SOLUBILITY METHOD OF ANALYSIS APPLIED TO DOSAGE FORMS OF CHLORAMPHENICOL

BY

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Submitted in partial fulfilment of the requirements for the degree of Master of Science in Pharmacy (Pharmaceutical Chemistry) in the School of Pharmacy of the American University of Beirut.

Beirut, Lebanon

1968

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Date of thesis representation: Aug. 15, 1968

ACKNOWLEDGEMENT

The author wishes to extend his sincere appreciation and thanks to the Jordanian Government Ministry of Health, in giving the chance for a higher education.

Thanks and appreciations are also due to the World Health Organization for the finacial support of education and maintenance during the two years period of advamced study at the School of Pharmacy of the American University of Beirut.

The author wishes to extend further his thanks to the following firms for their kind cooperation and assistance in supplying pure chemicals needed for the experimental work:

Parke. Davis and Co.

Roussel Corp.

Scierlabs.

ABSTRACT

The proposed analytical solubility approach for chloramphenicol in many pharmaceutical formulations proved to be efficient. Its main objective is to eliminate interfering impurities and analyze the pure chloramphenicol residue spectrophotometrically.

The following dosage forms were thoroughly investigated:

1- Chloramphenicol in capsule formulation whether simple, vitaminized or mixed with other antibiotics or antibacterials.

2- Chloramphenicol in oleogenous bases, such as ointments, suppositories ...etc.

3- Chloramphenicol in parenterals such as chloramphenicol sodium succinate injections.

Chloramphenicol in oral suspension or drops for topical use were also investigated but not as thoroughly as the above three items.

The results obtained from the above analysis were comparable to other dependable methods for chloramphenicol determination, e.g chromatography. This method proved to afford the seperation of all interfering impurities as well as degradation products of chloramphenicol which could have coexisted with chloramphenicol samples.

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INTRODUCTION

The conventional phase-solubility method is seldom applied to the analysis of pharmaceutical preparations. This method was first introduced in 1929 primarily for the analysis and purification of some amino-acids (1). Had it not for the fact that the method involves tedious and time consuming techniques it could have, long ago, served as a powerful analytical tool in the hands of chemists.

This work is a modification of the conventional method; so simplified, to make it better fit as a quick and efficient analytical means for the analysis of some pharmaceutical preparations. The fundamental principle involved in any solubility method of analysis can be concisely stated as follows: 'The equilibrium solubility of a chemical substance in a given solvent, at given temperature and pressure, is a quantity characteristic of the substance, and may, therefore, be utilized as a criterion of identity and purity.'

The standard phase-solubility method of analysis as practised at present was introduced by Northorp and Kunitz (1). Moore (2) and Herriot (3) applied the method to protein preparations.

Kunitz (1) applied it successfully to binary mixtures, while Thorp (4) extended it further to multicomponent mixtures. Reeve and Adams (5), Stenger (6) and Bennett (7) introduced different modifications of the method. Webb (8), Madder (9), and Higuchi and Connors (10) have reviewed the method extensively. Because of the inherent accuracy and dependability of the method, the Pharmacopeial Boards of U.S.P. (11), B.P. (12) and I.P. (13) adopted the phase-solubility technique as the official method of analysis for mecamylamine HCl.

The extensive use and increased prevalance of antibiotics and antibiotic mixtures necessitates the development of quick analytical procedures. At present, physicochemical methods are finding increased acceptance over the bioassay methods for the routine analysis of several antibiotics. The present work is mainly elaborated to add to the inherent precision of solubility analysis, the ease and quickness of chemical methods for the assay of some chloramphenicol dosage forms.

Solubility analytical techniques, ideally considered, are based on the premise that the solubility of components in a given solvent should be additive; or in other words, the solubility

of each component in a given solvent should be uninfluenced by the presence of the other substances. Most systems do not exhibit this ideal solubility behavior and hence, inaccuracies are introduced into the analytical results. This solution interaction, responsible for the nonadditive solubility behavior of solutes is an unfortunate and a disagreeable phenomenon (10).

Often, the effect of minute impurities on the solubility of the major component is negligible. Furthermore, the effect of appreciably large amounts of interfering substances, on the solubility of the major component may be rendered to be negligible by the choice of an appropriate solvent so modified as to attain the ideal criteria. This effect depends on the physicochemical nature of the components as well as those of solvents used. The following are illustrative experimental results obtained to confirm the above statements:

Example 1:

Tarpley and Yudis (14) gave the following table for the solubility of three samples of cortisone acetate in benzene

at 25°C;

Per cent impurity	0.8	4.5	14
Solubility in mg/ml	1.36	1.32	1.3

Example 2:

Solubility of chloramphenicol in water was determined in the presence of one of its normal soluble excipients used in the preparation of capsules e.g. lactose; at 25°C. The results obtained are tabulated as follows:

Weight of lactose in mg/ml H_2O	0	1	2	4	8
Solubility of chloramphenicol in mg/ml H_2O	3.83	3.82	3.81	3.82	3.83

FUNDAMENTAL PRINCIPLES OF CONVENTIONAL PHASE-SOLUBILITY ANALYSIS

Theoretical

The mechanism of solubility involves severing of the forces that hold the ions or molecules of the solute together, the separation of the molecules of the solvent to create a space into which the solute can be filled, and the ultimate response of solute and solvent to whatever forces of interaction that may exist between them.

In the process of solution there is an energy exchange in any system exposed to solvent-solute interaction. This energy appears in the form of temperature change of the system. The quantity of heat evolved or absorbed when one mole of solute is dissolved in sufficient solvent so that further dilution produces no additional heat effect is known as heat of solution. This quantity is either negative, if heat is absorbed or positive if heat is given off. A large negative value is suggestive of little solvent-solute interaction while a large positive value implies

extensive solute-solvent interaction. The larger the positive amount of heat of solution, the greater the solubility of a substance in a certain solvent and the larger the negative amount of heat of solution, the lower the solubility. This is based on that the released heat is an energy supplier required for the fundamental process of separating the molecules or ions of solute one from the other. Since the process of solubility is usually followed by a net rise or a net fall of the temperature of the system a constant temperature bath is required to act as a heat reservoir to establish an equilibrium condition of isothermal heat transfers.

The solubility of a solute in a given solvent depends on the temperature, the nature of the solute and the nature of the solvent. Substances vary greatly in the rate at which they dissolve; some are capable of producing saturation quickly; others require serval hours or days for attaining saturation. The time required to attain equilibrium saturation depends upon a number of factors:

(1) The ease with which solute is "wetted" by the solvent.

(2) The quantity of solute per unit solvent.

(3) The size of the solute particles.

(4) The rate of stirring.

Therefore, this time varies with the conditions and must be determined experimentally in each case. It can be shorten by using a "wetting agent", if the solute is not properly wetted by the solvent; or exposing more solute surface to solvent action. This can be accomplished by either: adding large quantity of solute, powdering large particles into fine powder and finally increasing the rate of stirring or shaking.

In general, slightly soluble electrolytes have a constant solubility product and, therefore, common ion effect will drop their solubility in water. But, in the case of organic solutes, the inorganic electrolytes usually cause salting-out; while salts of various organic acids or organic substituted ammonium salts cause salting-in (15). No explanation is given for this phenomenon.

In the case of nitrogenous bases, organic acids or amphoteric compounds, pH has marked effect on the solubility of these substances in water. But, compounds which do not react with either acids or bases are slightly, if at all, influenced in their aqueous solubility by pH variations; provided there is no acid-base catalysed decomposition reactions.

General Techniques

Conventional phase-solubility method of anaylsis briefly consists of the following steps (9, 10):

(1) Mixing various quantities of dried material, under investigation, with equal or known quantities of solvent, in suitable containers, and shaking at constant temperature until equilibrium is attained.

(2) Separating of the solid phase from the solution at the specified equilibrium temperature.

(3) Determination of the concentration of the material dissolved in the various aliquots.

(4) Plotting the concentration of dissolved material (solution concentration in mg/gm of solvent) against the total solid added per unit weight (system concentration in mg/gm of solvent).

Since it is essential to analyze the total dissolved substance in solution, the analytical method should respond to all possible components of the mixture. The most general method is gravimetric where an aliquot of the supernatant liquid is evaporated to constant weight and the residue is weighed. This implies the absence of any volatile components in the sample under consideration.

In applying this method for the analysis of any substance, one should take into consideration the following:

(1) The solvent: the solvent should fulfill the following criteria:

(a) It should be chemically inert and have no chemical reaction whatsoever on the substance concerned.

(b) The substance analyzed should be very slightly soluble in it (1-10) mg/gm of solvent.

(c) It should be of known purity and composition,

(d) Boiling point should be 65^{o}-100^{o}. If it is low evaporation during filtration is undesirable because it changes concentration, and if high, drying is more difficult and decomposition or losses of compound or impurities are more likely to occur.

(2) Weight of the sample per tube: given that the approximate solubility of the major component is known the equilibration tubes are so prepared that at least one tube should result in complete solution and other tubes containing increasing quantities of material per unit solvent. For compounds known to be impure a large number of tubes is required to plot the solubility diagram adequately.

(3) Equilibration: Unless proof is obtained that equilibrium has been attained this type of analysis is erroneous and useless. In order to ascertain this, one of the tubes is warmed so that a supersaturated solution is obtained. After equilibration of this tube with the other tubes the plotting of this tube should fall on the same diagram as the plotting of the tubes in

which equilibrium was approached from the unsaturated side. The time required to obtain equilibrium varies with each compound and solvent system. Generally, trial and error is the only available method for determining the time required for equilibration.

(4) Particle size: large crystals or lumps of material should be finely powdered. On the other hand very fine particles may produce colloidal suspensions.

(5) Temperature: Adequate temperature control is necessary. Usually, 25-30° within 0.5°C control is chosen.

(6) Drying: Freeze-drying is the best where losses due to pumping or spattering are minimized.

Phase solubility diagrams depend on the principle that when a substance of limited solubility is added to a fixed quantity of solvent, in varying quantities and under a fixed temperature and pressure, giving rise to a continuous sequence of unsaturated solution followed by a single saturated solution; the added substance thereafter remaining as excess in the form of a characteristic phase.

Phase solubility diagrams are different for different samples. To illustrate this let us, take pure analytical reagents A,B, and C. Figure 1 represents the three phase-solubility profiles in one diagram system. ABC represents the phase solubility diagram of substance A with equilibrium solubility S_1. A mixture of substances A and B will have the phase solubility diagram of AB'C'D with solubility of A as S_1 and solubility of B as $(S_1 + S_2) - S_1$. When the three substances A,B and C are mixed, their phase-solubility diagram is AB"C"D'E with solubility of A and B as above and that of C as $(S_1 + S_2 + S_3) - (S_1 + S_2)$.

When the substance analyzed consists of a major component and a small amount of soluble impurities; its phase solubility diagram is represented by ABC in Figure 2.

