RSD) was found to be 1.51%, 1.96% and 3.05% when phenacetin, benzoic acid and aspirin were used respectively (Table 3.13). Comparing these to the (RSD) standard value (2 %), only aspirin value which was higher than the standard. Therefore any

effort to improve this method would be to improve the precision of the aspirin method. The RSD of the replicates provides the analysis variation or how precise the test method was. The mean of the replicates, expressed as percentage label claim, indicates how accurate the test method was (47).

With UV detectors, it is difficult to assure the detection precision of low level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer. At low levels, assurance is needed that the detection and quantitation limits are achievable with the test method each time. The detection limit was estimated early in the method development-validation process (49). Refer table 3.11

The concentrations were varied to ascertain its effect on the constant K. Refer fig 3.1 It can be deduced that there was no relation between the concentration and the constant K. This means change in concentration has no effect on the constant K. The statistical analysis also confirmed this by the p-value being insignificant compared with the standard p-value, meaning that, the constant obtained could be used irrespective of the concentration prepared for a given assay of paracetamol tablet. Refer to Table 3.21

After all these were done to obtain the constant, nine samples of paracetamol bought from nine manufacturers were analysed, both with the standard methods and the developed method. The standard methods used were the BP method (which was UV analysis) and the USP method, (which was HPLC), these were then compared with the developed HPLC method. It was observed that the difference between the BP method and developed method, and between the USP method and developed method using the aspirin, benzoic acid and

phenacetin as surrogate reference standards at 99% confidence limit were not significant. (Refer to Tables 3.22 & 23).

4.2 Conclusion

The maximum retention times for each standard used during the analysis were 10 minute for aspirin and 15 minutes each for phenacetin and benzoic. This is very convenient for analytical work using High Performance Liquid Chromatography (HPLC). The constant K, when the pure standards were used was: aspirin 18.23 ± 0.048, phenacetin 1.15 ± 0.051 and benzoic acid 11.66 ± 0.251. The concentration of the pure paracetamol powder was observed to have no effect on the constant K.

The results presented in the study suggest that the surrogate reference standard can be used for the analysis of paracetamol without the use of pure paracetamol powder as reference. Hence the developed method can replace the standard method in the absence of a pure standard for analysis.

4.3 Recommendations

1. This method was only used in the analysis of paracetamol tablets; it should also be tried with the syrups.
2. Use of test method in analysis of a combination two drugs like paracetamol-caffeine, aspirin-caffeine and paracetamol-aspirin or a combination of all three drugs should be explored.
3. The test method should be tried on other drugs and validated.

