Assay Procedure for Pharmaceutical Combinations of Aspirin, Phenacetin, Caffeine, and Itobarbital with Phenothiazine Derivatives I

By PAUL TURI

A procedure is presented for the quantitative determination of combinations of aspirin, phenacetin, caffeine, and itobarbital with salts of phenothiazine derivatives. The powdered mixture is dissolved and passed through a series of partition chromatography columns. The ingredients are separated by specific trap segments, eluted by selective solvents, and assayed by any suitable procedure. The assay technique offers a rapid and accurate determination of the five ingredients.

MIXTURES OF acetylsalicylic acid, acetophenetidin, and caffeine—by themselves or in combination with other drugs—are among the most popular pharmaceutical preparations. To assay quantitatively the components of these mixtures, a number of authors (1–6) applied modified partition chromatography as a very convenient and adequate technique. Most recently, Heuermann and Levine (5) published two procedures: one variation was adapted for assaying APC with barbituric acid derivatives, the other variation for analyzing APC combined with organic bases.

Our objective was to develop a single method which facilitates an assay for APC compounds combined with both barbituric acid derivatives and certain organic bases, particularly with phenothiazine derivatives.

The first and most important problem was to find a stationary phase system providing a selective entrapment for the individual ingredients. The combination column containing a 7.5% tartaric acid–Celite segment and a 10% sulfuric acid–Celite segment which had been described to trap simultaneously caffeine and many organic bases (5), was unsuitable for the phenothiazine derivative containing mixtures for the following reasons: (*a*) phenothiazine derivatives degrade rapidly in presence of mineral acids (such as 10% sulfuric acid) by forming strongly colored products; and (*b*) solubilities of the phenothiazine derivatives and the caffeine are very similar. For this reason, chloroform, which can be used as a selective eluant of caffeine from its mixtures with many stronger bases, cannot be considered for the separation of caffeine-phenothiazine derivative combinations. A part of this study aimed therefore to find a trap segment, which separates the phenothiazines from the other ingredients, particularly from the caffeine.

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In selecting the proper solvent system, chloroform appeared to be superior by its ability to dissolve quantitatively the salts of the phenothiazine derivatives as well as the aspirin, phenacetin, caffeine, and itobarbital. However, in order to achieve transfer of the phenothiazine derivative and the caffeine to the immobile phase in the proper columns, the addition of a more polar solvent (such as diethyl ether) was needed. The distribution coefficients of the solutes are thereby shifted to favor the aqueous phases. The appropriate solvent ratio was investigated.

Once the satisfactory stationary phase and mobile phase had been determined, there was no difficulty in recovering the individual ingredients by the conventionally used eluants.

EXPERIMENTAL

Apparatus

Chromatographic Columns.—Glass tubes ($8 \times {}^{3}/_{4}$ in.) with $3 \times {}^{5}/_{16}$ in. stems were used. The stationary phase solutions were mixed with Celite 545 and packed into the columns in the conventional manner (7). A scheme for the assembly of the chromatography tube series is shown in Fig. 1.

Spectrophotometer.—Assays were performed on a Cary 11 recording spectrophotometer, using 1-cm. cylindrical silica cells.

pH Meter.—Beckman Zeromatic was used.

Reagents

The list of reagents is presented in Table I.

Procedures

Ethyl Ether: Chloroform Solvent Ratio.—Earlier

	Aqueous stationary phase: Composition	ml.	Celite 545: Gm.
I.	Tartaric acid, 1% (w/v)	3	3
II.	Sulfuric acid, 12% (v/v)	3	3
III.	Sodium bicarbonate, 8.4% (w/v)	2	2
IV.	Tripotassium phosphate, 22.1% (w/v)	3	3

Fig. 1.—Schematic assembly of chromatography column series.

TABLE I.—DESCRIPTION OF REAGENTS

Name	% Composition, in water	Grade
Tartaric acid sol.	1 (w/v)	U.S.P. XVI
Sulfuric acid sol.	12 (v/v)	U.S.P. XVI
Sodium bicarbonate sol.	8.4 (w/v)	U.S.P. XVI
Tripotassium phosphate sol.	22.1 (w/v)	U.S.P. XVI
Chloroform		ACS water-washed
Ethyl ether		ACS, redistd., water-washed
Isooctane		U.S.P. XVI

TABLE II.—DISTRIBUTION RATIO OF THIORIDAZINE HYDROCHLORIDE

Phase I	1% Tartaric Acid—		
Phase II	Chloroform	Ether:Chloroform 4:1	Ether:Chloroform 8:1
α^a	0.1	5.6	6.3

[a] α = solute concn. in phase I/solute concn. in phase II.

experimental results indicated that a complete separation of the ingredients can be achieved only by a properly selected solvent system. In this study, the distribution ratio of thioridazine hydrochloride between 1% tartaric acid and several organic solvents has been measured at 25° by shaking extensively 10.0 mg. of this substance with equal volumes of the solvent pair and, after a complete separation, determining the concentrations spectrophotometrically (Table II).