Phase-solubility diagrams of more than three component mixtures are possible and in this case the number of sloping segments is equal to the number of independent substances in the sample. The order in which the components reach saturation depends upon their solubilities in the solvent chosen and their

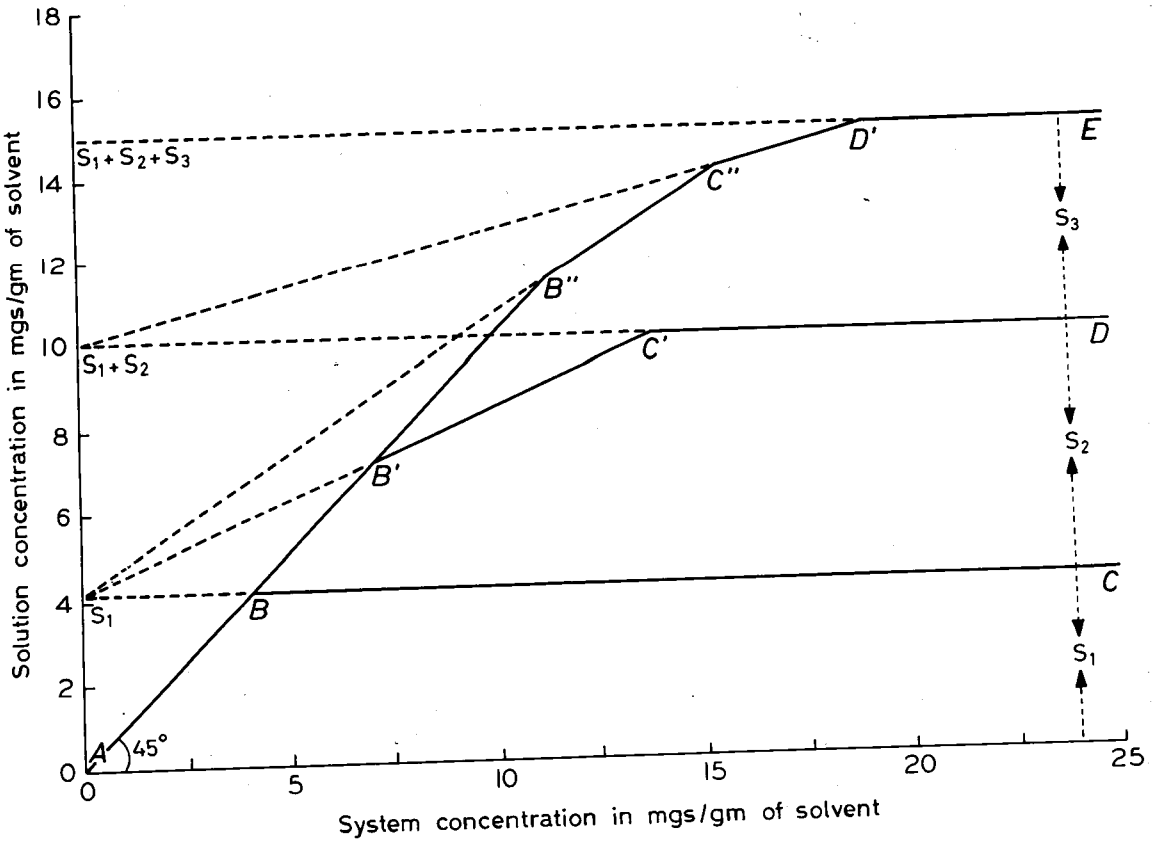

Figure 1

ABC, AB'C'D and AB"C"D'E are phase-solubility diagrams of one, two and three component solid mixtures respectively.

concentration in the original sample.

Adequate interpretation of the above diagrams is very essential. In Figure 1 although ABC represents the phase-solubility diagram of a pure compound, it can be a mixture of two or more components in direct proportion to their solubilities, at the given conditions. This case is just theoretical and can be easily excluded if the material under study has a constant phase-solubility diagram in several different solvents or at different temperatures.

AB'C'D and AB"C"D'E result from components which form simple mixture in the solid state and their solubilities are independent one from the other. Each time there is an abrupt change in slope a new solid phase appears.

A mixture of d-and l - optical isomers in any proportion other than 1:1 would be expected to have a curve of the type AB'C'D. (16,9). A racemic or a mixture of tagged isomers would behave as a single component even in several solvent. (16).

The phase-solubility diagram is constructed by plotting

solution concentration in mg/gm solvent on the y-axis vs the system concentration in mg/gm of solvent, on the x-axis. Along the first segment, AB, AB' and AB" in figure 1 and AB in figure 2 all the solid added to the system dissolves and is found in solution. And, therefore, the slopes of AB, AB' and AB" in figure 1 and AB in figure 2 (the first segment of each diagram) are unity slopes and the angle is 45^{o}.

Some authors label the axis as mg/ml of solution for solution concentration (y-axis) and mg/ml of solvent for system concentration (x-axis). In this case the slopes of AB, AB' and AB", in figure 1 and AB in figure 2 will deviate from unity because the concentration bases for the two axes are slightly different. But, when the solubility is very low the deviation is negligible. To be on the safe side the concentration bases for the two axes should be identical in their expression units (mg/gm solvent).

At point B, B', B" in figure 1 and B in figure 2 the solution becomes saturated with respect to the first component, and further addition of the sample cannot lead to an increase in

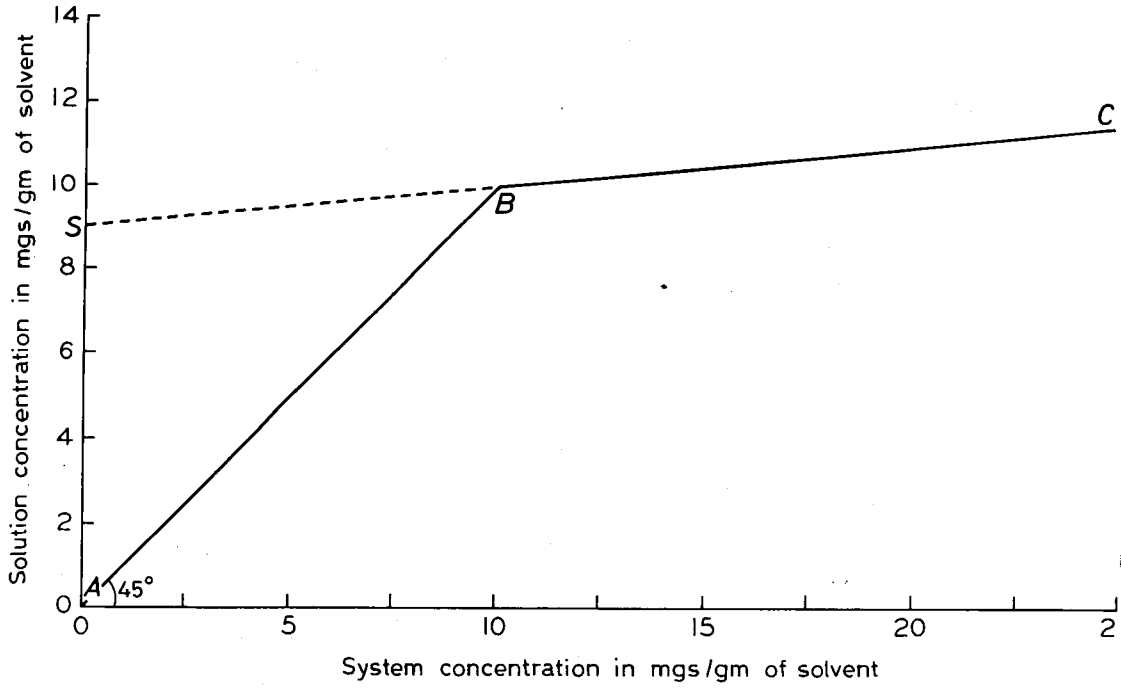

Figure 2

ABC is a phase-solubility diagram of an impure substance

its solution concentration.

Along BC, B'C', B"C", in Figure 1 and BC in Figure 2 all components other than the first component pass into solution. Therefore, the slopes of these segments are equal to the fraction of all components excluding the first one, and the y-intersept of these segments is the equilibrium solubility of the first component to reach saturation.

Similarly the slopes of C"D' is equal to the fractional composition of the sample excluding the first two components. Extrapolation of C'D and C"D' to the y-axis gives the equilibrium solubility of the first two components to reach saturation and the D'E y-intersept gives the sum of the three solubilities of the mixture components.

Solid solutions (14, 16) do not have well defined segmented phase-solubility diagrams and therefore, phase-solubility technique cannot be used for their quantitative analysis, on the other hand, this method can be utilized for the purification of some biologically active products which are solid solutions in nature, such as pepsin and diphtheria antitoxin.

Another distinct class of phase-solubility diagrams is the complexation type: these are most commonly used to study the stoichiometry of the complexation reactions and to determine the stability constants of the products (10,18,19,17).

Related Methods

These methods are related to the typical phase-solubility analysis in principle only, but techniques, apparatus and experimental features are different. They are as follows:-

(1) Recrystallization method: Bennett in 1948 (7) worked on two types of differential solubility methods, in the first of which the sample is extracted with a solution saturated with respect to the major component and in the second one component is extracted with a solvent saturated with respect to all other components. This requires knowledge of the nature and the number of components in the sample. Dicophane (D.D.T) B.P. '63 is assayed by the first method.

(2) Solubility temperature method: Reeve and Adams in 1950 (5) defined the solubility temperature as the temperature at

which a solid dissolves in a given amount of solvent. To apply this method you should have the pure analytical standard in order to establish the solubility temperature composition diagram. It is excellent for characterization of compounds with unsatisfiactory melting points, e.g. amino acids; but it is used only when other methods fail completely due to its tediousness, requirement of large sample and difficulty in expression of results.

(3) Extraction solubility method: Stenger et al (6) in 1953 devised this method in which they could easily distinguish between pure and 99.5% pure compounds where the classical phase-solubility method fails to distinguish, due to the difficulty in determining the slope of the second segment of the diagram. They extracted the sample from tarred extraction thimbles in soxhlet apparatus by a solvent. They determined the following fractions to estimate the absolute purity of the substance under consideration.

(a) Insoluble impuirties

(b) Soluble impurities and the amount of pure substance required to sturate the solvent used.

(c) Pure residue.

Advantages and Disadvantages

Advantages:

(1) Simple and inexpensive equipment is required.

(2) It is applicable to all species of molecules.

(3) The sample is not destroyed and can be easily recovered(9).

(4) It can give the equilibrium solubility of an impure substance.

(5) Steroids are difficult to purify and consequently difficult to analyze otherwise (14).

(6) The phase-solubility diagram indicates a method for separating the components of a mixture or purifying an impure sample.

(7) Sufficiently sensitive to distinguish between d- & l- optical isomers not in 1:1 ratio (16, 9).

(8) Very useful in determining the identity and purity of synthetic and naturally occurring biologically active products with minimum efforts (16)

Disadvantages:

(1) It is time consuming; but modifications can be introduced for routine work which enables one to obtain dependable results within reasonably short time.

(2) Experimental conditions especially, long time of shaking, are liable to induce solvent-solute interaction or in the case of optical isomers racemization may be encountered (16).

(3) Interfering substances such as other components of a mixture or impurities may produce salting-out or salting-in effects. This nonideality can be eliminated or minimized by working in very dilute solutions, which requires the solubility of the major component in the solvent to be very low (1-10 mg/gm). This leads to the use of smaller samples, thus minimizing the amount of impurity to dissolve in the solvent and exert its undesirable effect.

(4) Non effective for the quantitative analysis of components 99.5% pure or more due to the difficulty in

interpreting the slope for such a small value.

(5) Any volatile impurities present in the original sample render the method erroneous and misleading for analytical purposes. To avoid this effect, dry the sample before analysis under the same conditions of drying at the termination of the experiment. However, this drying does not help if the major component is volatile where the method fails completely.

(6) Not applicable for solid solutions and mixtures in the ratio of their solubilities. (14, 16).