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APPENDIX

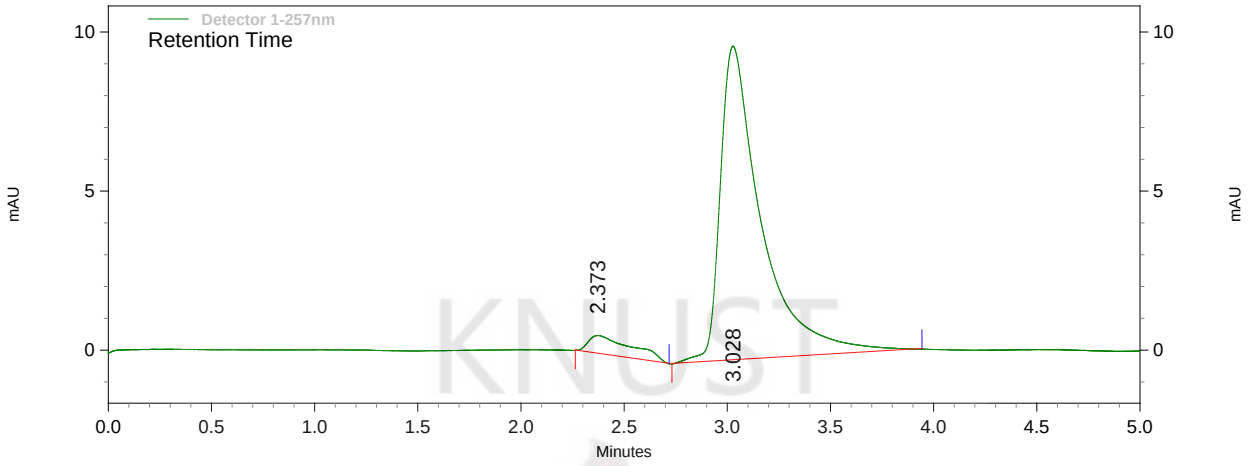

Fig A.1 Paracetamol standard

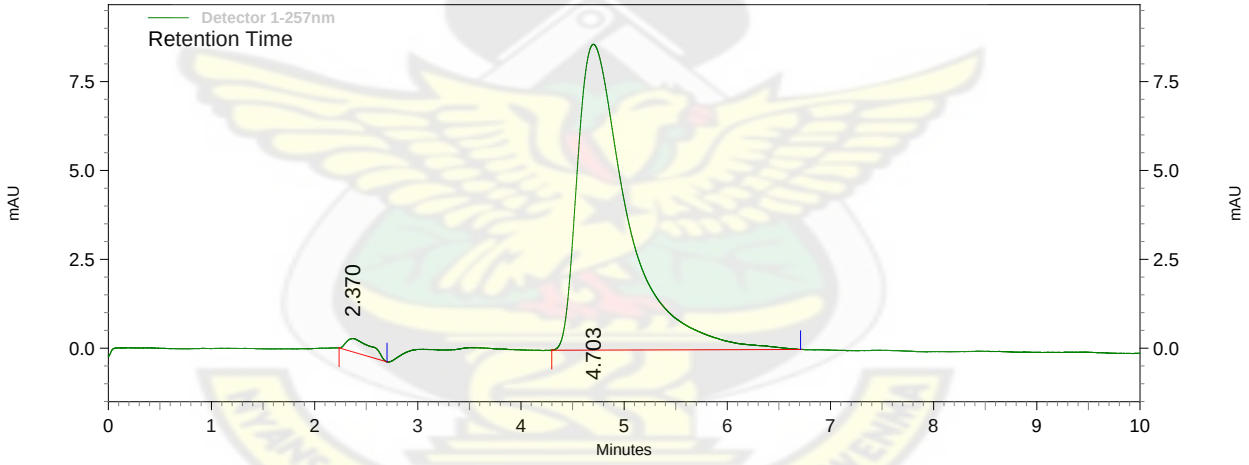

Fig A.2Caffeine standard

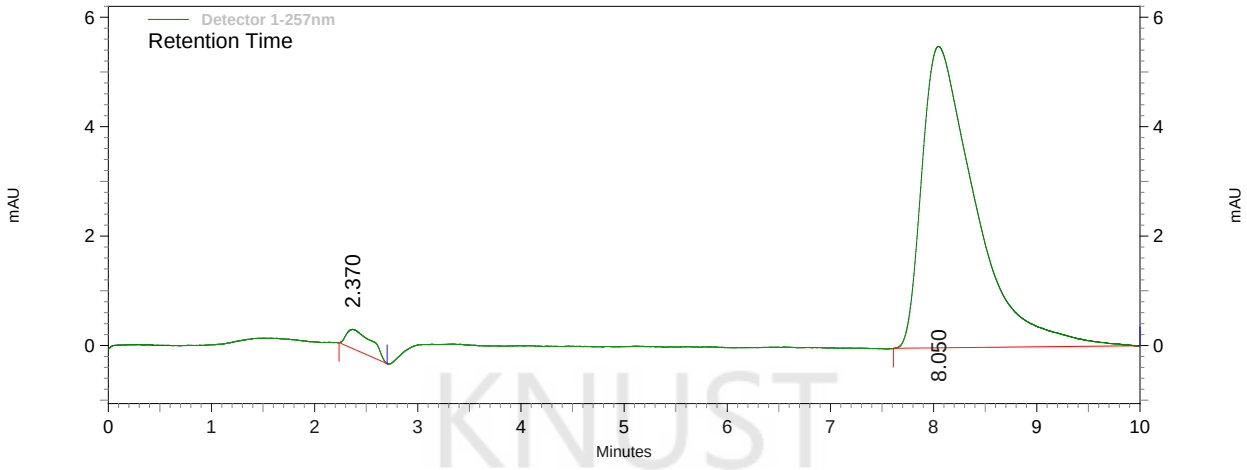

Fig A.3 Aspirin standard

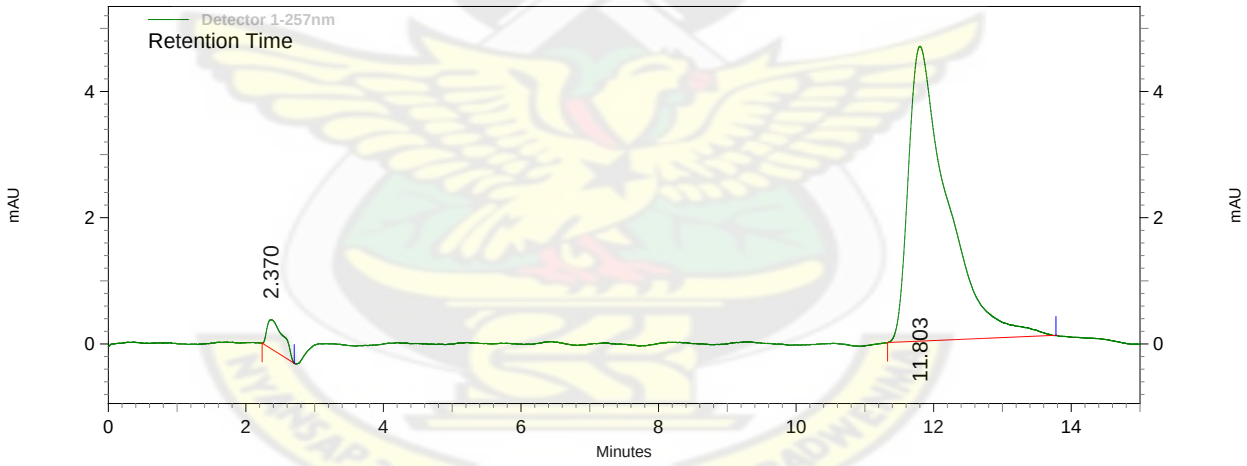

Fig A.4 Benzoic acid standard

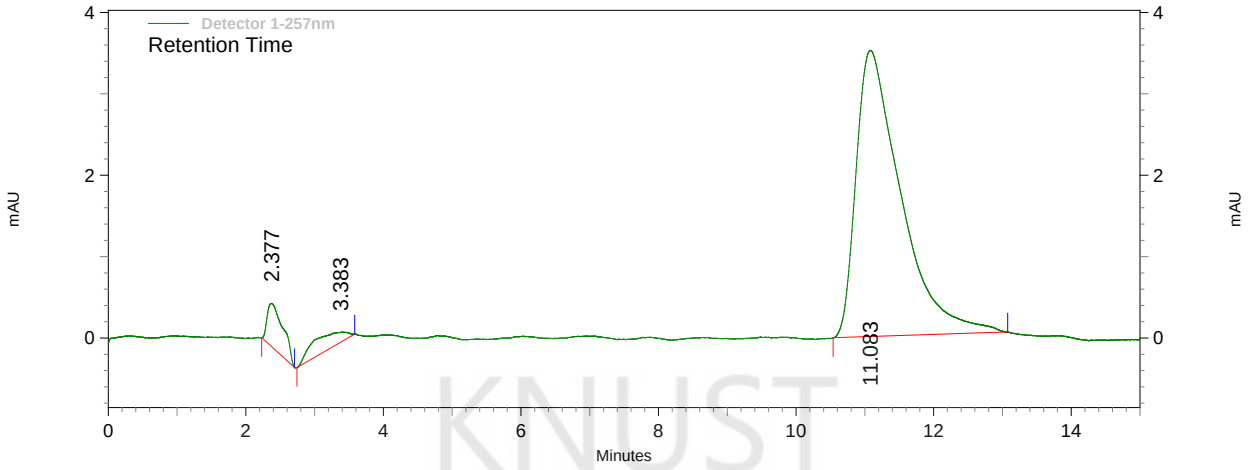

Fig A.5 Phenacetin standard

Table A.1 Percentage deviation of tablets; *DN*

Number	individual weight	Deviation	% deviation
1	0.5627	-0.0217	-4.0110
2	0.5523	-0.0113	-2.0887
3	0.5509	-0.0099	-1.8299
4	0.5739	-0.0329	-6.0813
5	0.5721	-0.0311	-5.7486
6	0.5709	-0.0299	-5.5268
7	0.5713	-0.0303	-5.6007
8	0.5585	-0.0175	-3.2347
9	0.5582	-0.0172	-3.1793
10	0.5822	-0.0412	-7.6155
11	0.5729	-0.0319	-5.8965
12	0.5805	-0.0395	-7.3013
13	0.5745	-0.0335	-6.1922
14	0.5640	-0.0230	-4.2514
15	0.5805	-0.0395	-7.3013
16	0.5670	-0.0260	-4.8059
17	0.5785	-0.0375	-6.9316
18	0.5702	-0.0292	-5.3974
19	0.5776	-0.0366	-6.7653
20	0.5775	-0.0365	-6.7468

Table A.2 Percentage deviation of tablets; *EC*

Number	individual wt	Deviation	% deviation
1	0.5600	0.0013	0.2316
2	0.5641	-0.0028	-0.4988
3	0.5626	-0.0013	-0.2316
4	0.5708	-0.0095	-1.6925
5	0.5608	0.0005	0.0891
6	0.5574	0.0039	0.6948
7	0.5709	-0.0096	-1.7103
8	0.5663	-0.0050	-0.8908
9	0.5639	-0.0026	-0.4632
10	0.5614	-1E-04	-0.0178
11	0.5602	0.0011	0.1960
12	0.5720	-0.0107	-1.9063
13	0.5649	-0.0036	-0.6413
14	0.5668	-0.0055	-0.9799
15	0.5667	-0.0054	-0.9621
16	0.5689	-0.0076	-1.3540
17	0.5662	-0.0049	-0.8730
18	0.5595	0.0018	0.3207
19	0.5605	0.0008	0.1425
20	0.5637	-0.0024	-0.4276

Table A.3 Percentage deviation of tablets; *ET*

Number	individual wt	Deviation	% deviation
1	0.6032	0.0094	1.5344
2	0.6046	0.0080	1.3059
3	0.6173	-0.0047	-0.7672
4	0.6011	0.0115	1.8772
5	0.614	-0.0014	-0.2285
6	0.5723	0.0403	6.5785
7	0.6204	-0.0078	-1.2732
8	0.6124	0.0002	0.0326
9	0.6053	0.0073	1.1916
10	0.6244	-0.0118	-1.9262
11	0.5808	0.0318	5.1910
12	0.5983	0.0143	2.3343
13	0.6017	0.0109	1.7793
14	0.6111	0.0015	0.2449
15	0.6066	0.006	0.9794
16	0.6028	0.0098	1.5997
17	0.6299	-0.0173	-2.8240
18	0.604	0.0086	1.4039
19	0.6066	0.006	0.9794
20	0.6031	0.0095	1.5508

Table A.4 Percentage deviation of tablets; *GR*

Number	individual wt	Deviation	% deviation
1	0.5610	0.0110	1.9231
2	0.5613	0.0107	1.8706
3	0.5686	0.0034	0.5944
4	0.5753	-0.0033	-0.5769
5	0.5724	-0.0004	-0.0699
6	0.5608	0.0112	1.9580
7	0.5583	0.0137	2.3951
8	0.5725	-0.0005	-0.0874
9	0.5576	0.0144	2.5175
10	0.5808	-0.0088	-1.5385
11	0.5716	0.0004	0.0699
12	0.5749	-0.0029	-0.5070
13	0.5572	0.0148	2.5874
14	0.5731	-0.0011	-0.1923
15	0.5671	0.0049	0.8566
16	0.5734	-0.0014	-0.2448
17	0.5730	-0.0010	-0.1748
18	0.5677	0.0043	0.7517
19	0.5726	-0.0006	-0.1049
20	0.5582	0.0138	2.4126

Table A.5 Percentage deviation of tablets; *KP*

Number	individual wt	deviation	% deviation
1	0.5334	0.0166	3.0182
2	0.5483	0.0017	0.3091
3	0.5476	0.0024	0.4364
4	0.5517	-0.0017	-0.3091
5	0.5543	-0.0043	-0.7818
6	0.5433	0.0067	1.2182
7	0.5603	-0.0103	-1.8727
8	0.5411	0.0089	1.6182
9	0.5526	-0.0026	-0.4727
10	0.5455	0.0045	0.8182
11	0.5523	-0.0023	-0.4182
12	0.5437	0.0063	1.1455
13	0.5463	0.0037	0.6727
14	0.5617	-0.0117	-2.1273
15	0.5480	0.0020	0.3636
16	0.5573	-0.0073	-1.3272
17	0.5530	-0.003	-0.5455
18	0.5552	-0.0052	-0.9455
19	0.5497	0.0003	0.0545
20	0.5523	-0.0023	-0.4182