Subsequently, 10.0-mg. amounts of several phenothiazine derivatives had been dissolved in 100.0-ml. portions of chloroform. A 5.0-ml. aliquot of each solution was mixed with 40 ml. of ethyl ether and passed through a chromatography column containing 3 ml. of 1% tartaric acid on 3 Gm. of Celite. The eluates were collected into glass-stoppered Erlenmeyer flasks. To simulate assay conditions, the columns were then washed with consecutive 20, 20, and 10-ml. portions of an ether:chloroform (8:1) solvent, and these washings were also combined in the receiving flasks. The eluates were then evaporated to incipient dryness, the residues dissolved in suitable solvents, and the released amounts were determined spectrophotometrically (Table III).

Separation of Phenothiazine Derivatives from Caffeine.—Thioridazine hydrochloride[1] was used as a model in the following experiments. A mixture of 20.0 mg. of caffeine and 5.0 mg. of thioridazine hydrochloride was dissolved in 50.0 ml. of chloroform. Aliquots of 5.0-ml. of this solution were thoroughly mixed with 40-ml. portions of ethyl ether and passed through separate Celite columns[2] containing aqueous solutions of tartaric acid in different concentrations. (The pH values of these solutions had been measured at 25°.) After collecting the effluents in 100-ml. volumetric flasks, each column was washed with consecutive 20, 20, and 10-ml. portions of the ether:chloroform (8:1) solvent. These eluates were also collected into the same receivers which were finally adjusted to volume with the solvent mixture at 20°. Aliquots of 25.0 ml. of these solutions were then evaporated to incipient dryness, the residues redissolved in 25.0-ml. volumes of chloroform, and the ultraviolet absorption curves were measured (Table IV).

[1] Marketed as Mellaril Hydrochloride by Sandoz Pharmaceuticals.

[2] Before introducing the sample solutions, the columns had been rinsed with 20-ml. portions of ether. The rinsing solvents were then discarded.

TABLE III.—RETENTION OF PHENOTHIAZINE DERIVATIVES

Mobile Phase: Ether:Chloroform (8:1); Stationary Phase: 1% Tartaric Acid–Celite

Compound	% Released	% Retained (calcd.)
Thioridazine HCl	0.24	99.76
Thiethylperazine dimaleate	0.35	99.65
Chlorpromazine HCl	0.32	99.68
Promethazine HCl	0.22	99.78

TABLE IV.—SEPARATION OF THIORIDAZINE AND CAFFEINE

Mobile Phase: Ether:Chloroform (8:1); Stationary Phase: 3 ml. of Tartaric Acid + 3 Gm. of Celite

Concn., % of the tartaric acid sol.	pH	Released caffeine, %	Thioridazine entrapment
7.5	1.35	67.50	complete
3.0	1.70	83.70	complete
1.0	2.07	99.87	complete
0.5	2.25	101.25	complete

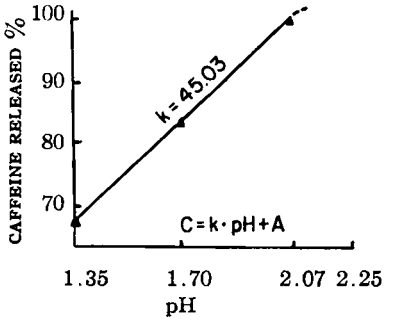

Fig. 2.—pH dependence of the caffeine release.

Quantitative Assay Procedure for the Five Active Ingredients.

Preparation of the Sample.—The average weight of the tablets or capsules was determined. A representative number of tablets was reduced to a fine powder, or a similar number of capsules was emptied and homogenized. In our experiments, the following typical mixture was used: acetylsalicylic acid (200 mg.), caffeine (40 mg.), acetophenetidin (130 mg.), itobarbital[3] (50 mg.), phenothiazine derivative salt (5–20 mg.). In some of the experimental mixtures, inactive ingredients such as stearic acid, talc, sucrose, starch, and lactose were also added; these excipients did not interfere with the assay procedures.