COMMENTS ON ANALYTICAL METHODS USED FOR THE DETERMINATION OF CHLORAMPHENICOL

The chemical structure of chloramphenicol involves the p-nitrophenyl group as an absorbing chromophore for direct spectrophotometric assay of pure samples or samples that are free from absorbing species, primarily at 278 mu. The accepted spectrophotometric analytical methods for pharmaceutical formulations containing chloramphenicol (20-23) does not yield valid results (24) when applied to partially degraded samples. Therefore, direct ultra-violet spectrophotometric determinations on chloramphenicol, its derivatives and their various dosage forms are not acceptable for quantitative purposes. To overcome this difficulty and improve the situation it is desirable to find a method which would quantitatively separate the chloramphenicol from its degradation products as well as other interfering substances. Various chromatographic methods (25-29) have already been developed for the effective separation of chloramphenicol from its decomposition products and other absorbing species in a

given formulation. In addition to the chromatographic separation, paper electrophoresis (30) and solvent extraction schemes (31) have also been proposed for various dosage forms.

These techniques, although, often effective in separation, they are complicated and time consuming. The simplicity and quickness of the method proposed by this work may render it preferable in the routine analysis of chloramphenicol dosage forms.

Polarographic methods (32-37) of analysis suggested for chloramphenicol are also subject to similar criticism because of their nonspecificity. Chloramphenicol degradation product (1-p-nitrophenyl -2- amino- 1,3 - propanediol) will interfere by producing a well defined wave which corresponds closely in half-wave potential ($E_{\frac{1}{2}}$), to that obtained by the chloramphenicol (32), since the electroreduction of nitro group to amino group is the principle involved in the polarographic determination. Polarographic analysis is also not applicable to chloramphenicol palmitate because of its unfavourable solubility characteristics (32, 38).

Chloramphenicol can also be estimated colorimetically (38-41) by different chemical approaches. The most important approach makes use of the p-NO_2 functional group (42-45) which upon reduction, diazotiation and azo-compound formation produces a well defined colored substance that can be determined photometrically. p-NO_2 group when reduced to - NH_2 group can be determined nitrometrically (46). The second approach utilizes color reactions based on dichloracetic acid (47) which is also a hydrolysis product of chloramphenicol. After hydrolysis, chloralphenicol may also be quantitatively oxidized with periodic acid yielding p-nitrobenzaldehyde; and with subsequent conjugation with 2, 4- dinitrophenyl-hydrazine yielding a colored substance to be determined colorimetrically (48-49). The yellow color occurring when chloramphenicol is heated with 40% NaOH solution is sometimes used for photometric determinations (50).

Other physicochemical means of chloramphenicol analysis are feasible. These include the following methods: titrimetry (51-53), argentometry (54-56), potentiometry (57) and

measurement of optical rotation (58-59). Spectrophotometric analysis for mixtures of chloramphenicol with sulfa drugs (60) or tetracyclines (61) are also reported.

The above chemical and instrumental methods of quantitative assay of chloramphenicol are rather nonspecific. Nearly all such procedures are essentially, methods for determining the amounts of certain functional groups present in the sample. The p-nitrophenyl moeity is the most important. It makes very little difference whether this group is determined by conversion to an azo-compound or to an amino group. These are still functional group analysis and not specific methods for the determination of the whole chloramphenicol per se. They are unreliable in the presence of its degradation product or irrelevant impurities containing such functional groups.

Some chemical methods may be specific and the chloramphenicol hydrolysis products do not interfere. When the amide group of the molecule is subjected to the action of hydroxylamine hydrochloride, the resulting hydroxamic acid is measured after

color development by ferric chloride (62). Only esters in this case may interfere; but the choice of the proper wave length may minimize this interference.

Although chloramphenicol capsules in the B.P. '63 monograph are assayed absorptiometrically at 278 mu; U.S.P. XVII is adopting the microbiological method published by the Food and Drug Administration as the only method of assay for all chloramphenicol dosage forms.

Biological methods (63-69) are ideally suited for the evaluation of chloramphenicol activity and concentration since they are insensitive to the presence of similar but inactive contaminants or degradation products. They are in most cases very sensitive and capable of detecting drugs present in extremely minute concentrations. In spite of specificity and sensitivity characteristics of these procedures, however, their adoption as analytical procedures is usually accepted with reluctance and chosen as a last resort, because of their inherent low degree of precision (25). They are often used to compliment

the validity of the results obtained by the analytical techniques. Biological methods cannot, also, be utilized for the analysis of chloramphenicol palmitate since the unhydrolyzed ester lacks the antibacterial activity (37), but it is applied only after hydrolysis of the product (70-71).

EXPERIMENTAL

The difficulty faced in the analysis of chloramphenicol capsules by column chromatography (72) initiated the idea to think of some other simpler analytical method for its quantitation. This type of analysis, proposed by this work, originated from the ideal assumption that water or any solvent at a given temperature will dissolve a fixed amount of any chemical substance even in the presence of other interfering substances.

Reagents

l- Chloramphenicol, Roussel UCLAF: (D-(-)- threo-2- dichloro-acetamide - 1 - (p-nitrophenyl)- 1,3 propanediol. (Active isomer)

d-Chloramphenicol, Parke, Davis & Co.:(L-(+)- threo-2-dichloro-acetamide -1- (p-nitrophenyl)-1,3- Propanediol. (inactive isomer).

Chloramphenicol base, Parke, Davis & Co.: L-(+)- threo-2- amino -1-(p-nitrophenyl)-1,3- propanediol). (chloramphenicol degradation product.)

Chloramphenicol hemisuccinate, Roussel UCLAF.

Chloramphenicol palmitate, (Chloromycetin palmitate, Parke, Davis and Co.)

Instruments and Apparatus

Mettler Balance, Type H16.

Burrell Mechanical Shaker, Model 88.

Water Bath Shaker, WCLID, Model 2156.

Ultraviolet-Visible Spectrophotometer, Perkin-Elmer, Model 202.

Quartz Cells, 1cm. (matched).

Extraction-Filter Tubes (figure 3A).

Volumetric flasks, pipets, glass stoppered Erlenmeyer flasks (125 ml), 30 ml sintered glass funnels, screw capped test tubes and other necessary glass ware or apparatus needed. All volumetric glass ware are of grade A quality.

Devising of an appropriate apparatus (figure 3A):

The preliminary exploration of chloramphenicol analysis

using solubility method was first performed in volumetric flasks. A known weight of standard chloramphenicol and a representative aliquot of a capsule treated as unknown were simultaneously subjected to identical experimental conditions. The respective accurate weight of each was treated with equal volumes of water adjusted in volumetric flasks, and allowed to stand for 3 -4 hours at room temperature (about 25°C), with seldom shaking of both. After the specified time the supernatants of both were filtered and the residue recovered quantitatively and analyzed spectrophotometrically for its chloramphenicol contents. Based on the theory that both standard and sample lost an equal quantity of chloramphenicol, the content of the sample was assessed by summing the residue and the amount lost in supernatant water (obtained from standard flask). The results were very encouraging.

Volumetric flasks were found not suitable for shaking due to their shape. Therefore, they were replaced by screw capped test tubes of convenient size and subjected to continuous shaking by the use of mechanical shaker, at room temperatute.

Repeating the above mentioned experiment under these modified conditions gave better results compared to the first trial.

At this stage the evaluation of equilibrum solubility and the optimum conditions to attain it were explored. Shaking excess chloramphenicol with water from one week to 15 minutes, produced supernatants almost of identical concentrations. Therefore, shaking for 30 minutes was thought to be sufficient for equilibration (pending further reconfirmation).

Glass stoppered Erlenmeyer flasks were found to be convenient for shaking purposes. But, these were satisfactory only when one extraction was to be performed. Due to the complex nature of many sample formulations the need of more than one extraction would have been required for the elimination of all interfering soluble substances. This necessitated for each extraction centrifugation and decantation through filter paper with minimum transfer of residue. Due to convenience of the subsequent steps to be followed for quantitation, filter papers were replaced by sintered glass funnels.

Instead of working solubility experiments at approximate values as indicated by 'room temperature',the constant water bathe shaker was adopted for further experimentation. This shaker has a reciprocation speed of 50-200 cycles per minute (c.p.m.) three stroke length settings (1/2", 1" and 1 1/2" and a temperature sensitivity of $\pm 0.5\ ^{o}C$. The working specifications are 1 1/2" stroke length setting, 150 c.p.m. speed and 25^{o} C temperature.

When the saturated supernatant liquid is decanted from the flask or tube into the sintered glass funnel the temperature change may cause some alteration in the concentration of the filtered supernatant; although such hazards are not very significant due to the little gap between the room temperature and the chosen bath temperature. If an apparatus could be somehow devised to do both jobs, shaking and filtering without the additional transfering step; the analytical technique could be greatly improved.

The tube like apparatus illustrated in figure (3A)

was devised to serve this purpose. It is made by fusing a coarse type sintered glass funnel (15 ml capacity) with a screw capped test tube both with identical 20 mm internal diameters.The resulting tube' will have a capacity of 50 ml. While in operation the funnel stem is tightly closed with a short rubber tubing fused from one side. Such a designed apparatus hereafter, will be called 'extraction-filter' tube. This naming justifies the dual function of the apparatus because it serves both for extraction and filtration without transfer of sample, thus avoiding any possible loss of residue especially when subjected to more than one extraction. This apparatus is ideal in determining equilibrium solubilities. After equilibration, filtration is performed quickly by suction and the saturated liquid is analyzed.

However, the 'extraction-filter' tube originally designed was slightly different from the one described. It is represented in figure (3B), where the modification of using tephlon-lined screw cap fitting tightly to the sintered glass,

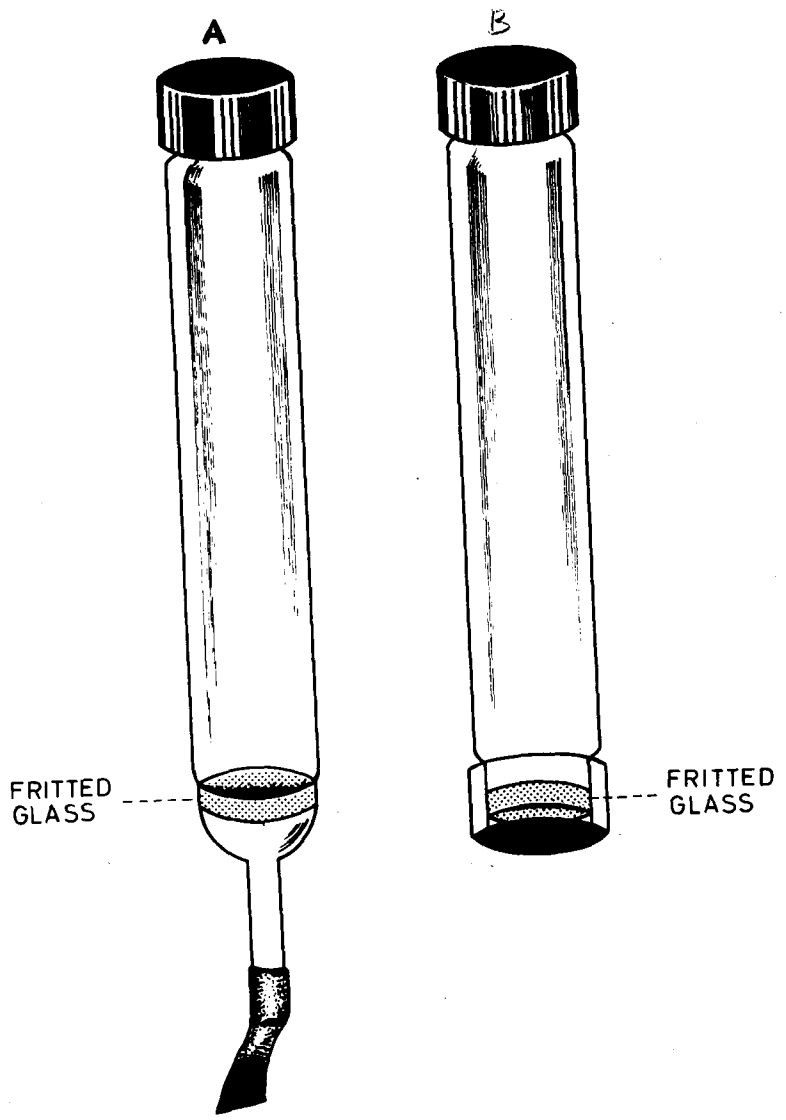

Figure 3

The 'extraction-filter' tubes

thus avoiding the possibility of solvent leakage and the probability of effecting the rubber when organic solvents are to be used.