Table A.6 Percentage deviation of tablets; *LP*

Number	individual wt	deviation	% deviation
1	0.5975	-0.0035	-0.5892
2	0.5726	0.0214	3.6027
3	0.6570	-0.0630	-10.6060
4	0.5775	0.0165	2.7778
5	0.5928	0.0012	0.2020
6	0.5998	-0.0058	-0.9764
7	0.5997	-0.0057	-0.9596
8	0.5917	0.0023	0.3872
9	0.5980	-0.0040	-0.6734
10	0.5932	0.0008	0.1347
11	0.6144	-0.0204	-3.4343
12	0.5868	0.0072	1.2121
13	0.6032	-0.0092	-1.5488
14	0.6075	-0.0135	-2.2727
15	0.5942	-0.0002	-0.0337
16	0.6120	-0.0180	-3.0303
17	0.5733	0.0207	3.4848
18	0.5840	0.0100	1.6835
19	0.5921	0.0019	0.3199
20	0.5773	0.0167	2.8114

Table A.7 Percentage deviation of tablets; *MG*

Number	individual wt	deviation	% deviation
1	0.5797	-0.0034	-0.5900
2	0.5765	-0.0002	-0.0347
3	0.5642	0.0121	2.0996
4	0.5764	-1E-04	-0.0174
5	0.5735	0.0028	0.4859
6	0.5700	0.0063	1.0932
7	0.5959	-0.0196	-3.4010
8	0.5835	-0.0072	-1.2493
9	0.5651	0.0112	1.9434
10	0.5907	-0.0144	-2.4987
11	0.5798	-0.0035	-0.6073
12	0.5717	0.0046	0.7982
13	0.5729	0.0034	0.5900
14	0.5732	0.0031	0.5379
15	0.5762	1E-04	0.0174
16	0.5810	-0.0047	-0.8155
17	0.5723	0.0040	0.6941
18	0.5695	0.0068	1.1799
19	0.5723	0.0040	0.6941
20	0.5805	-0.0042	-0.7288

Table A.8 Percentage deviation of tablets;*PR*

Number	individual wt	deviation	% deviation
1	0.5523	0.0019	0.3428
2	0.5549	-0.0007	-0.1263
3	0.5542	0	0
4	0.5491	0.0051	0.9202
5	0.5529	0.0013	0.2346
6	0.5492	0.0050	0.9022
7	0.5595	-0.0053	-0.9563
8	0.5496	0.0046	0.8300
9	0.5574	-0.0032	-0.5774
10	0.5506	0.0036	0.6496
11	0.5566	-0.0024	-0.4331
12	0.5551	-0.0009	-0.1624
13	0.5523	0.0019	0.3428
14	0.5490	0.0052	0.9383
15	0.5545	-0.0003	-0.0541
16	0.5562	-0.0020	-0.3609
17	0.5612	-0.0070	-1.2631
18	0.5482	0.0060	1.0826
19	0.5572	-0.0030	-0.5413
20	0.5608	-0.0066	-1.1909

Table A.9 Determination of K using aspirin as the standard at paracetamol concentration of 0.0003

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration of aspirin	concentration of paracetamol	K
200423	107776	300640	0.666654	0.3584886	0.009767	0.0002984	17.60098
206341	107814	306484	0.673252	0.3517769	0.009767	0.0002984	17.1022
198689	108542	309383	0.64221	0.3508338	0.009767	0.0002984	17.88078
203842	109881	301152	0.676874	0.3648689	0.009767	0.0002984	17.64377
206573	108997	313323	0.659297	0.3478742	0.009767	0.0002984	17.27044
180160	107907	303778	0.593065	0.3552166	0.009767	0.0002984	19.6044
185800	105097	307662	0.603909	0.3415989	0.009767	0.0002984	18.51429
186555	105821	307886	0.605922	0.3437019	0.009767	0.0002984	18.56638
185377	106329	302884	0.61204	0.3510552	0.009767	0.0002984	18.77406
179606	107904	306528	0.585937	0.35202	0.009767	0.0002984	19.66433

Table A.10 Determination of K using aspirin as the standard at paracetamol concentration of 0.0004

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration of aspirin	concentration of paracetamol	K
182371	142846	288811	0.631454	0.4946003	0.009767	0.0003979	19.22647
185873	139414	291159	0.63839	0.4788243	0.009767	0.0003979	18.411
174684	140011	286004	0.610775	0.4895421	0.009767	0.0003979	19.67416
176402	136975	283956	0.62123	0.4823811	0.009767	0.0003979	19.06009
187352	139660	290994	0.643835	0.4799412	0.009767	0.0003979	18.29789
200570	141900	287325	0.69806	0.4938658	0.009767	0.0003979	17.36615
200267	140384	288645	0.693818	0.4863552	0.009767	0.0003979	17.20662
200210	145655	291254	0.687407	0.5000961	0.009767	0.0003979	17.85776
198592	141239	285869	0.694696	0.494069	0.009767	0.0003979	17.45742
198240	142392	277700	0.713864	0.5127548	0.009767	0.0003979	17.63119

Table A.11 Determination of K using aspirin as the standard at paracetamol concentration of 0.0005

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	concentration of paracetamol	K
189923	175781	276786	0.686173	0.63507909	0.009767	0.0004974	18.17397
197076	171294	277651	0.709798	0.61693997	0.009767	0.0004974	17.06726
194799	173252	270188	0.720976	0.64122759	0.009767	0.0004974	17.46413
193826	171387	272799	0.710508	0.62825377	0.009767	0.0004974	17.36286
195658	176896	275563	0.71003	0.64194395	0.009767	0.0004974	17.75317
185660	177059	279165	0.665055	0.63424498	0.009767	0.0004974	18.72643
181159	170540	278812	0.649753	0.61166664	0.009767	0.0004974	18.4851
180383	172183	277135	0.650885	0.62129648	0.009767	0.0004974	18.74347
179059	172433	274865	0.651443	0.62733706	0.009767	0.0004974	18.90948
177551	173334	275662	0.64409	0.62879178	0.009767	0.0004974	19.16973

Table A.11 Determination of K using aspirin as the standard at paracetamol concentration of 0.0006

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	concentration of paracetamol	K
182106	214680	278600	0.653647	0.77056712	0.009767	0.0005968	19.293
175855	216267	286283	0.61427	0.75543081	0.009767	0.0005968	20.12648
178340	212371	282774	0.63068	0.75102732	0.009767	0.0005968	19.48852
181479	207801	282849	0.641611	0.73467115	0.009767	0.0005968	18.73931
180183	209934	282401	0.63804	0.74338972	0.009767	0.0005968	19.06783
193866	205084	279320	0.694064	0.73422598	0.009767	0.0005968	17.31261
194871	203994	278139	0.700625	0.73342465	0.009767	0.0005968	17.13178
197624	203710	270392	0.73088	0.75338767	0.009767	0.0005968	16.86961
194127	206059	280197	0.692823	0.73540759	0.009767	0.0005968	17.37153
204129	205796	274291	0.744206	0.75028346	0.009767	0.0005968	16.49926

Table A.12 Determination of K using aspirin as the standard at paracetamol concentration of 0.0007

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	concentration of paracetamol	K
195688	240445	279264	0.700728	0.86099533	0.009767	0.0006963	17.2352
194150	238512	281893	0.688737	0.84610828	0.009767	0.0006963	17.23208
193828	237237	285729	0.678363	0.83028674	0.009767	0.0006963	17.16843
194685	245682	287572	0.676996	0.85433213	0.009767	0.0006963	17.70132
197208	243435	288483	0.683604	0.84384522	0.009767	0.0006963	17.31503
181175	246116	289017	0.626866	0.85156236	0.009767	0.0006963	19.05489
179205	245344	285872	0.626871	0.85823026	0.009767	0.0006963	19.20393
178711	240564	286168	0.624497	0.84063907	0.009767	0.0006963	18.88183
180294	256677	286314	0.629707	0.89648777	0.009767	0.0006963	19.96965
179205	245344	285872	0.626871	0.85823026	0.009767	0.0006963	19.20393

Table A.13 Determination of K using benzoic acid as the standard at paracetamol concentration of 0.0002.

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration of benzoic acid	concentration of paracetamol	K
192756	75641	282384	0.68260241	0.26786574	0.006067	0.0001989	11.96984614
203031	76516	281859	0.72032825	0.27146907	0.006067	0.0001989	11.49553311
197994	72746	286806	0.69034121	0.25364183	0.006067	0.0001989	11.20717879
186621	72843	280273	0.66585436	0.25990017	0.006067	0.0001989	11.90601768
184152	73807	282715	0.65136975	0.26106503	0.006067	0.0001989	12.22532251
192088	72690	279439	0.68740584	0.26012833	0.006067	0.0001989	11.54286579
194597	75350	280673	0.69332283	0.26846188	0.006067	0.0001989	11.81099071
188194	72749	278072	0.67678155	0.26161929	0.006067	0.0001989	11.79126681
194926	73974	283098	0.68854602	0.26130174	0.006067	0.0001989	11.57573414
186675	73063	283545	0.65836111	0.25767691	0.006067	0.0001989	11.93852168

Table A.14 Determination of K using benzoic acid as the standard at paracetamol concentration of 0.0003.