Assay.—A portion of the homogenized powder (equivalent to the weight of a quarter or a half of a dosage unit) was placed into a 50-ml. amber volumetric flask and shaken mechanically with 25 ml. of chloroform for 15 minutes. The solution was then adjusted to volume with chloroform at 20°, mixed

[3] Marketed as Sandoptal by Sandoz Pharmaceuticals.

TABLE V.—ASSAY RESULTS

Sample	% of Label Claim				Phenothiazine Derivative	
	Aspirin	Phenacetin	Caffeine	Itobarbital	Type[a]	% of Label Claim
A1	92.09	104.46	96.94	97.93	T	98.57
A2	92.09	101.92	95.42	100.27	T	99.45
B1	95.10	103.07	99.80	99.66	T	102.20
B2	94.75	103.69	99.80	102.53	T	99.40
C	95.85	100.92	98.16	104.34	T	92.86
D	96.73	99.23	96.84	97.87	TP	108.40
E1	100.70	100.33	97.62	96.85	C	104.12
E2	101.00	100.83	96.35	96.14	C	106.86
F	96.23	99.23	94.82	101.42	P	98.59

[a] Symbols: T = thioridazine hydrochloride, TP = thiethylperazine dimaleate, C = chlorpromazine hydrochloride, P = promethazine hydrochloride.

well, and a 5.0-ml. portion was transferred (without filtration) into a beaker containing 40 ml. of ethyl ether. (Sample solution.)

The assembled column series (Fig. 1) was washed with 35 ml. of ether and the washing discarded. A 250-ml. glass-stoppered Erlenmeyer flask was placed under the column series. The sample solution was quantitatively transferred onto column I, allowed to flow through the column series, and collected in the receiver. After the passage of the first eluate, the column system was eluted with consecutive 20, 20, and 10-ml. portions of the ether:chloroform (8:1) solvent. The eluates were combined in the receiver and evaporated to dryness. The residue which constituted the phenacetin portion of the mixture was redissolved completely in 5.0 ml. of chloroform, diluted with 45.0 ml. of isooctane, and assayed spectrophotometrically.

Immediately after the passage of the last ether:chloroform eluate, the column series was disassembled and column III was eluted first to minimize the time of residence of the aspirin on the column. (A prolonged stay would result in a partial *in situ* degradation of the acetylsalicylic acid.) Elution of the column and the determination of the aspirin were performed in the usual manner (7).

To recover the phenothiazine derivative,[4] the tartaric acid containing column I was eluted with consecutive 20-ml. portions of ammonia-saturated chloroform; the eluates were collected in a 100-ml. volumetric flask and adjusted to volume with the eluant at 20°. An aliquot of the eluate was then evaporated to dryness, redissolved in a suitable solvent, and assayed by the ultraviolet absorption curve.

In the further course, caffeine and itobarbital were eluted from their respective columns and assayed by the technique described in the literature (5, 6).

RESULTS AND DISCUSSION

Martin and Synge (8) in their basic theoretical work on the column partition chromatography developed an equation which expressed the mathematical relation between the movements of the solute and the mobile phase

$$R = \frac{A}{A_m + \alpha A_s} \qquad \text{(Eq. 1)}$$

where R = movement of chromatography band/movement of developing fluid, A = the cross-section area of the column, A_m = the cross-section area of the mobile phase, A_s = the cross-section area of the stationary phase, and α = partition coefficient (solute concentration in the stationary phase/solute concentration in the mobile phase).

Equation 1 indicates that the speed of the solute movement is inversely proportional with the partition coefficient. The distribution ratio of thioridazine hydrochloride between 1% tartaric acid solution and some organic solvents were experimentally determined (Table II).

The ratio with an ether:chloroform (8:1) mixture is more than 60 times higher than that with pure chloroform. Consequently, by the use of this ether:chloroform (8:1) solvent mixture, a low R value and a satisfactory retention of the phenothiazine derivative band were anticipated.

The quantitative entrapment of several phenothiazine derivatives by the selected system is shown in Table III.

The released amounts were found invariably less than 0.35%, indicating a practically complete entrapment of each substance.

The next experimental step was to study the selectivity of the phenothiazine derivative entrapment in the presence of caffeine.