Exploratory Experiments

1 - Evaluation of equilibrium conditions:

The preliminary test mentioned previously, about the evaluation of chloramphenicol equilibrium solubility is confirmed by the use of the new apparatus at 25°C where a series of 'extraction-filter' tubes in which excess chloramphenicol standard was shaken with water were exposed to different conditions. Tube No. 1 was first heated in boiling water bath and then left to stand for an overnight, at 25°C, to equilibrate. And, finally it was shaken with the other tubes for one hour. Tubes Nos. 2, 3, 4, and 5, were shaken at the same specified temperature for 15, 30, 45, and 60 minutes respectively. The filtrates were analyzed spectrophotometrically. The analytical results are given below:-

Tube No.	1	2	3	4	5
Solubility of chloramphenicol in mg/ml water	3.88	3.87	3.87	3.87	3.89

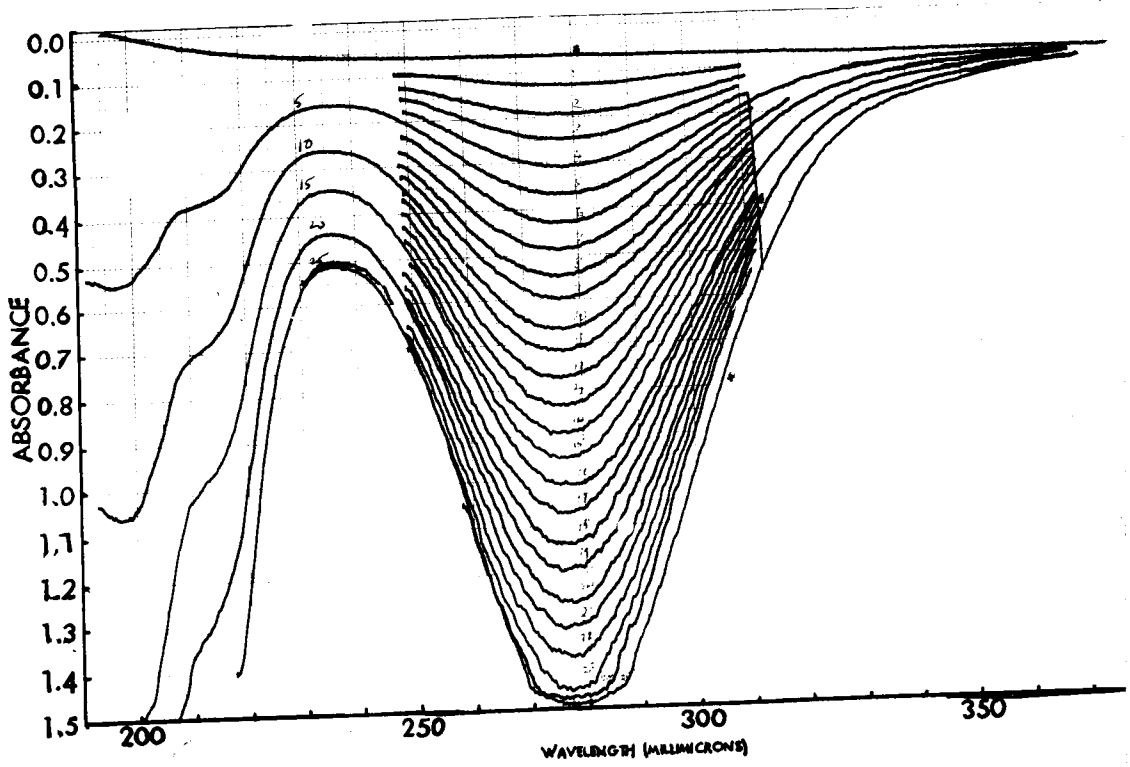

Figure 4

Spectra of chloramphenicol concentrations ranging from 2×10^{-4} to 50×10^{-4} gm/100 ml water.

These results ensure that equilibrium solubility of chloramphenicol in water is attained within a short time (15 minutes). However to be on the safe side shaking for 30 minutes is adopted for all chloramphenicol equilibration techniques used in all subsequent experiments.

2- Determination of $A^{1\%}_{1cm}$ for chloramphenicol in water:

Chloramphenicol standard (50.00mg), dried at 105°C, was dissolved in 1000.0 ml of water using a one liter volumetric flask. From this solution 1.0, 2.0, 3.0, 4.0, up to 25.0 ml were pipetted into 25.0 ml volumetric flasks and made up to volume by water.

Figure 4 represents the spectra taken by the recording spectrophotometer and figure 5 represents the calibration curve drawn by plotting the absorbance at 278 mu vs the concentration of solutions in gm/100 ml water. These values are tabulated in table 1.

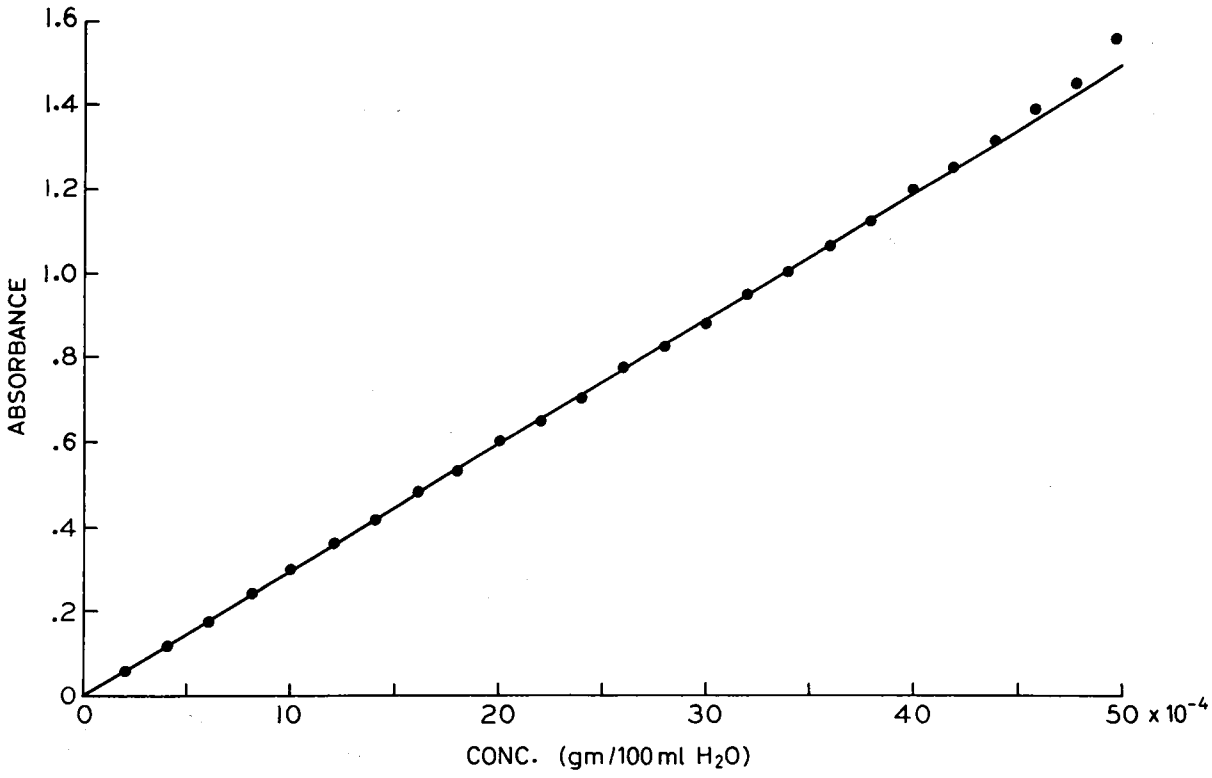

Figure 5

Absorbance at 278 mu vs concentration of chloramphenicol

Table 1

Data obtained for concentrations of chloramphenicol and their corresponding absorbance at 278 mu.

Conc. in gm/100 ml water	A	Conc. in gm/100 ml water	A
2×10^{-4}	0.06	28×10^{-4}	0.828
4×10^{-4}	0.125	30×10^{-4}	0.882
6×10^{-4}	0.180	32×10^{-4}	0.952
8×10^{-4}	0.243	34×10^{-4}	1.005
10×10^{-4}	0.302	36×10^{-4}	1.070
12×10^{-4}	0.361	38×10^{-4}	1.125
14×10^{-4}	0.42	40×10^{-4}	1.20
16×10^{-4}	0.479	42×10^{-4}	1.255
18×10^{-4}	0.53	44×10^{-4}	1.318
20×10^{-4}	0.60	46×10^{-4}	1.39
22×10^{-4}	0.648	48×10^{-4}	1.45
24×10^{-4}	0.705	50×10^{-4}	1.55
26×10^{-4}	0.778		

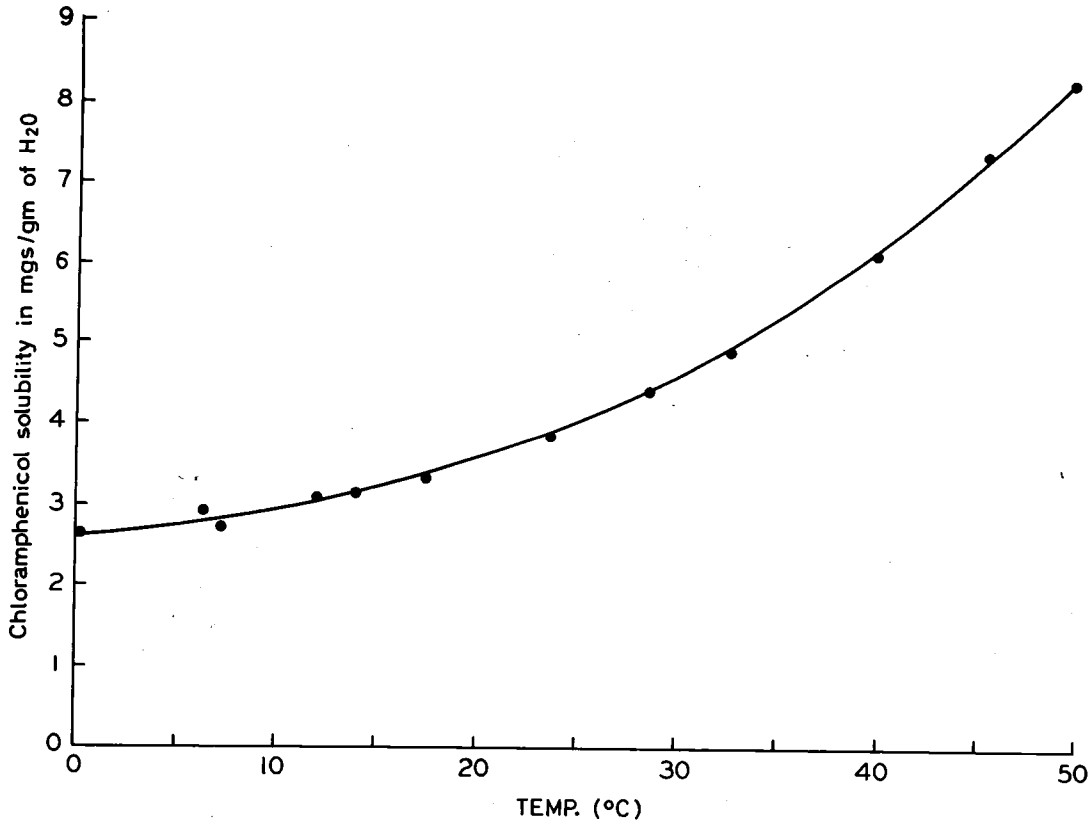

Figure 6

Chloramphenicol solubility-temperature profile

The slope of the calibration curve (Figure 5) was found to be 292. Therefore, $A^{1\%}_{1cm}$ should be the same. This value is used for all chloramphenicol spectrophotometric determinations.