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration of benzoic acid	concentration of paracetamol	K
186199	107903	304720	0.61104949	0.35410541	0.006067	0.0002984	11.78233459
190541	110370	309403	0.61583436	0.35671923	0.006067	0.0002984	11.77708416
187280	110417	304724	0.61458894	0.36235085	0.006067	0.0002984	11.9872543
196948	108355	306859	0.64181921	0.35311006	0.006067	0.0002984	11.18594186
194057	107214	305211	0.6358126	0.3512783	0.006067	0.0002984	11.23304147
190588	113012	306786	0.62124086	0.36837405	0.006067	0.0002984	12.05602623
194687	108500	302466	0.64366573	0.358718	0.006067	0.0002984	11.33099274
189294	120719	317272	0.59663002	0.38049056	0.006067	0.0002984	12.96623704
192441	107553	303554	0.63395969	0.35431258	0.006067	0.0002984	11.36318559
191651	109286	307163	0.62393908	0.35579155	0.006067	0.0002984	11.59387509

Table A.15 Determination of K using benzoic acid as the standard at paracetamol concentration of 0.0004

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration of benzoic acid	Concentration Of paracetamol	K
192199	152304	329900	0.58259776	0.46166717	0.006067	0.0003979	12.0826
185324	135613	311135	0.59563855	0.43586546	0.006067	0.0003979	11.15757
190281	133759	302792	0.62842149	0.44175209	0.006067	0.0003979	10.71834
192968	137349	311151	0.62017477	0.44142233	0.006067	0.0003979	10.85276
190569	133739	311609	0.61156449	0.4291885	0.006067	0.0003979	10.70054
185613	136665	305060	0.60844752	0.44799384	0.006067	0.0003979	11.22662
183288	139844	312416	0.5866793	0.44762112	0.006067	0.0003979	11.63349
190884	152269	334023	0.57146963	0.45586382	0.006067	0.0003979	12.16304
190376	134605	307959	0.61818619	0.4370874	0.006067	0.0003979	10.78075
190647	139651	313125	0.60885269	0.44599122	0.006067	0.0003979	11.169

Table A.16 Determination of K using benzoic acid as the standard at paracetamol concentration of 0.0005.

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration of benzoic acid	Concentration Of paracetamol	K
186393	175261	283074	0.65846033	0.61913493	0.006067	0.0004974	11.46896
183317	179644	283775	0.64599419	0.63305083	0.006067	0.0004974	11.95303
184681	189703	303735	0.60803332	0.62456747	0.006067	0.0004974	12.52911
189261	190493	302659	0.62532751	0.6293981	0.006067	0.0004974	12.27683
195861	180525	293464	0.66741065	0.61515211	0.006067	0.0004974	11.24236
185607	185958	301453	0.61570792	0.61687228	0.006067	0.0004974	12.22049
191171	187355	304355	0.62811848	0.61558049	0.006067	0.0004974	11.95395
189501	190274	307615	0.61603303	0.61854591	0.006067	0.0004974	12.24718
196093	186367	298666	0.65656285	0.62399804	0.006067	0.0004974	11.59245
194231	187625	295484	0.6573317	0.63497516	0.006067	0.0004974	11.78258

Table A.17 Determination of K using benzoic acid as the standard at paracetamol concentration of 0.0006.

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration of benzoic acid	concentration of paracetamol	K
186148	216194	300677	0.61909624	0.71902407	0.006067	0.0005968	11.80675
186326	218183	299763	0.62157771	0.72785167	0.006067	0.0005968	11.90399
188327	215607	295416	0.63749763	0.72984199	0.006067	0.0005968	11.63846
188479	215991	295892	0.63698579	0.72996566	0.006067	0.0005968	11.64978
194865	216829	291761	0.66789256	0.74317335	0.006067	0.0005968	11.31172
184846	216129	296567	0.6232858	0.72876955	0.006067	0.0005968	11.88634
184131	217703	282859	0.65096391	0.76965202	0.006067	0.0005968	12.0194
194820	204596	275175	0.70798583	0.74351231	0.006067	0.0005968	10.67601
190370	217102	292834	0.65009528	0.74138249	0.006067	0.0005968	11.59339
184096	218543	303348	0.60688055	0.72043659	0.006067	0.0005968	12.06807

Table A.18 Determination of K using phenacetin as the standard at paracetamol concentration of 0.0002.

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration of phenacetin	concentration of paracetamol	K
165213	92576	324432	0.509238	0.28534793	0.0004989	0.0001989	1.405507
165892	82629	311611	0.532369	0.26516715	0.0004989	0.0001989	1.249355
163585	78350	311386	0.525345	0.25161696	0.0004989	0.0001989	1.201363
159292	75178	305645	0.521167	0.24596509	0.0004989	0.0001989	1.183792
159368	79236	300186	0.530898	0.26395635	0.0004989	0.0001989	1.247097
159429	79694	309717	0.514757	0.25731232	0.0004989	0.0001989	1.253825
159781	78541	308241	0.518364	0.25480387	0.0004989	0.0001989	1.232963
156824	79795	306178	0.512199	0.26061637	0.0004989	0.0001989	1.276268
166300	79806	303863	0.547286	0.2626381	0.0004989	0.0001989	1.20371
159292	75178	305645	0.521167	0.24596509	0.0004989	0.0001989	1.183792

Table A.19 Determination of K using phenacetin as the standard at paracetamol concentration of 0.0003.

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration of phenacetin	concentration of paracetamol	K
162902	107138	280601	0.580547	0.38181617	0.0004989	0.0002984	1.099593
164509	108719	275460	0.597216	0.39468162	0.0004989	0.0002984	1.104919
157666	113680	293405	0.537366	0.38745079	0.0004989	0.0002984	1.205482
158297	108860	275591	0.574391	0.39500564	0.0004989	0.0002984	1.149768
160668	106060	273302	0.587877	0.38806888	0.0004989	0.0002984	1.103664
157318	111284	275186	0.571679	0.40439557	0.0004989	0.0002984	1.182685
155956	110078	279650	0.557683	0.39362775	0.0004989	0.0002984	1.180085
164197	110339	280905	0.584529	0.39279828	0.0004989	0.0002984	1.123514
157883	110732	277450	0.56905	0.39910615	0.0004989	0.0002984	1.172607
161923	110653	277151	0.584241	0.39925167	0.0004989	0.0002984	1.142535

Table A.20 Determination of K using phenacetin as the standard at paracetamol concentration of 0.0004.

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration of phenacetin	concentration of paracetamol	K
160105	147869	278893	0.574073	0.5302	0.0004989	0.0003979	1.158009
162013	143892	273931	0.591437	0.525286	0.0004989	0.0003979	1.113593
161277	145997	274670	0.587166	0.531536	0.0004989	0.0003979	1.13504
159916	145357	281366	0.568356	0.516612	0.0004989	0.0003979	1.139682
162297	142123	278059	0.583678	0.511125	0.0004989	0.0003979	1.097977
165798	153794	292375	0.567073	0.526016	0.0004989	0.0003979	1.163053
155999	151085	290357	0.537266	0.520342	0.0004989	0.0003979	1.214337
161220	141335	272633	0.591344	0.518408	0.0004989	0.0003979	1.099184
163105	154125	293878	0.555009	0.524452	0.0004989	0.0003979	1.184801
161629	138804	273212	0.591588	0.508045	0.0004989	0.0003979	1.076768

Table A.21 Determination of K using phenacetin as the standard at paracetamol concentration of 0.0005.

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration of phenacetin	concentration of paracetamol	K
161697	183234	297765	0.543036	0.615364	0.0004989	0.0004974	1.136611
162286	185213	303906	0.534001	0.609442	0.0004989	0.0004974	1.144717
155021	186110	309678	0.500588	0.600979	0.0004989	0.0004974	1.204167
161411	185695	302519	0.533557	0.613829	0.0004989	0.0004974	1.153918
161285	182044	299597	0.53834	0.60763	0.0004989	0.0004974	1.132114
156677	173125	282243	0.555114	0.61339	0.0004989	0.0004974	1.108313
165371	173065	280771	0.588989	0.616392	0.0004989	0.0004974	1.049682
161462	170276	282641	0.571262	0.602446	0.0004989	0.0004974	1.057769
161219	175010	282235	0.571223	0.620086	0.0004989	0.0004974	1.088816
156605	176930	287140	0.545396	0.61618	0.0004989	0.0004974	1.133192

Table A.22 Determination of K using phenacetin as the standard at paracetamol concentration of 0.0006.