As the results in Table IV demonstrate, by using the ether:chloroform (8:1) mobile phase on tartaric acid–Celite columns of various acid concentrations, the thioridiazine component of the mixture was quantitatively trapped on each column, whereas the release of the caffeine appeared to be a function of the acidity of the stationary phase.

Plotting the data of Table IV in a coordinate system, a graphical interpretation of the pH dependency of the caffeine release was obtained as shown in Fig. 2.

The curve is linear between 1.35 to 2.07 pH values and, by determining the slope of this line, an empirical equation was found

$$C = k \cdot \text{pH} + A \qquad \text{(Eq. 2)}$$

where C is the released percentage of caffeine. (The values of k and A were found to be 45.03 and 6.71, respectively.)

Based on the favorable results of the preliminary model experiments, a number of assays had been carried out with five-component mixtures. Results of these determinations are listed in Table V.

Recoveries of the materials are reported as percentages of the theoretical amounts. Samples with identical letter designation were duplicates. The

[4] The column, receiver, and all glassware containing the phenothiazine derivative must be made of low actinic glass (or protected by a dark cloth) to prevent the photo-catalyzed degradation of the substance.

relatively small deviations between the parallel readings indicate a promising accuracy of the described technique.

The operation of the four-column system imposed no technical difficulty, and the total assay of the five ingredients can be performed by an experienced operator in about 2 hours.

Preliminary experiments for some further simplification of the procedure are now in progress; the results will be reported in a subsequent publication.

SUMMARY

The mechanism of separation of some pharmaceutical derivatives of phenothiazine from mixtures with aspirin, phenacetin, caffeine, and itobarbital has been investigated.

A modified partition chromatography procedure was developed which facilitates a simultaneous quantitative assay of the five active ingredients with satisfactory accuracy.

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Absorption and Incorporation of Methionine-S^{35} into Hair

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Administration of methionine-S^{35} to guinea pigs by topical, oral, and intramuscular routes resulted in the incorporation and utilization of the S^{35}-radioactivity by the growing dorsal and ventral hair. Oral or intramuscular administration of methionine-S^{35} led to utilization by the growing hair of between 13 and 21 per cent of the total amount administered. Metabolic utilization by these routes was confirmed by the fact that roughly twice as much nonmethionine-S^{35} as methionine-S^{35} was found. The amount utilized was approximately proportional to the amount administered. A greater amount of S^{35}-radioactivity was detected in the dorsal hair than in the ventral hair even after allowing for the greater rate of hair growth in the dorsal area. When the methionine-S^{35} was applied topically to the dorsal area, approximately 10 per cent of the total amount applied was utilized by the total body hair. At the site of topical application, two-thirds of the S^{35} in the hair in the area appeared to be derived locally and the other one-third systemically.

THE CHEMISTRY and physiology of hair growth has been investigated to gain insight into the factors that control growth and differentiation of hair follicles and formation of keratin. Of special interest is the amino acid composition of and uptake into the proteins and other components of the variety of cells that constitute keratinizing structures and keratin.

This report describes the results of radiotracer investigations initiated to study the influence of various routes of administration on the incorporation of amino acids into hair protein. The amino acid methionine, indispensable for all animals, was chosen for study because it is incorporated into proteins and is converted, after demethylation, into cysteine and cystine which are vital components of keratinized structures.

EXPERIMENTAL

Hartley strain male guinea pigs, adequately adjusted to laboratory conditions and diet, were employed. The diet consisted of pregnant rabbit chow supplemented with fresh lettuce and water *ad libitum*. The animals were housed in individual wire mesh cages that were cleaned daily to prevent the accumulation of any radioactive excreta which could possibly be ingested or adsorbed to the hair of the animal. Prior to the administration of the labeled compound, an area of 2 sq. in. was shaved from the dorsal and ventral surfaces of each animal with an electric clipper. Care was taken in this and all subsequent clippings to prevent abrasions of the skin.

The animals were separated into three groups of six animals. Group *A* received methionine by topical application, group *B* by oral administration, and group *C* by intramuscular injection. Three of the animals in each group received a total dose of 500 mcg. of methionine-S^{35}, and three of the animals received a total dose of 250 mcg.

Radioactive *dl*-methionine-S^{35} was obtained from the Radiochemical Centre, Amersham, England, with a specific activity of 55.9 millicuries per millimole (mc./m*M*). The labeled methionine, shown by paper chromatographic methods to be of radiochemical homogeneity, was diluted with sufficient nonlabeled *dl*-methionine so that the animals at the 250-mcg. level were exposed to a total of 52 μc. and

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