3- Establishment of chloramphenicol solubility - temperature curve:

The constant temperature water bath was filled with crushed ice and shaken until the temperature was constant. Solubilities of chloramphenicol were determined experimentally for a range from about zero to 50°C. 'Tubes' containing excess chloramphenicol in water were subjected to shaking for 1 hr. at different temperatures but at the same shaking conditions. Filtrates from the respective 'tubes' were analyzed spectrophotometrically. The corresponding values are tabulated in table 2.

Table 2

Chloramphenicol equilibrium solubilities at different temperatures (From 0 to 50°C)

Bath temp.	Solubility in mg/ml H2O	Bath temp.	Solubility in mg/ml H2O
0.2	2.68	23.7	3.83
6.4	2.70	28.6	4.40
7.2	2.90	32.7	4.86
12.3	3.06	40.0	6.12
14.1	3.13	45.6	7.35
17.4	3.30	49.9	8.28

These results are expressed graphically in figure 6.

4- The phase-solubility diagrams of chloramphenicol samples:

Three samples of chloramphenicol were subjected to phase-solubility analysis. The first sample was pure chloramphenicol, the second was vitaminized capsules and the third was a mixture of chloramphenicol and tetracycline Hcl. Increasing quantities of each sample were incorporated with 10 gm of water

in hermatically closed 20ml vials. They were equilibrated together at 25°C for 3 days. Supernatants were analyzed gravimetrically for their solid contents. Table 3 represents the data obtained from such analysis.

Table 3

Data of solution and system concentrations of chloramphenicol samples.

Sample	Vial No.	system Conc. in mg/gm water	solution Conc. in mg/gm water
Pure chloramphenicol	1	5.20	3.97
	2	10.59	4.03
	3	15.17	4.02
	4	20.36	4.04
	5	25.04	4.05
Vitaminized Chloramphenicol	1	4.65	4.67
	2	10.86	6.14
	3	14.62	6.98
	4	20.18	7.90
	5	22.50	8.67
Chloramphenicol and tetracycline HCl mixture (3:2)	1	5.60	5.6
	2	9.87	7.65
	3	14.87	9.55
	4	20.06	11.54
	5	24.77	13.35

The graphical representation of the above data is given in figure 7, from which one could easily compute the percentage of chloramphenicol in the respective samples as well as assessing the solubility at the specified temperature. These values are given in table 4

Table 4

Phase-solubility data of chloramphenicol samples.

Sample	Slope of 2nd segment	% purity computed	Solubility in mg/gm H20
1 - Pure chloramphenicol	.005	99.5	3.89
2 - Vitaminized "	0.216	78.4	3.87
3 - Chloramphenicol + Tetracycline	0.384	61.6	3.87

These values obtained here reconfirm the solubilities determined previously by a different approach.

5 - Interference of d-chloramphenicol

As previously mentioned l-and d-isomers of amino acids not in 1:1 ratio behave as though they were two different

substances (10, 16) and have a phase-solubility diagram similar to AB' C'D (Figure 1) which indicates that their solubilities are additive. However, this was not found to be the case with d- and l- chloramphenicol. They behaved exactly as if they were one substance and consequently this method failed to separate d-isomer from the active chloramphenicol. The following physical properties were determined simultaneously for both isomers.

Isomer	l-isomer	d-isomer
Solubility in mg/ml H20 at 25°	3.92	3.92
Computed $A^{1\%}_{1cm}$ in H20	292	292

Different relative proportions of each were equilibrated with water and respective solubilities were determined at 25°. The results were found to be identical with either d- or l- isomers.

Analysis for active chloramphenicol in the presence of known quantities of d-isomer gave additive results. The

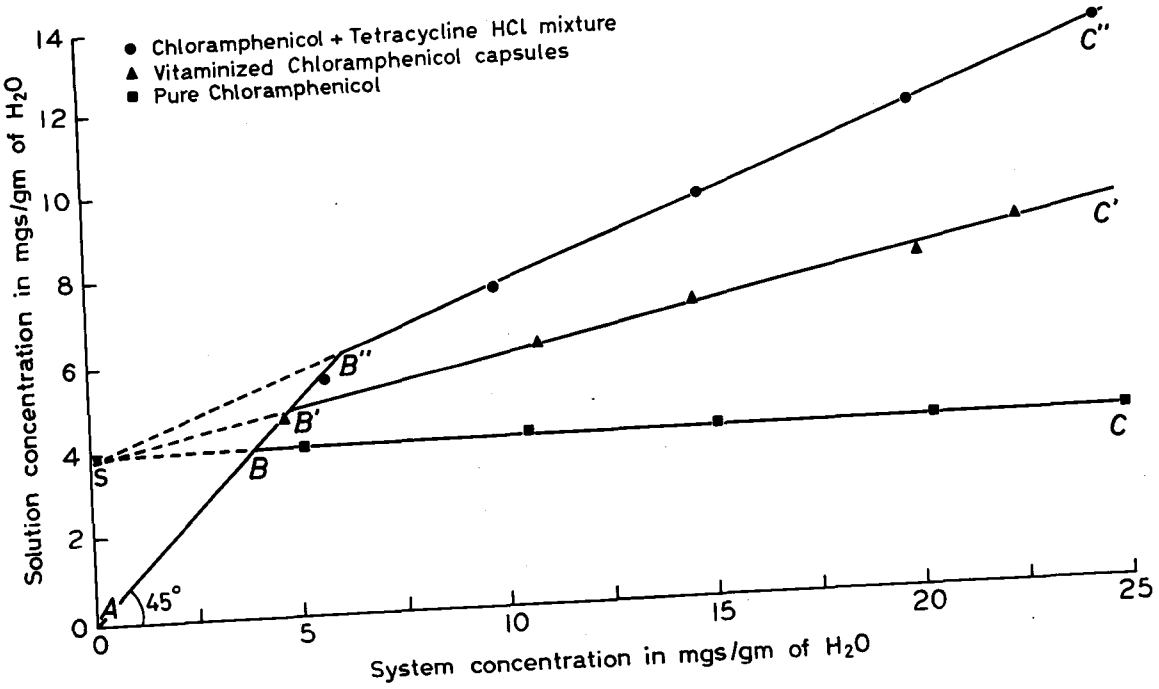

Figure 7

Phase-solubility profiles

1 - ABC is the phase-solubility diagram of pure chloramphenicol.

2 - AB'C' is the phase-solubility diagram of chloramphenicol in vitaminized capsules.

3 - AB"C" is the phase-solubility diagram of chloramphenicol mixed with tetracycline HCl.

following are experimental data.

Weight of chloramphenicol (l-isomer)	89.35 mg
Weight of d-isomer	15.43 mg
Recovery	104.7 mg

6 - Interference of chloramphenicol degradation product:

Increasing amounts of chloramphenicol base were incorporated with known samples of chloramphenicol and the resulting mixtures were analyzed by the proposed method for their chloramphenicol contents. Since chloramphenicol base is very slightly soluble in water, its presence in more than 20% in any sample would interfere, thus, producing higher analytical results. But, since it is an amine, advantage is taken of the fact that it can form salts with mineral acids, thus becoming completely soluble in water. The analysis, for chloramphenicol contents of samples containing up to 70% of the mentioned base

had negligible effects when 0.1N HCL was used as the extracting solvent. Table 5 reveals a picture of the analytical results of known samples of chloramphenicol admixed with known amounts of chloramphenicol base, when water and 0.1N HCL were used as solvents for extraction.

Table 5

Analytical results of known mixtures of chloramphenicol and chloramphenicol base.

Extracting solvents	H20			0.1N HCl		
Weight of chloramphenicol in mgs.	116.35	117.6	116.85	118.25	119.2	118.95
Weight of chloramphenicol base in mgs.	80	60	40	200	100	50
Chloramphenicol recovered by analysis, in mgs.	144	120.3	117.8	117.55	119.15	117.45
% recovery	127%	105%	100.8%	99.4%	100%	99%

Therefore, it is safer to use 0.1N HCl as a solvent in the analysis of chloramphenicol samples suspected to contain appreciable amounts of the degradation product, provided the presence of the HCl in the medium does not produce any decomposition of the chloramphenicol (Pending confirmation)

7 - Hydrochloric acid effect:

To verify the effect of HCl on the decomposition of chloramphenicol, the following set of experiment was performed. Excess pure chloramphenicol was shaken with different solvents having gradual increase of HCl in water. Solubilities were determined and then known samples were analyzed by the proposed method. Results are shown in table 6.

Table 6

Solubilities and analytical results

of chloramphenicol in acidic solvents

Solvent. Solvents	1N HCl	0.5N HCl	0.1N HCl	0.01N HCl	0.001N HCl	0.0001N HCl	H_2O
Solubility of chloramphenicol (mg/ml solvent):	4.14	3.85	3.81	3.87	3.85	3.89	3.91
Weight of sample analyzed in mgs.:	53.13	47.67	58.35	101.65	99.1	99.9	46.5
Weight recovered in mgs.	49.41	46.6	57.85	102.6	100.57	100.46	46.5
% Recovery:	93%	98%	101%	101%	101.5%	101.6%	100%

These results indicate that up to 0.5N HCl could be used as the extracting solvent without any danger of chloramphenicol decomposition. Above this concentration decomposition is liable to occur. To be on the safe side, 0.1N HCl solution is routinely adopted as the solvent for analysis of simple chloramphenicol samples suspected to contain appreciable amounts of chloramphenicol degradation product.

Analytical Procedure Followed

After these preliminary investigations the following general procedure is adopted in the analysis for chloramphenicol. An accurate weight of standard chloramphenicol is carefully transfered to an 'extraction-filter' tube and an equivalent amount of chloramphenicol sample, is also, accurately weighed, and transfered to another 'tube'. Close the funnel stems of the 'tubes', tightly by short fused rubber tubings. Pipet equal volumes of the proper solvent into both 'tubes'. Close the tubes with their screw caps, tightly. Immerse, horizontally, both tubes in the water bath shaker at 25°C and shake for 30 minutes. Remove the tubes, wipe and filter quickly by suction. At this stage, the solvent is supposed to extract equal quantities of chloramphenicol from the sample and the standard, in addition to any soluble impurities or interfering substances. These extraction and filtering processes may be repeated whenever necessary. Wash the residue in both tubes by about 1 ml of the filtrate received from the standard once or more, if necessary.

Filter quickly by suction and continue suction until the residue on the fritted glass is almost dry.