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration of phenacetin	concentration of paracetamol	K
140136	191657	250918	0.558493	0.76382324	0.0004989	0.0005968	1.143299
143417	192985	249386	0.57508	0.77384055	0.0004989	0.0005968	1.124884
145939	190364	249803	0.584216	0.7620565	0.0004989	0.0005968	1.090431
142387	191282	244047	0.583441	0.78379165	0.0004989	0.0005968	1.123022
145617	190623	244900	0.594598	0.77837076	0.0004989	0.0005968	1.094329
147073	193597	252596	0.582246	0.7664294	0.0004989	0.0005968	1.100399
147845	206131	273369	0.540826	0.75403941	0.0004989	0.0005968	1.165524
148499	190034	245702	0.604387	0.77343286	0.0004989	0.0005968	1.069775
144303	191659	254628	0.566721	0.75270198	0.0004989	0.0005968	1.110295
139037	200443	258235	0.538413	0.77620385	0.0004989	0.0005968	1.205161

Table A.23 Percentage content of using aspirin as the standard sample one

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	A concentration of paracetamol	N concentration of paracetamol	percentage
193775	173031	292836	0.661719	0.59088022	0.009767	0.000478395	0.0004974	96.17908
188615	175699	292099	0.645723	0.60150497	0.009767	0.000499061	0.0004974	100.33386
195299	175842	291500	0.669979	0.60323156	0.009767	0.000482373	0.0004974	96.978852
196907	185680	309364	0.63649	0.60019912	0.009767	0.000505201	0.0004974	101.56835
193115	180207	301300	0.640939	0.59809824	0.009767	0.000499938	0.0004974	100.51019
174878	171046	291390	0.600151	0.58700024	0.009767	0.000524008	0.0004974	105.34943
174584	186635	286541	0.609281	0.65133785	0.009767	0.000572729	0.0004974	115.14447
174195	178435	293880	0.592742	0.60716959	0.009767	0.000548788	0.0004974	110.33132
170041	171410	290442	0.585456	0.59016947	0.009767	0.000540061	0.0004974	108.57678
173695	173387	292232	0.594374	0.59331969	0.009767	0.000534798	0.0004974	107.51861

Table A.24 Percentage content of using aspirin as the standard sample two

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	A concentration of paracetamol	N concentration of paracetamol	percentage
190083	169411	280263	0.678231	0.60447151	0.009767	0.000477484	0.0004974	95.99592
189497	162676	280770	0.674919	0.57939239	0.009767	0.000459919	0.0004974	92.46462
190618	162487	277526	0.686847	0.58548388	0.009767	0.000456683	0.0004974	91.814052
192303	163116	280429	0.685746	0.58166595	0.009767	0.000454434	0.0004974	91.361863
193174	165969	281312	0.68669	0.58998194	0.009767	0.000460297	0.0004974	92.540693
157139	161758	281119	0.558977	0.57540757	0.009767	0.000551496	0.0004974	110.87566
156471	199427	293513	0.533097	0.67944861	0.009767	0.000682826	0.0004974	137.27914
157689	161329	280452	0.562267	0.57524639	0.009767	0.000548114	0.0004974	110.19591
155178	158448	278950	0.556293	0.56801577	0.009767	0.000547037	0.0004974	109.97933
156291	161774	277673	0.56286	0.58260616	0.009767	0.000554543	0.0004974	111.48828

Table A.25 Percentage content of using aspirin as the standard sample three

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	A concentration of paracetamol	N concentration of paracetamol	percentage
185277	174660	284322	0.651645	0.6143035	0.009767	0.000505047	0.0004974	101.53748
184008	174085	286562	0.642123	0.60749506	0.009767	0.000506856	0.000503	100.76667
182213	183292	286097	0.636892	0.64066383	0.009767	0.00053892	0.000503	107.14117
188032	185188	282436	0.665751	0.65568129	0.009767	0.000527644	0.000503	104.89948
189903	171541	283442	0.669989	0.60520671	0.009767	0.000483945	0.000503	96.211803
169524	173420	287068	0.590536	0.60410774	0.009767	0.00054806	0.000503	108.95828
168873	187236	279064	0.605141	0.67094287	0.009767	0.000594004	0.000503	118.09224
167065	172448	282918	0.590507	0.6095335	0.009767	0.00055301	0.000503	109.94233
165314	169403	286638	0.576734	0.5909998	0.009767	0.000548999	0.000503	109.14496
165916	192562	278666	0.595394	0.69101361	0.009767	0.000621788	0.000503	123.61596

Table A.26 Percentage content of using aspirin as the standard sample four

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin		N concentration of paracetamol	percentage
185249	176067	274686	0.674403	0.64097551	0.009767	0.000509193	0.0004974	102.3709
186448	167744	282244	0.660592	0.59432264	0.009767	0.000482003	0.000503	95.825591
187098	193955	303221	0.617035	0.63964897	0.009767	0.000555382	0.000503	110.41398
187592	178287	283135	0.662553	0.62968902	0.009767	0.000509173	0.000503	101.22729
188708	169483	281783	0.669693	0.60146638	0.009767	0.000481167	0.000503	95.659492
157500	207959	303265	0.519348	0.6857336	0.009767	0.000707388	0.000503	140.6337
152024	190641	275516	0.551779	0.69194167	0.009767	0.000671838	0.000503	133.56615
155481	168230	280049	0.555192	0.6007163	0.009767	0.000579677	0.000503	115.24401
154294	167989	279298	0.552435	0.60146868	0.009767	0.0005833	0.000503	115.96423
153594	169404	280802	0.546983	0.6032863	0.009767	0.000590894	0.000503	117.47397

Table A.27 Percentage content of using aspirin as the standard sample five

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	A concentration of paracetamol	N concentration of paracetamol	percentage
182178	195852	301175	0.604891	0.65029302	0.009767	0.00057596	0.000503	114.50497
179550	182427	293889	0.610945	0.62073436	0.009767	0.000544332	0.000503	108.21711
183615	184833	287036	0.639693	0.64393665	0.009767	0.000539301	0.000503	107.21699
182128	172516	285162	0.638683	0.60497542	0.009767	0.000507473	0.000503	100.88925
186373	200440	311023	0.599226	0.64445395	0.009767	0.000576185	0.000503	114.54962
165223	172265	288050	0.573591	0.59803853	0.009767	0.000558582	0.000503	111.05006
163982	173189	281713	0.582089	0.61477106	0.009767	0.000565828	0.000503	112.49063
164608	171428	290754	0.566142	0.58959808	0.009767	0.000557945	0.000503	110.92337
164407	171497	289113	0.56866	0.59318329	0.009767	0.000558852	0.000503	111.10368
166181	202965	302139	0.550015	0.67176035	0.009767	0.000654335	0.000503	130.08644

Table A.28 Percentage content of using aspirin as the standard sample six

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin		N concentration of paracetamol	percentage
182162	193248	296860	0.613629	0.65097352	0.009767	0.000568352	0.000503	112.99246
179937	167430	283900	0.633804	0.58974991	0.009767	0.000498509	0.000503	99.107163
181875	169970	283017	0.642629	0.60056463	0.009767	0.000500679	0.000503	99.538595
182400	183342	283917	0.642441	0.64575915	0.009767	0.000538514	0.000503	107.06053
182880	180754	285879	0.639711	0.63227449	0.009767	0.000529519	0.000503	105.27226
165026	206015	307871	0.536023	0.66916014	0.009767	0.000668816	0.000503	132.96542
165294	203803	300714	0.549672	0.67773034	0.009767	0.000660562	0.000503	131.32449
163486	205041	306391	0.533586	0.66921352	0.009767	0.000671924	0.000503	133.58337
162158	173535	287849	0.563344	0.60286817	0.009767	0.000573336	0.000503	113.98323
162435	208094	307837	0.527666	0.67598762	0.009767	0.000686341	0.000503	136.44957

Table A.29 Percentage content of using aspirin as the standard sample seven

Area for aspirin	Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	A concentration of paracetamol	N concentration of paracetamol	percentage
167139	168185	291689	0.573004	0.5765901	0.009767	0.0005391	0.000503	107.177
167409	168677	287664	0.58196	0.5863681	0.009767	0.000539805	0.000503	107.3172
162533	171801	298256	0.544945	0.5760186	0.009767	0.000566297	0.000503	112.5839
167881	167699	288166	0.582584	0.5819528	0.009767	0.000535167	0.000503	106.395
167131	165979	289727	0.576857	0.5728807	0.009767	0.000532055	0.000503	105.7763
179229	170213	287740	0.622885	0.5915514	0.009767	0.000508797	0.000503	101.1525
181020	198465	307568	0.588553	0.6452719	0.009767	0.000587378	0.000503	116.7749
181707	181197	289409	0.627855	0.6260932	0.009767	0.000534244	0.000503	106.2115
182193	196801	314043	0.580153	0.626669	0.009767	0.000578703	0.000503	115.0503
182462	175560	306061	0.596162	0.5736111	0.009767	0.000515482	0.000503	102.4815

Table A.30 Percentage content of using aspirin as the standard sample eight

Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin	A concentration of paracetamol	N concentration of paracetamol	Percentage
167890	284699	0.623072	0.589710536	0.009767	0.000507062	0.0004974	101.942443
167288	282341	0.630454	0.592503391	0.009767	0.000503498	0.0004974	101.225962
168822	285748	0.618507	0.590807285	0.009767	0.000511755	0.0004974	102.885935
167667	277340	0.650004	0.604553977	0.009767	0.000498287	0.0004974	100.178324
168434	283858	0.622325	0.593374152	0.009767	0.000510824	0.0004974	102.698866
203784	293758	0.507285	0.693713873	0.009767	0.000732637	0.0004974	147.293263
170213	282396	0.521704	0.60274579	0.009767	0.000618971	0.0004974	124.441382
210293	302142	0.491329	0.696007175	0.009767	0.00075893	0.0004974	152.579481
174831	293435	0.494631	0.595808271	0.009767	0.000645336	0.0004974	129.741759
169750	284693	0.521811	0.596256318	0.009767	0.000612181	0.0004974	123.076186

Table A.31 Percentage content of using aspirin as the standard sample nine

Area for paracetamol	Area for caffeine	PAR for aspirin	PAR for paracetamol	concentration aspirin		N concentration of paracetamol	Percentage
171142	271477	0.527643	0.630410679	0.009767	0.000640094	0.0004974	128.6878779
175773	273121	0.519015	0.643571897	0.009767	0.00066432	0.0004974	133.5584244
179188	273401	0.515711	0.655403601	0.009767	0.000680867	0.0004974	136.8852251
186275	281485	0.503274	0.661758175	0.009767	0.000704458	0.0004974	141.6281285
173735	269948	0.52649	0.643586913	0.009767	0.000654903	0.0004974	131.6652856
179676	271726	0.65459	0.661239631	0.009767	0.00054119	0.0004974	108.8038472
173573	267919	0.657591	0.647856255	0.009767	0.000527817	0.0004974	106.115187
176038	272411	0.651115	0.646222069	0.009767	0.000531721	0.0004974	106.9001355
175594	272765	0.642531	0.643755614	0.009767	0.000536769	0.0004974	107.9148743
205822	282089	0.621144	0.729634973	0.009767	0.000629323	0.0004974	126.5224286

Table A.32 Percentage content of using benzoic acid as the standard sample one

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
183723	139636	285588	0.6433148	0.488942	0.006067	0.000395436	0.0003979	99.38066
181210	152751	299457	0.6051286	0.510093	0.006067	0.000438575	0.0003979	110.2224
181702	137676	279602	0.6498594	0.4924	0.006067	0.000394222	0.0003979	99.07556
184127	136871	279328	0.6591785	0.490001	0.006067	0.000386755	0.0003979	97.19903
185452	145335	285447	0.6496898	0.509149	0.006067	0.000407737	0.0003979	102.4723
172527	141853	280060	0.6160358	0.506509	0.006067	0.000427783	0.0003979	107.5101
190446	144558	284073	0.6704122	0.508876	0.006067	0.000394923	0.0003979	99.25177
188663	142331	282818	0.6670827	0.50326	0.006067	0.000392514	0.0003979	98.64629
193456	144394	281138	0.6881176	0.513605	0.006067	0.000388337	0.0003979	97.59665
183723	139636	285588	0.6433148	0.488942	0.006067	0.000395436	0.0003979	99.38066

Table A.33 Percentage content of using benzoic acid as the standard sample two

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
185183	135290	285716	0.6481366	0.473512	0.006067	0.000380108	0.0003979	95.52841
191225	138102	284028	0.6732611	0.486227	0.006067	0.000375748	0.0003979	94.43289
183754	137374	276792	0.6638703	0.496308	0.006067	0.000388964	0.0003979	97.75427
181650	134522	279739	0.6493553	0.480884	0.006067	0.000385301	0.0003979	96.83356
181822	134082	283727	0.6408343	0.472574	0.006067	0.000383677	0.0003979	96.42553
186107	133678	277345	0.6710307	0.481992	0.006067	0.000373714	0.0003979	93.92154
184330	129854	271082	0.6799788	0.479021	0.006067	0.000366523	0.0003979	92.11435
181466	133609	272581	0.6657324	0.490163	0.006067	0.000383074	0.0003979	96.27387
180122	132524	272719	0.6604674	0.485936	0.006067	0.000382798	0.0003979	96.20458
180098	135933	274262	0.6566641	0.495632	0.006067	0.000392697	0.0003979	98.69247

Table A.34 Percentage content of using benzoic acid as the standard sample three

Area for benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
187471	159643	285147	0.65745387	0.5598621	0.006067	0.000443055	0.0003979	111.34835
180796	139226	275422	0.65643267	0.5055006	0.006067	0.000400658	0.0003979	100.69305
187808	144738	273244	0.68732708	0.5297024	0.006067	0.000400969	0.0003979	100.77121
185028	142744	274431	0.67422412	0.5201453	0.006067	0.000401386	0.0003979	100.87613
174280	143968	277229	0.62864996	0.5193108	0.006067	0.000429794	0.0003979	108.01558
188796	148206	275031	0.68645353	0.5388702	0.006067	0.000408427	0.0003979	102.64575
187678	144144	275156	0.68207853	0.5238628	0.006067	0.0003996	0.0003979	100.42716
187369	147351	275255	0.68071061	0.5353254	0.006067	0.000409164	0.0003979	102.83083
185970	144895	277485	0.67019839	0.5221724	0.006067	0.000405371	0.0003979	101.87755
183827	146579	278659	0.65968442	0.5260157	0.006067	0.000414863	0.0003979	104.26305

Table A.35 Percentage content of using benzoic acid as the standard sample four

Area for Benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
179765	134767	268504	0.6695059	0.501918	0.006067	0.00039005	0.0003979	98.02716
177357	134141	266795	0.6647688	0.502787	0.006067	0.000393509	0.0003979	98.89656
178202	133621	268541	0.6635933	0.497581	0.006067	0.000390125	0.0003979	98.04606
185252	136843	268502	0.6899464	0.509654	0.006067	0.000384328	0.0003979	96.589
183307	139525	264760	0.6923516	0.526987	0.006067	0.000396018	0.0003979	99.52701
184956	142549	282438	0.6548552	0.504709	0.006067	0.000400994	0.0003979	100.7775
186026	137130	270702	0.6871985	0.506572	0.006067	0.000383531	0.0003979	96.38885
180095	138682	274119	0.6569957	0.505919	0.006067	0.000400646	0.0003979	100.69
191611	142396	271828	0.704898	0.523846	0.006067	0.000386651	0.0003979	97.17294
180826	140212	270169	0.669307	0.518979	0.006067	0.000403428	0.0003979	101.3893

Table A.36 Percentage content of using benzoic acid as the standard sample five

Area for Benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
184322	152940	276319	0.66706234	0.55349071	0.006067	0.000431704	0.0003979	108.49554
187147	141699	271821	0.68849353	0.52129526	0.006067	0.000393936	0.0003979	99.003808
184388	146076	268083	0.68780191	0.54489095	0.006067	0.000412181	0.0003979	103.58913
190567	149234	269289	0.70766723	0.55417785	0.006067	0.000407438	0.0003979	102.3972
182424	147442	275436	0.66230994	0.53530403	0.006067	0.000420515	0.0003979	105.68351
159623	138991	264687	0.60306324	0.52511457	0.006067	0.000453036	0.0003979	113.85686
165454	142110	266746	0.62026797	0.53275401	0.006067	0.000446878	0.0003979	112.30921
169521	142016	269267	0.62956471	0.52741702	0.006067	0.000435869	0.0003979	109.54227
167879	156616	285704	0.58759765	0.54817573	0.006067	0.00048538	0.0003979	121.98537
165331	146352	267304	0.618513	0.54751145	0.006067	0.00046056	0.0003979	115.7477

Table A.37 Percentage content of using benzoic acid as the standard sample six

Area for Benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
167750	144754	274584	0.61092416	0.52717565	0.006067	0.000448962	0.0003979	112.83297
170535	138793	266906	0.63893281	0.52000704	0.006067	0.000423444	0.0003979	106.4197
162627	140003	270842	0.60044971	0.51691761	0.006067	0.000447906	0.0003979	112.56741
170514	140877	270703	0.62989328	0.52041167	0.006067	0.000429855	0.0003979	108.03091
154130	141640	267812	0.57551566	0.52887847	0.006067	0.000478124	0.0003979	120.16188
159623	138991	264687	0.60306324	0.52511457	0.006067	0.000453036	0.0003979	113.85686
165454	142110	266746	0.62026797	0.53275401	0.006067	0.000446878	0.0003979	112.30921
169521	142016	269267	0.62956471	0.52741702	0.006067	0.000435869	0.0003979	109.54227
167879	156616	285704	0.58759765	0.54817573	0.006067	0.00048538	0.0003979	121.98537
165331	146352	267304	0.618513	0.54751145	0.006067	0.00046056	0.0003979	115.7477