Dissolve the residue, by the help of distilled water and collect it quantitatively into a convenient volumetric flask and analyze it spectrophotometrically using the formule previously stated ($A^{1\%}_{1cm} = 292$). From these results obtained, the weight of chloramphenicol in both residues and consequently in the whole sample, can be easily computed. Like any analytical technique it is desirable to run the analysis, by this proposed method, in duplicate.

Analysis of Chloramphenicol Capsules

A review of many chloramphenicol capsule formulations revealed the following as the most occurring excipients: Lactose, starch, magnesium, stearate and talc. During the review of commercial products the presence of bismuth carbonate was also found in some formulations in addition to the above mentioned common excipients. The solubility of chloramphenicol in

water in presence of different concentrations of the excipients and bismuth carbonate revealed that they did not interfere whatsoever with the solubility of chloramphenicol, within their occurence range in capsule formulations. Table 7 shows the effect of various excipients and bismuth carbonate used in capsule formulations.

Table 7

Chloramphenicol solubility in water in presence of excipients and bismuth carbonate

Concentration of excipient in the system in mg/ml H_2O		0	1	2	4	8
Chloramphenicol solubility (mg/ml H_2O), in the presence of:						
1	Lactose	3.83	3.82	3.81	3.82	3.83
2	Starch	3.83	3.83	3.87	3.83	3.82
3	Mag. Stearate	3.85	3.82	3.75	3.66	3.56
4	Talc	3.85	3.83	3.83	3.84	3.81
5	Bismuth carb.	3.85	3.82	3.85	3.83	3.81

Before attempting any analysis of chloramphenicol

capsules, the average weight of the capsule should be ascertained. This is achieved by weighing accurately a minimum of 10 capsules. They are cut by a sharp blade and their contents emptied in a clean dry mortar. Brush the empty capsules while holding with a pair of forceps, to clean them from any adhering powder. Powder the contents as finely as possible and immediately store in tightly closed amber bottle. Label the bottle as chloramphenicol capsule 'sample to be analyzed', with the trade name clearly appearing on the label. Weigh the empty capsules accurately and compute the average weight per capsule.

To estimate the amount of sample required for analysis the following approach is convenient. The amount of chloramphenicol in the sample should be approximately 10 to 15 mg plus 4 mg per ml of solvent by which the sample is to be extracted.

The following will illustrate a sample analysis and subsequent calculations involved for computing the chloramphenicol content in capsule formulations:

	Sample	Standard
1 - Net average weight of one capsule content (mg)	293.2	-
2 - Label claim of chloramphenicol (mg)	250	-
3 - Weight of sample subjected to analysis (mg)	113.1	102.15
4 - Volume of aqueous solvent to extract with (ml)	20.0	20.0
5 - Volumetric flask in which residue is received (ml)	250.0	250.0
6 - Volume of aliqnot per 100.0 ml of water	25.0	25.0
7 - Absorbance at 278 mu	0.44	0.62
8 - Chloramphenicol conc. in the final dilution(calculated,in mg%)	1.52	2.14
9 - Wt. of residue (mg):(Item 8x10)	15.2	21.4
10- Loss of chloramphenicol in the extracting solvent(mg)(Item3)-(Item9) of standard	80.75	80.75
11- Weight of pure chloramphenicol in the sample (mg)(Item 9) + (Item 10) of sample	95.95	-
12- Calculated capsule content of pure chloramphenicol (mg)	248	-
13- Percent deviation from the label claim	(-)0.8%	-

About 30 different proprietory capsule formulations containing chloramphenicol were subjected to the proposed method of analysis. For convenience, these capsules can be classified into the following arbitrarily assigned classes:

1 - Simple capsules:

These capsule formulations may contain pure chloramphenicol or admixed with excipients only, with no other active ingredients. The following table (Table 8) is a result of the analysis of 10 different capsule formulations belonging to 10 different pharmaceutical firms. The final averages are results of 5 determinations. In order not to reveal proprietory names these capsules are labelled by numbers from 1 - 10. All of these formulations are powders in gelatin capsules except No. 6 which is a sugar coated tablet (dragee).

2 - Vitaminized capsules:

These capsule formulations contain in addition to chloramphenicol and possible excipients, various proportions of individual vitamins especially vitamin B-complex, Vit. C and menadione. Before running the analysis of vitaminized capsules,

the following preliminary experimental studies were perfomed.

(a) Interference of vitamins: the most commonly occurring vitamins in capsule preparations were studied spectrophotophotometrically and by the proposed method of analysis. Absorbance values of a 2 mg% aqueous solution of all the individual possible vitamins occurring in chloramphenicol formulations were determined at 278 mu. The values are represented in table 9.

Table 8

Results of analysis of simple chloramphenicol capsules

Capsule No.	1	2	3	4	5	6	7	8	9	10
Average net weight of 1 capsule (mg)	293	349	304	470	306	611	266	270	346	306
Label claim of chloramphenicol (mg/caps.)	250	250	250	250	250	250	250	250	250	250
1st. Determination (mg/caps.)	251	262	250	250	261	250	260	258	240	262
2nd. Determination (mg/caps.)	245	258	246	251	261	249	261	270	240	264
3d. Determination (mg/caps.)	248	258	249	245	261	254	262	272	234	264
4th. Determination (mg/caps.)	248	257	250	252	258	254	263	272	241	262
5th. Determination (mg/caps.)	248	258	251	253	261	251	260	262	241	263
Average (mg/ caps.)	248	258	249	250	260	251	261	266	239	263

Table 9

Spectrophotometric Study for Vitamin Interference at 278 mu.

Name of vitamin	Average occurrence per caps (mg)	Amount present /caps. in%	Absorbance at 278 mu of 2 mg% aq. sol.	Absorbance contribution per sample aliquot analyzed
1. Thiamine HCl (Vit.B1)	4	1.6	0.33	0.005
2. Riboflavine (Vit.B2)	3	1.2	0.96	0.011
3. Pyridoxine HCl (Vit.B6)	3	1.2	0.34	0.004
4. Nicotinamide (Vit.B3)	15	6.0	0.06	0.004
5. Folic Acid (Vit.Bc)	1	0.4	0.51	0.002
6. PABA (Vit.Bx)	10	4	1.60	0.060
7. Ascorbic acid (Vit.C)	50	20	0.12	0.024
8. Biotin (Vit.H)	10	4	0.00	0.000
9. Menadione (Vit.K3)	5.2	2.1	0.49	0.010
10. Ca-Pantothenate (Vit.B5)	3.5	1.4	0.00	0.000
Total	104			0.12

From this table one can estimate the collective effect of the absorbance values of all vitamins possibly occurring in an average capsule formulation. This total absorbance value sums up to about 20% re-evaluated in terms of chloramphenicol. Therefore, it is imperative that vitamin removal is ascertained for assessing the absolute concentrations of chloramphenicol in vitaminized samples. Aqueous solvents were found to remove all the above mentioned vitamins, axcept for riboflavine, menadione and biotin. These three vitamins are water insoluble to the extent that they may not be eliminated by the volume of the aqueous solvent used in the extraction purposes. Their solubilities expressed in mg/ml H20 at 25°C are 0.1, 0.1, 0.5 respectively. The complete extraction of biotin is immaterial because its presence does not interfere with the subsequent assay of chloramphenicol at 278 mu. However, the vitamins B2 and K3, do interfere intensely at that wave length (see table 9). Therefore, their complete removal is important.

Experimentally 0.5N Na2 CO3 solution was found to be

very effective in the removal of riboflavine but not menadione. The removal of menadione was efficiently effected by dilute solutions of $NaHSO_3$. To substantiate this statement and to assess the optimum conditions under which menadione could be efficiently removed; the following simple experiment was performed.

A 10 mg. sample of menadione was subjected to extraction using 25 ml of 4, 3, 2, 1, 0.5, 0.25 and 0.1% of aqueous $NaHSO_3$ for 30 minutes. The results revealed that any concentration below 1% was not sufficient to extract the 10 mg menadione taken. Therefore, the 1% $NaHSO_3$ solution was adopted for extraction purposes for samples labelled to contain menadione, irrespective of its concentration, as well as other water soluble vitamins.

(b) Interference of $NaHSO_3$ and Na_2CO_3 solutions:

However, to evaluate the effect of $NaHSO_3$ on the solbility of chloramphenicol itself an additional experiment was performed. (One should not expect any decomposition of chloramphenicol by acid-base catalysis since $NaHSO_3$ is almost neutral

And yet, chloramphenicol solubility was appreciably effected by salting-out in the presence of $NaHSO_3$ aqueous solutions. The following are the results of solubility obtained by using 1% and 2% $NaHSO_3$ aqueous solutions and water: 3.62, 3.47 and 3.83 mg/ml respectively.

Similar solubility experiments were performed for the evaluation of the effects of various concentrations of Na_2CO_3 aqueous solutions on chloramphenicol. The results indicated that 0.1N to 0.5N, Na_2CO_3 solution had no decomposition effect on chloramphenicol. It is only a matter of salting-out, which does not interfere in the final determination of chloramphenicol, since both the sample and the standard are extracted with the same solvent.

In general, water soluble vitamins can be efficiently extracted with specified volumes of H_2O or dilute HCl solutions (0.1 - 0.5N) However, chloramphenicol formulations containing only riboflavine, in addition to the above stated water soluble vitamins, should be extracted twice. The first extraction, as mentioned above,

should be followed by a second extraction using a dilute solution of Na_2CO_3 (0.1 - 0.5N). However, capsules containing only menadione in addition to the water soluble vitamins should be extracted with 1% $NaHSO_3$.

If both riboflavine and menadione occur in the same sample the following order of extraction should be respected: first extraction should be performed by 1% solution of aqueous $NaHSO_3$ followed by a similar extraction using (0.1 - 0.5N) Na_2CO_3 solution. This sequence of extraction is important so as to avoid any possible precipitation, e.g. aneurine HCl.

After the removal of all interfering vitamins or other degradation products, by the suggested appropriate modification of the method the chloramphenicol residue is washed with the filtrate of the standard, as previously stated, and subjected to spectrophotometric analysis.

Table 10 consists of 10 different vitaminized chloramphenicol capsule formulations numbered from 11 - 20. They were analyzed by the above proposed method, using the proper solvent(s)

for extraction. Numbers 14 and 20 contain only water soluble vitamins. Numbers 11, 12, 15 and 18 contain riboflavine, and numbers 13, 16, 17 and 19 contain both riboflavine and menadione in addition to the water soluble vitamins. Only number 12 is a sugar coated tablet (dragee). The averaging is a result of five separate determinations.

Table 10

Results of analysis of vitaminized capsules

Capsules No.	11	12	13	14	15	16	17	18	19	20
Average net Wt. (mg/caps)	423	347	344	347	363	295	307	319	270	323
Label claim(mg/caps) of chloramphenicol	250	250	250	250	250	250	250	250	250	250
Ist. Determination (mg/caps.)	274	236	272	256	281	274	268	258	253	253
2nd. Determination (mg/caps.)	274	241	274	259	280	269	261	261	252	252
3rd. Determination (mg/caps.)	271	243	272	257	267	268	262	256	249	-
4th Determination (mg/caps.)	268	233	274	254	277	272	263	256	248	-
5th. Determination (mg/caps.)	267	236	274	255	276	270	263	258	237	-
Average (mg/caps.)	271	238	273	256	276	271	263	256	248	252

3- Capsules containing other antibiotics or antibacterials:

Many pharmaceutical formulations contain besides chloramphenicol other antibiotics such as tetracyclines and streptomycin sulfate or antibaterial agents such as sulfa drugs. These antibiotics or antibacterials should be removed before attempting any determination of the chloramphenicol contents in these formulations. Seven different capsule formulations are tested by the proposed method.