Table A.38 Percentage content of using benzoic acid as the standard sample seven

Area for Benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
189963	134233	277251	0.68516615	0.484157	0.006067	0.000367648	0.0003979	92.397083
181977	140598	283485	0.64192814	0.4959627	0.006067	0.00040198	0.0003979	101.02541
183426	136227	283357	0.64733181	0.480761	0.006067	0.000386406	0.0003979	97.111415
190378	139696	282311	0.67435559	0.4948302	0.006067	0.000381776	0.0003979	95.947839
184998	140008	284888	0.64937098	0.4914493	0.006067	0.000393757	0.0003979	98.958659
181268	137869	286526	0.63264067	0.4811745	0.006067	0.000395719	0.0003979	99.451988
191616	136720	282602	0.67804191	0.4837899	0.006067	0.000371229	0.0003979	93.297126
189272	139902	289258	0.65433627	0.4836582	0.006067	0.000384574	0.0003979	96.650819
190577	135693	281694	0.67653908	0.4817036	0.006067	0.000370449	0.0003979	93.101128
182752	150301	292656	0.62446012	0.5135757	0.006067	0.000427899	0.0003979	107.53943

Table A.39 Percentage content of using benzoic acid as the standard sample eight

Area for Benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
188079	140890	276640	0.67986914	0.5092901	0.006067	0.000389746	0.0003979	97.950768
185325	142544	273950	0.67649206	0.5203285	0.006067	0.000400181	0.0003979	100.57335
182496	145134	272998	0.66848841	0.5316303	0.006067	0.000413769	0.0003979	103.98814
180278	143135	272114	0.6625091	0.5260112	0.006067	0.00041309	0.0003979	103.81763
188053	143812	278443	0.67537342	0.5164863	0.006067	0.000397884	0.0003979	99.99605
186495	146247	282358	0.66049129	0.5179488	0.006067	0.000408001	0.0003979	102.53869
184444	145602	274082	0.67295189	0.5312352	0.006067	0.000410719	0.0003979	103.22165
187317	144051	276652	0.67708529	0.5206939	0.006067	0.000400111	0.0003979	100.55579
182676	143584	278189	0.65666148	0.5161383	0.006067	0.000408946	0.0003979	102.7762
184269	145499	274486	0.67132386	0.530078	0.006067	0.000410818	0.0003979	103.24659

Table A.40 Percentage content of using benzoic acid as the standard sample nine

Area for Benzoic acid	Area for paracetamol	Area for caffeine	PAR for benzoic acid	PAR for paracetamol	concentration benzoic acid	A concentration Of paracetamol	N concentration of paracetamol	Percentage
191518	145429	261226	0.73315061	0.5567172	0.006067	0.000395078	0.0003979	99.29089
185242	138088	258353	0.71701122	0.5344935	0.006067	0.000387845	0.0003979	97.473029
183953	141518	258404	0.7118814	0.5476618	0.006067	0.000400264	0.0003979	100.59417
187306	146695	265066	0.7066391	0.5534282	0.006067	0.000407479	0.0003979	102.40746
186101	139824	262569	0.70876989	0.5325229	0.006067	0.000390908	0.0003979	98.242861
188079	140890	276640	0.67986914	0.5092901	0.006067	0.000389746	0.0003979	97.950768
185325	142544	273950	0.67649206	0.5203285	0.006067	0.000400181	0.0003979	100.57335
182496	145134	272998	0.66848841	0.5316303	0.006067	0.000413769	0.0003979	103.98814
180278	143135	272114	0.6625091	0.5260112	0.006067	0.00041309	0.0003979	103.81763
188053	143812	278443	0.67537342	0.5164863	0.006067	0.000397884	0.0003979	99.99605

Table A.41 Percentage content of using phenacetin as the standard sample one

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
159412	142452	280427	0.5684617	0.50798247	0.0004989	0.000386193	0.0003979	97.057877
156103	141265	281451	0.5546365	0.50191685	0.0004989	0.000391093	0.0003979	98.289374
158010	146351	276200	0.5720854	0.52987328	0.0004989	0.000400284	0.0003979	100.59916
154492	142779	279671	0.5524062	0.51052487	0.0004989	0.000399407	0.0003979	100.3787
159025	143410	278399	0.5712125	0.51512398	0.0004989	0.000389737	0.0003979	97.948385
163385	142938	280200	0.5831014	0.51012848	0.0004989	0.000378088	0.0003979	95.020818
161238	141551	279578	0.5767192	0.50630236	0.0004989	0.000379405	0.0003979	95.351776
166668	141314	279062	0.5972436	0.50638926	0.0004989	0.000366429	0.0003979	92.090793
159762	144314	280273	0.5700228	0.51490511	0.0004989	0.000390384	0.0003979	98.111117
163892	145469	282441	0.5802699	0.51504208	0.0004989	0.000383592	0.0003979	96.404198

Table A.42 Percentage content of using phenacetin as the standard sample two

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
155776	133367	280250	0.5558466	0.47588582	0.0004989	0.000370003	0.0003979	92.988894
155535	135478	284552	0.5465961	0.47610981	0.0004989	0.000376442	0.0003979	94.607135
158520	131316	279014	0.5681435	0.47064305	0.0004989	0.000358006	0.0003979	89.973959
162870	134210	280639	0.5803541	0.47823004	0.0004989	0.000356124	0.0003979	89.500823
165014	135932	284839	0.5793238	0.47722398	0.0004989	0.000356007	0.0003979	89.471385
156189	129665	283716	0.5505118	0.45702393	0.0004989	0.000358781	0.0003979	90.16865
159836	131598	278581	0.5737505	0.47238685	0.0004989	0.000355821	0.0003979	89.424791
154729	128835	280920	0.5507938	0.45861811	0.0004989	0.000359848	0.0003979	90.436842
154085	128124	273533	0.5633141	0.46840418	0.0004989	0.000359358	0.0003979	90.313645
163259	134477	283514	0.5758411	0.47432226	0.0004989	0.000355982	0.0003979	89.465198

Table A.43 Percentage content of using phenacetin as the standard sample three

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
159846	145666	272292	0.5870389	0.53496247	0.0004989	0.000393834	0.0003979	98.978227
160608	145723	280103	0.5733891	0.52024791	0.0004989	0.000392119	0.0003979	98.547174
157327	147265	282060	0.5577785	0.52210523	0.0004989	0.000404533	0.0003979	101.66689
161546	147509	278366	0.5803367	0.52991026	0.0004989	0.00039462	0.0003979	99.175764
155242	146615	278848	0.5567262	0.52578824	0.0004989	0.000408156	0.0003979	102.57757
158997	145041	271714	0.5851631	0.53380025	0.0004989	0.000394239	0.0003979	99.079796
157541	141951	277653	0.5674025	0.51125325	0.0004989	0.000389405	0.0003979	97.865159
162358	148894	274121	0.5922859	0.54316889	0.0004989	0.000396333	0.0003979	99.606287
155448	144741	274267	0.5667762	0.52773757	0.0004989	0.000402405	0.0003979	101.13225
159518	145079	284751	0.5602017	0.50949426	0.0004989	0.000393054	0.0003979	98.782066

Table A.44 Percentage content of using phenacetin as the standard sample four

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
158656	144378	273878	0.579294	0.5271617	0.0004989	0.00039328	0.0003979	98.83887
155991	136914	273831	0.569662	0.4999945	0.0004989	0.00037932	0.0003979	95.33043
158556	138534	278253	0.569827	0.4978706	0.0004989	0.000377599	0.0003979	94.89797
152346	139350	270700	0.562785	0.5147765	0.0004989	0.000395306	0.0003979	99.34801
163246	136412	278597	0.585957	0.4896392	0.0004989	0.000361133	0.0003979	90.75975
161246	134869	268650	0.600208	0.5020249	0.0004989	0.000361477	0.0003979	90.84613
157063	137503	270525	0.580586	0.508282	0.0004989	0.000378352	0.0003979	95.08708
155304	133759	270427	0.574292	0.4946215	0.0004989	0.000372218	0.0003979	93.54565
151254	128833	268579	0.563164	0.4796838	0.0004989	0.00036811	0.0003979	92.51316
156264	134120	269275	0.580314	0.4980782	0.0004989	0.00037093	0.0003979	93.22188

Table A.45 Percentage content of using phenacetin as the standard sample five

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
159816	139023	273073	0.58525	0.5091056	0.0004989	0.000375944	0.0003979	94.48212
152047	140914	275074	0.552749	0.5122767	0.0004989	0.000400529	0.0003979	100.6606
158188	141523	272738	0.58	0.5188973	0.0004989	0.000386643	0.0003979	97.17101
165540	139043	268110	0.617433	0.5186043	0.0004989	0.000362997	0.0003979	91.22827
159567	141262	270636	0.5896	0.5219631	0.0004989	0.000382595	0.0003979	96.15359
157469	147099	268989	0.585411	0.5468588	0.0004989	0.000403712	0.0003979	101.4607
156837	144118	271120	0.578478	0.5315654	0.0004989	0.000397125	0.0003979	99.80515
158483	145937	264337	0.599549	0.5520869	0.0004989	0.00039796	0.0003979	100.0152
156103	145916	269104	0.580084	0.542229	0.0004989	0.00040397	0.0003979	101.5254
161460	153753	270226	0.5975	0.5689793	0.0004989	0.000411544	0.0003979	103.4289

Table A.46 Percentage content of using phenacetin as the standard sample six

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
159892	141874	281675	0.567647	0.5036798	0.0004989	0.000383472	0.0003979	96.37388
156419	138686	274965	0.568869	0.5043769	0.0004989	0.000383178	0.0003979	96.30002
159736	155890	289679	0.551424	0.5381474	0.0004989	0.000421767	0.0003979	105.9983
157047	144065	277968	0.564982	0.5182791	0.0004989	0.000396448	0.0003979	99.63504
161513	145104	288681	0.559486	0.5026448	0.0004989	0.000388266	0.0003979	97.57873
154618	138732	278806	0.554572	0.4975933	0.0004989	0.00038777	0.0003979	97.45404
160567	143559	281684	0.570025	0.5096456	0.0004989	0.000386395	0.0003979	97.10853
154957	135769	281629	0.550217	0.4820846	0.0004989	0.000378658	0.0003979	95.164
159213	137681	282613	0.56336	0.4871715	0.0004989	0.000373725	0.0003979	93.92447
159892	141874	281675	0.567647	0.5036798	0.0004989	0.000383472	0.0003979	96.37388

Table A.47 Percentage content of using phenacetin as the standard sample seven

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	A concentration of paracetamol	N concentration of paracetamol	Percentage
160945	144189	278557	0.5777812	0.5176283	0.0004989	0.000387179	0.0003979	97.305612
158716	143694	285343	0.5562288	0.5035834	0.0004989	0.000391269	0.0003979	98.333426
149166	143215	290074	0.5142343	0.4937188	0.0004989	0.000414931	0.0003979	104.28021
162908	142504	284889	0.5718297	0.5002089	0.0004989	0.000378044	0.0003979	95.009688
168382	142742	280889	0.599461	0.5081794	0.0004989	0.000366364	0.0003979	92.074498
166346	139675	284802	0.584076	0.4904284	0.0004989	0.00036288	0.0003979	91.198893
155982	132805	275407	0.566369	0.4822136	0.0004989	0.000367957	0.0003979	92.474755
155745	132185	266676	0.5840233	0.4956764	0.0004989	0.000366797	0.0003979	92.1831
146730	132413	272687	0.5380895	0.485586	0.0004989	0.000390004	0.0003979	98.015544
150697	132402	276844	0.5443391	0.4782549	0.0004989	0.000379706	0.0003979	95.427421

Table A.48 Percentage content of using phenacetin as the standard sample eight

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	Actual concentration of paracetamol	Nominal concentration of paracetamol	percentage
161360	141284	296813	0.543642	0.4760034	0.0004989	0.000378403	0.0003979	95.099962
162406	148653	294949	0.550624	0.5039956	0.0004989	0.000395575	0.0003979	99.415673
168623	162346	302505	0.5574222	0.5366721	0.0004989	0.000416085	0.0003979	104.57022
159546	145793	288428	0.5531571	0.5054745	0.0004989	0.000394919	0.0003979	99.250796
160006	145326	284064	0.5632745	0.511596	0.0004989	0.000392522	0.0003979	98.648457
171845	150716	292610	0.5872834	0.5150747	0.0004989	0.000379035	0.0003979	95.258931
159211	148676	287324	0.5541166	0.5174507	0.0004989	0.000403576	0.0003979	101.42641
160056	145838	287504	0.5567088	0.5072556	0.0004989	0.000393782	0.0003979	98.965082
161510	147483	290272	0.5564092	0.5080855	0.0004989	0.000394639	0.0003979	99.180386
159312	147630	290626	0.5481684	0.5079724	0.0004989	0.000400482	0.0003979	100.64898
161360	141284	296813	0.543642	0.4760034	0.0004989	0.000378403	0.0003979	95.099962

Table A.49 Percentage content of using phenacetin as the standard sample nine

Area for phenacetin	Area for paracetamol	Area for caffeine	PAR for phenacetin	PAR for paracetamol	concentration phenacetin	Actual concentration of paracetamol	Nominal concentration of paracetamol	percentage
154360	144753	275703	0.5598778	0.5250324	0.0004989	0.000405275	0.0003979	101.85352
158438	142887	278570	0.5687547	0.5129303	0.0004989	0.000389754	0.0003979	97.952745
154419	147911	273169	0.5652874	0.5414633	0.0004989	0.000413959	0.0003979	104.03584
154437	144820	277938	0.5556527	0.5210515	0.0004989	0.000405261	0.0003979	101.84986
161950	147231	277029	0.5845958	0.5314642	0.0004989	0.000392894	0.0003979	98.741917
160060	149814	281795	0.5680016	0.5316418	0.0004989	0.000404508	0.0003979	101.66064
148680	146634	280246	0.5305339	0.5232332	0.0004989	0.000426225	0.0003979	107.11872
147496	152602	281616	0.5237487	0.5418797	0.0004989	0.000447133	0.0003979	112.37332
165456	147563	277581	0.5960639	0.5316034	0.0004989	0.000385436	0.0003979	96.867525
165214	148400	285423	0.5788391	0.5199301	0.0004989	0.00038819	0.0003979	97.559665
154360	144753	275703	0.5598778	0.5250324	0.0004989	0.000405275	0.0003979	101.85352

Table A. 50 Validation of developed method

Aspirin			Benzoic acid			Phenacetin		
PAR for Paracetamol tablet	PAR for pure paracetamol powder	Percentage content	PAR for Paracetamol tablet	PAR for pure paracetamol powder	Percentage content	PAR for Paracetamol tablet	PAR for pure paracetamol powder	Percentage content
0.601505	0.628678	95.7	0.510093	0.444445	114.8	0.501917	0.521202	96.3
0.603232	0.628678	96.0	0.4924	0.444445	110.8	0.529873	0.521202	101.7
0.600199	0.628678	95.5	0.490001	0.444445	110.3	0.510525	0.521202	98.0
0.598098	0.628678	95.1	0.509149	0.444445	114.6	0.515124	0.521202	98.8
0.587	0.628678	93.4	0.506509	0.444445	114.0	0.510128	0.521202	97.9
0.651338	0.628678	103.6	0.508876	0.444445	114.5	0.506302	0.521202	97.1
0.60717	0.628678	96.6	0.50326	0.444445	113.2	0.506389	0.521202	97.2
0.590169	0.628678	93.9	0.513605	0.444445	115.6	0.514905	0.521202	98.8

Table A.51 Statistical analysis for varying K

		Sum of Squares	df	Mean Square	F	Sig.
VAR00007	Between Groups	.092	4	.023	.023	.999
	Within Groups	44.092	45	.980		
	Total	44.184	49			
VAR00008	Between Groups	2.525	4	.631	3.159	.023
	Within Groups	8.994	45	.200		
	Total	11.519	49			
VAR00012	Between Groups	.005	3	.002	.870	.466
	Within Groups	.063	36	.002		
	Total	.068	39			

Table A.52 Statistical analysis for developed method and standard methods

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	99% Confidence Interval of the Difference Lower	Upper			
ASPIRIN - BP	2.9152	7.02032	2.34011	-4.9368	10.7671	1.246	8	.248
BENZOIC ACID - BP	-1.6700	4.66772	1.55591	-6.8907	3.5506	-1.073	8	.314
BP - PHENACITIN	5.4589	2.15690	.71897	-3.0465	7.8713	7.593	8	.348
USP - ASPIRIN	-7.7952	8.27184	2.75728	-17.0469	1.4566	-2.827	8	.022
USP - BENZOIC ACID	-3.2100	7.09504	2.36501	-11.1455	4.7256	-1.357	8	.212
USP - PHENACITIN	.5789	5.26122	1.75374	-5.3056	6.4634	.330	8	.750