(a) Formulations containing tetracyclines:

Capsule formulations number 21 and 22 contain, in addition to chloramphenicol, tetracycline HCl and tetracycline phosphate respectively. Tetracycline HCl is water soluble and is easily extracted with water or dilute (0.1 - 0.5N) HCl.

Table 11

The effects of the presence of tetracycline HCl and tetracycline phosphate on chloramphenicol analysis

Tetracyclines admixed with chloramphenicol	Tetracycline HCl			Tetracycline phosphate		
Wt. of chloramphenicol(mg) in the samples	104	100.2	104	91.2	89.5	89.48
Wt. of tetracycline(mg)	00	100	200	00	100	200
Solubility of chloramphenicol in mg/ml of solvent used*	3.84	3.86	3.84	3.85	3.86	3.89
Chloramphenicol recovered(mg)	104	99.6	103.80	91.2	89.35	88.2
Percent recovery	100%	99.3%	99.7%	100%	99.8M	98.5%

* In case of tetracycline HCl pure H20, and in case of tetracycline phosphate 0.5N HCl were used as extracting solvents. In the analysis of commercial products the use of 0.5N HCl is recommended; because sometimes they use an equivalent quantity of tetracycline base instead of its hydrochloride salt.

Table 11 proves that the presence of tetracycline HCl in chloramphenicol did not interfere with the accuracy of the method for the analysis of chloramphenicol in the presence of as much as 60 - 70% of the amount of the whole sample.

However tetracycline phosphate complex is very insoluble in water. To remove it from chloramphenicol fomulations it was found that 0.5N HCl rendered it completely soluble. Assuming that during the treatment with 0.5N HCl the tetracycline phosphate is changed to its HCl, thus liberating metaphosphoric acid (HPO_3); it is therefore, important to evaluate any possible effect of the liberated HPO_3 on the solubility of chloramphenicol. Experiments were run to determine the solubility of chloramphenicol in various proportions of HPO_3. The results of such experiments indicated that when up to 30% of HPO_3 were incorporated with the chloramphenicol standard (much above the quantity which could be liberated) has no effect whatsoever on the solubility of chloramphenicol. The following are experimental findings.

Percent HPO_3 in chloramphenicol sample	0%	15%	30%
Solubility of chloramphenicol mg/ml	3.9	3.93	3.89

Synthetic known mixtures containing different proportions of chloramphenicol and tetracyclines were subjected to analysis by the proposed method. The analytical results are given in table 11. The extraction solvent used was 0.5N HCl.

Therefore, it is important to note that all samples containing tetracycline phosphate in chloramphenicol formulations should be subjected to 0.5N HCl extraction rather than H_2O. The results of 5 determinations of capsules numbers 21 and 22 are given in table 13.

(b) Formulations containing sulfa drugs:

Sulfadiazine (S.D.), sulfaguanidine (S.G.) and phthalylsulfathiazole (P.S.T.) are sometimes incorporated with chloramphenicol formulations. These sulfa drugs may be as much as in equal quantities or more compared to chloramphenicol. However, certain formulations may contain in addition to the above, bismuth carbonate in appreciable quantities. The best solvent for the removal of sulfa drugs were as follows: P.S.T. and S.D. were found to be easily removed by 0.5N Na_2CO_3 and S.G. was easily removed by

0.5N HCl. Equal quantities of these three sulfa drugs were mixed individually with almost the same weights of chloramphenicol. These synthetic sample mixtures were subjected to the analysis by the proposed method. The results are tabulated in table 12.

Capsule No. 23 contains only S.G. in addition to chloramphenicol, it is extracted once or twice with 0.5N HCl alone because there are no interfering substances or vitamins. Capsule No. 24 formulation contains S.D. and P.S.T. in addition to vitamins including menadione; extract this sample first with 1% $NaHSO_3$ followed by 0.5N Na_2CO_3 aqueous solution. The results of 5 determinations are indicated in table 13.

(c) Formulations containing streptomycins:

The presence of dihydrostreptomycin (D.H.S.) sulfate in capsule formulations (Nos. 25, 26 and 27) as such should not present any difficulty for chloramphenicol analysis because of its extreme solubility in H_2O. However, its effect on the solubility of chloramphenicol should be evaluated quantitatively before adopting the analytical technique. This was tested by known

Table 12

The effect of the presence of sulfa drugs on chloramphenicol analysis

Sulfa drug mixed with known sample of chloramphenicol:	Phthatyl sulfa thiazole		Sulfa diazine		Sulfaguanidine	
Wt. of chloramohenicol in the sample (mg).	90.65	96.35	87.85	99.75	93.25	96.8
Wt. of sulfa drug in the sample (mg).	0.00	100	0.00	100	0.00	100
Solubility of chloramphenicol in mg/ml solvent	3.2	3.23	3.15	3.18	3.82	3.85
Recovery of chloramphenicol (mg)	90.65	95.3	87.85	99.05	93.25	96.05
Percent recovery	100%	99%	100%	99.4%	100%	99.25%

synthetic mixtures using pure chloramphenicol and D.H.S. sulfate in various proportions. The solubility of chloramphenicol in water was not effected whatsoever by concentrations of D.H.S. sulfate, in the chloramphenicol samples, up to 70%. However, in such formulations, equivalent quantities of D.H.S. base can be used instead of its sulfate salt. In such cases it is advisable to adopt the use of 0.5N HCl as the extraction solvent. This precaution of using 0.5N HCl as the extraction solvent will also remove the S.G. which is sometimes included in similar formulations. Certain formulations of chloramphenicol and D.H.S. sulfate (No.27) are dispersed in an oily medium. These samples cannot be analyzed by the regular procedure so far proposed. The oily excipient should be removed prior to the analysis of such samples. To achieve this, various solvents were tested and the best among these were found to be n-hexane and petroleum ether. The solubility of chloramphenicol in these two solvents are almost negligible. They were found to solubilize chloramphenicol to the extent of less than 1 mg per 100 ml of solvent. Petroleum ether being much easily available and

cheaper was adopted for such extractions. To overcome any discrepancies of chloramphenicol solubility during such extraction the precaution of using saturated solution of this solvent with respect to chloramphenicol at room temperature was adopted. After complete extraction of the oil the residue was subjected to the proposed extraction method followed by the subsequent analysis.

The result of the analysis of three capsule formulations indicated above (Nos. 25-27) are shown in table 13.

4 - Confirmatory experiments:

In the analysis of pharmaceutical products containing chloramphenicol; various capsule formulations so far tested by the proposed method, were further subjected to other analytical techniques claimed to be reliable and reproducible. Among these techniques, the two worth mentioning are the ones proposed by Kassems and el-Nimr (27) using thin-layer chromtography and Cosi (28) resorting to paper chromatography. These methods were repeated using known samples and found to be dependable for simultaneous estimation of chloramphenicol in pharmaceutical formulations.

Table 13

Results of analysis of chloramphenicol capsules containing other antibiotics or antibacterials

Other active constituents admixed.	Tetracycline		Sulfa Drugs		D.H.S.		
Variety	HCl	Phosph.	S.G.	P.S.T & S.D.	Sulfate & S.G.		Sulfate
No.	21	22	23	24	25	26	27
Average Net weight(mg/caps.)	411	411	549	335	399	388	
Label claim(mg/caps.) of chloramphenicol	150	200	60	100	125	220	125
Ist. Determination (mg/caps.)	147	209	64	100	131	214	115
2nd. Determination (mg/caps.)	149	208	65	95	127	218	112
3rd. Determination (mg/caps.)	147	212	64	97	128	225	117
4th. Determination (mg/caps.)	149	210	64	96	130	228	112
5th. Determination (mg/caps.)	146	213	63	-	132	220	114
Average (mg/caps.)	148	210	64	97	130	221	114

They were adopted by us as a supplementary checking on the reliability of the proposed method. Several commercial samples, chosen at random, which were already analyzed by our method were resubjected for checking by T.L.C. chromatography (27) and paper chromatography (28) techniques refered to above. In addition to the above methods one of the capsules was further analyzed by phase-solubility method. These results of the three methods are shown in table 14.

A comparative study of the analytical results obtained in this table reconfirm favorably the accuracy and reproducibility of the proposed method.

Table 14

Comparative analytical results obtained by various methods (27-28).

Capsule No.	2	6	11	19	20	22	25
Average net weight per capsule (mg)	394	611	423	270	323	411	323
Label claim of chloramphenicol (mg/caps.)	250	250	250	250	250	200	125
Proposed method (Average of 5 determinations(mg/caps.)	259	252	271	248	252	210	130
The conventional phase solubility method(one determination mg/caps.)	-	-	-	-	253	-	-
Thin-layer chromatography(Average of two determinations) mg/caps.)	256	250	271	244	-	211	126
Paper chromatography(Average of 2 determinations)mg/caps.)	256	254	247	246	-	205	127

Analysis for Chloramphenicol in Oleogenous Bases

Under this heading the following dosage forms of chloramphenicol may commonly be included: ointments, suppositories and ovules. A survey of various formulations of these dosage forms revealed the use of the following bases: vaselin, wool fat, cocoa butter, as well as B.P. '63 and U.S.P. XVII ointment bases. The solubility of chloramphenicol in the above mentioned bases were tested as follows: transfer an accurate weight of the base to the 'extraction-filter' tube containing excess chloramphenicol. Shake in the constant water bath at 60°C for 30 minutes. Filter the melted base quickly by suction. Any appreciable residue of oleogenous bases congealing in the tube during filtration is reheated at 60°C in an oven refiltered until no conceivable amount remain in the 'tube'. The chloramphenicol residue in the 'tube' is further subjected to washing with organic solvents to remove completely traces of such oleogenous bases. The most convenient solvent to be used was found to be petroleum

ether which has been previously saturated with chloramphenicol at room temperature. The purified residue thus obtained was analyzed by the usual spectrophotometric method. From the results of the analysis of the residue, the loss is estimated and consequently the solubility of chloramphenicol in the base concerned is computed. This was found to be 0.18, 0.65 and 0.67 mg. per gm at 60°C of vesalin, wool fat and cocoa butter respectively.

However, upon further solubility investigations it was found that when these bases were exposed to excess of chloramphenicol mixed in the 'tubes' and the mixture heated directly in an oven at about 60°C or at higher temperatures,as the case may need, until the base is melted. These were immediately filtered by suction. This process of heating and filtration could be repeated until the residue seemed to be free from oleogenous bases. When this method applied to a series of bases it was found that the solubility of chloramphenicol in the bases tested were reduced greatly compared to the shaking process. The comparative results of solubilities by these two methods are shown in table 16. These are the average of two determinations.

Table 15

Solubility of chloramphenicol in melted ointment bases at 60°C

Ointment base	Solubility of chloramphenicol in mg/gm base	
	Ist Method*	2nd Method**
1) B.P. '63 ointment bases:		
a) Paraffin ointment	0.259	0.03
b) Simple "	0.39	0.03
c) B.P. eye "	0.25	0.03
2) U.S.P. XVII Ointment bases:		
a) Hydrophylic ointment	Very soluble	
b) Polethylene glycol ointment	Very soluble	
c) White ointment	0.145	.013

* Shaking at 60°C for 30 minutes.

** Melting directly in an oven followed by filtration.

The above mentioned ointment bases were prepared carefully according to pharmacopeial specifications and the solubility of chloramphenicol in each was determined by the first approach. The second approach was used to determine the possible loss of chloramphenicol per gram of base during the analysis.

Therefore, for manipulatory convenience and due to the negligible loss of chloramphenicol in the melted base the second method was followed in all subsequent analysis of these dosage forms. As stated in table 15, melted, polyethylene glycol ointment (U.S.P.) and hydrophyllic ointment (U.S.P.) dissolves chloramphenicol to a great extent. Consequently the above two nonoleogenous ointment bases render the proposed method not applicable for the analysis of chloramphenicol in them.

The following procedure is followed in the analysis of chloramphenicol in oleogenous bases: Transfer an accurately weighed sample from the collapsible tube directly into the "extraction-filter" tube. Melt the base in an oven at 60°C or more if required. While hot, filter the melted base quickly by suction. Reheat and filter until no conceivable traces of the base are noticeable. Wash the chloramphenicol residue with petroleum ether already saturated with chloramphenicol, until it is free from any traces of oleogenous base. The above sample residue and an equivalent weight of chloramphenicol standard are subjected to

the proposed method of extraction and quantiation.

A number of proprietory products; some of which contain only chloramphenicol in oleogenous bases while others contained besides chloramphenicol either cortisone alone or admixed with peru balsam, were analyzed by following the above indicated method.

Cortisone does not represent any difficulty in the analytical method adopted because it has no absorption, whatsoever, at 278 mu, while the presence of peru balsam represents significant hazards when absorption at 278 mu is considered for the analysis; because the presence of benzoic and cinnamic acids or their esters absorb greatly at the chosen wave length. Therefore, their removal before spectrophotometric determination is imperative. This removal can be achieved completely using twice 0.5N Na_2CO_3 as the extraction solvent.

The analysis of suppositories presented no difficulty; since the two oleogenous suppository formulations analyzed were simple and contain only chloramphenicol in the base.

The analytical results of 3 eye ointments, one dermic ointment and two suppositories are given in table 16.

Table 16

Analytical results of chloramphenicol dosage forms

containing oleogenous bases

Product analyzed	Ointments Ophthalmic Oint.			Skin Oint.	Suppositories	
Sample No.	1	2	3*	4**	5	6
Label claim of chloramphenicol.	1%	1%	1%	2%	200mg/ Supp.	250mg/ Supp.
Ist. Determination	1.0%	0.91%	1.04%	2.04%	198mg.	239 mg.
2nd. Determination	0.983%	0.94%	0.985%	1.92%	148mg.	234 mg.
3rd. Determination	1.01%	0.38%	-	-	206mg.	242 mg.
Average	1.001%	0.93%	1.012%	1.98%	201mg.	238 mg.

* Active ingredients are chloramphenicol in addition to conrtisone.

** Active ingredients are chloramphenicol in addition to cortisone and peru balsam.

Analysis for Chloramphenicol in Parenterals

Chloramphenicol sodium succinate happened to be the most important salt occurring in all such formulations. Therefore, the objective here is to devise a method by which chloramphenicol succinate could be analyzed by the proposed method. To achieve this we had first to convert the sodium salt to its acid form by acidifying the medium with HCl solution (0.1N). The resulting mixture containing the precipitated chloramphenicol succinic acid derivative was subjected to the analysis by the proposed method. Before running the analysis the following preliminary quantitative tests were to be performed on known samples to assess the solubility of chloramphenicol succinic acid in the extracting solvent. Excess of the succinic acid derivative was shaken for different lengths of time at 25°C using 0.1N HCl as the solvent. The solubility values were found to be 3.05, 3.055, 3.05 and 3.05 mg/ml 0.1N HCl when shaken for 2 hours, 1 hour, 30 min. and 15 min. respectively. As the above results indicate, idental values of solubilities were obtained regardless of time; whether shaken for 2 hurs. or 15 min.

Therefore, 30 minutes of shaking as suggested in the proposed method is readopted.

The spectral profile of chloramphenicol sodium succinate revealed a peak in H_2O at 276 mu instead of 278 mentioned for chloramphenicol. The $A^{1\%}_{1cm}$ value for this compound, at this wave length, was computed to be 222 instead of 292 for chloramphenicol.

The following procedure is adopted for the analysis of chloramphenicol succinate - Na vials (labelled 1 gm/vial). The vial contents are dissolved quantitatively into 25.0 ml volumetric flask by the help of H_2O. 5.00 ml of this solution (representing 200 mg) are carefully transfered to an 'extration-filter' tube, using appropriate technique. A solution of the chloramphenicol hemisuccinate (acid form) standard is prepared in 10.0 ml volumetric flask by dissolving about 380 mg of the standard drug with a calculated amount of 0.1N NaOH. 5.00 ml of this solution are transfered to another tube using the same technique as previously stated. Into both, sample and standard, pipet 10.0 ml of 0.1N HCl and let stand for few minutes until precipitation is complete. An additional

10.00 ml of H20 were added to the above tubes' and were shaken under the experimental specifications adopted for 30 minutes, and then filtered immediately under suction. The residues were washed by the filtrate obtained from the standard and then analyzed spectrophotometrically.

The results obtained from the analysis of one vial, in duplicate are as shown below:

Sample	Chloramphenicol sodium succinate
Label claim	1 gm/vial
Results obtained	1.11 gm and 1.08gm/vial
Average	1.095 gm/vial

Supplementary Notes on Other Dosage forms

This class includes suspensions for oral use and solutions for topical use.

1. Suspensions: Chloramphenicol palmitate is always provided in suspensions for oral use.

The following preliminary investigations were run on chloramphenicol palmitate standard: hydroalcoholic solutions with various concentrations of alcohol (10-95% v/v) were used in this preliminary test to assess the optimum conditions under which a solvent could be found best fit for extraction purposes. 'Tubes' containing excess of chloramphenicol palmitate were treated with different concentrations of hydroalcoholic solutions and shaken mechanically, at room temperature, for about 1hr. Clear supernatants of each tube were analyzed spectrophotometrically for chloramphenicol palmitate content. These analytical results revealed that it is almost completely insoluble in up to 50% alcohol. Above this concentration the solubility of chloramphenicol palmitate

gradually increased under the test conditions. It was found that solubility of chloramphenicol palmitate in 80% alcohol at 25°C was about 4 mg/ ml. Alcohol will best serve for extraction purposes accounting for its solubilizing effect of several impurities and decomposition products which could have possibly occurred in the samples. The optimum time of shaking to achieve equilibration at 25°C was also found to be 30 minutes.

The $A^{1\%}_{1cm}$ in 95% alcohol was found experimentally to be 170 at 273 mu.

But, the chloramphenicol palmitate has to be separated from the syrup before analysis. This was accomplished by either; the U.S.P. XVI method followed by evaporation of the CHCl3 using a jet of nitrogen or by following the method given by Masterson (38). The residue obtained by either method and a standard were simultaneously extracted with 80% alcohol in (125ml) conical glass stoppered flasks, by shaking for 30 minutes at 25°C. (However specifying 80% alcohol is not critical in this type of solubility analysis. Similar concentrations of hydroalcoholic solutions

(± 5%) could have been used safely provided sample and standard are treated with identical solvent). The residues obtained in each case were determined spectrophotometrically and initial concentrations of chloramphenicol palmitate computed.

Two chloramphenicol syrups were subjected to the analysis by the proposed method, and by the U.S.P. XVI method. Three determinations were almost reproducible but very low in results compared to U.S.P. XVI method. These results require certain explanation pending upon the additional experimental work to prove its validity. From the results obtained it can be deduced that the syrups are not prepared from pure chloramphenicol palmitate, but rather from mixture of both the palmitate and chloramphenicol. Since the U.S.P. XVI method analyses the $CHCl_3$ extract spectrophotometrically; it does not distinguish between both. But, our method purifies the chloramphenicol palmitate from all interfering substances including chloramohenicol itself.

2. Solutions: Since chloramphenicol is very slightly soluble in water, it is difficult to obtain appropriate concentrated aqueous

solutions for medicinal use. Sodium borate is known to have appreciable solubilizing effect on chloramphenicol in aqueous solutions. To analyze such products by the proposed method, it is imperative that the solubilizing effect of borate should be neutralized so as to permit the excess of chloramphenicol to precipitate. Among the borate precipitants ZuSO4 aqueous solution was found to be the most suitable. It is able to precipitate the borate ion from chloramphenicol-borate complex without any interference.

The above statement is substantiated by the following experiment: excess chloramphenicol was treated with the following solvents and the corresponding solubilities were determined:

Solvent	H_2O	0.1N sod.bor.	0.1N ZuSO4	0.2N ZuSO4
Solubility of chloramphenicol in mg/ml solvent	3.9	18.3	3.76	3.52

Salting out effect is noticed in the last two cases.

However, under these experimental conditions when only a slight excess of ZuSO4 was added to 0.1N sodium borate solution the chloramphenicol solubility in the solvent dropped to about

3.9 mg/ml. (comparable to that of water)

Although no samples were analyzed due to the lack of time, the following procedure can be proposed for the analysis of borate buffered dosage forms. An equivalent amount of ZuSO4 should be used to precipitate the anticipated borate ions present in the sample preparations. And then this sample and a standard are subjected to the analysis by the proposed method, by extracting them with 0.1N ZuSO4 solution.

D I S C U S S I O N

Chloramphenicol is one of the most widely used antibiotics. Its administration in proper doses for treatment is very essential. Therefore, strict analytical control on numerous formulations containing chloramphenicol is worth serious consideration. As previously indicated most chemical or instrumental methods used in chloramphenicol determinations are not specific. They are primarily functional group quantitations. Therefore, it is obvious and significant that any interfering substance(s) containing similar functional groups as well as degradation products of chloramphenicol itself should be separated prior to any analytical procedure adopted for chloramphenicol estimation.

Chromatographic and the like techniques, recently developed are sometimes effective in separating such interfering substances as well as degradation products of chloramphenicol (25-31). Procedure-wise, these are lengthy and tedious due to the very many steps involved in the course of analysis.

The analytical approach developed by this work may be

considered preferable to most quantitation methods, for chloramphenicol, yet available. It is simple and straight forward that as much as 10 samples can be simultaneously analyzed in about 4 - 5 hrs.

This proposed method as well as all other nonbiological methods of analysis are inferior to the bioassay method because d-chloramphenicol is not excluded from the picture. Since this is biologically inactive it can only be detected by the bioassay method. But, due to the inherent low degree of precision (25) complexities, expense and time consuming characters of this method, it is only resorted to as for reconfirmation purposes. It is too tedious to adopt as a routine analytical technique for chloramphenicol preparations.

Being a solubility type of analysis this proposed method is superior to the conventional phase-solubility method of analysis. Timewise, it is a matter of hours compared to days. It is as precise and exact as the above mentioned method in the case of the analysis of substances below 99.5% pure where conventional

method does not distinguish between 100% pure substances and 99.5% pure ones; due to the difficulty in distinguishing between the slopes of the second segments of the phase-solubility diagrams of these two substances. By this proposed method the purer the substance the less the interference of impurities and consequently the better the precision. The modification introduced by Stenger and his co-workers (6) to fulfill this purpose is complex, tedious and requires large amount of sample (about 10 gms).

The proposed method can be extended further to the analysis of many complex pharmaceuticals.

The development of the 'extraction filter' tube, especially type B (Fig. 3), can serve as an effective tool for separation and purification techniques, especially when multiple extractions are required. Due to the lack of glass blowing facilities type A 'tube' was adopted. However 'tube' B may be found more proficient for such analytical work as well as for purification purposes.

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