temperature the solution is stable at these temperatures by virtue of the high activation energy of the hydrolytic reaction.

SUMMARY

Linear nonisothermal kinetic data from a single experiment can be used to obtain activation energy, reaction rate, and shelf-life prediction. The method described in this report involves a comparison of model degradation curves to experimental data for the determination of activation energy. Calculation of reaction rate and shelf-life predictions at a specific temperature are made utilizing the total experimental degradation and activation energy. The advantages of the method over the classical isothermal method lie in the use of a single experimental unit, the analysis of one set of samples, the shorter time required for completion of the experiment, and the ability to change the temperature range of the experiment without interruption of the study. The advantages over other nonisothermal methods lie in the ease of analysis of data, simplicity of time-temperature relationship, and the use of readily available laboratory equipment. The approach is limited only by assay precision, constancy of activation energy, and applicability of the Arrhenius equation. The method is proposed and is currently being evaluated for preformulation and formulation screening of ampul solutions.

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Keyphrases

Stability studies—linear, nonisothermal
Nonisothermal method—stability prediction
Kinetic equations—nonisothermal hydrolysis
Acetaminophen, procainamide HCl—test compounds
Column chromatography—separation
UV spectrophotometry—analysis

Separation and Determination of Chlorpheniramine and Its Dealkylated Metabolites from Urine

By K. ALBERT and J. J. WINDHEUSER

Application of ion-pair extraction to partition chromatographic techniques has been used effectively to separate chlorpheniramine and its dealkylated metabolites from one another in urine. Gas-liquid chromatography was employed to detect the amines. The ion-pair partition column employed selectively separated the amines prior to detection by gas chromatography. The chromatograms indicated symmetric peaks with little or no tailing typical of the secondary and primary amine metabolites. Recovery studies from urine demonstrated that at the parts-per-million level, complete recovery of chlorpheniramine and its metabolites was achieved after certain precautions against loss were taken. On the basis of these studies, an analytical procedure to separate chlorpheniramine and its metabolites from urine was developed. Recoveries of chlorpheniramine and its dealkylated metabolites were quantitative. The method offers the advantage of complete isolation of all components from one another and suggests the possibility of using ion-pair partition columns as a method to quantitatively separate other nitrogen-containing compounds from one another.

THE SEPARATION AND DETECTION of drugs from mixtures of closely related compounds is becoming more and more important. Of particular interest is the analysis of drugs and their metabolites in blood and urine in microgram quantities. This communication deals with a model system utilizing the concept of ion-pair separation of related amines from urine.

The suitability of ion-pair extraction as a process for separation and isolation of nitrogen-containing compounds in analytical samples was recently demonstrated by Higuchi *et al.* (1, 2).

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These researchers, using dextromethorphan and chlorpheniramine as examples of drugs containing one and two nitrogen centers, respectively, studied solvation effects and anion polarizability as factors responsible for the extractive equilibrium of ion pairs.

Kamm and Van Loon (3) recently reported that in dogs and rats, the major metabolite of chlorpheniramine was the *N*-desdimethylated analog while the minor metabolite was *N*-desmonomethylchlorpheniramine. Kabasakalian *et al.* (4, 5), in their study of the urinary excretion of chlorpheniramine, brompheniramine, and pheniramine in humans, reported that *N*-demethylation accounted for the metabolic degradation of the drugs.

On the basis of the above results, it was considered of interest to study the use of ion-pair formation as a method to separate and quantify nitrogen-containing compounds that undergo *N*-demethylation from their metabolites in urine. Chlorpheniramine was chosen as a model of such a drug. The general method would be applicable to other drugs of the chlorpheniramine type.

EXPERIMENTAL

All spectrophotometric measurements were made on a recording spectrophotometer (Cary model 11) using 5-cm. cells. A gas chromatograph (Barber-Coleman model 5000) equipped with a flame-ionization detector and a 1-mv. recorder was used with the following conditions: 182.9 cm. (6 ft.) × 4 mm. borosilicate column containing 2% polyethylene glycol[1] and 2.5% KOH on 100/120 mesh Gas-Chrom Q operated at 235°; detector temperature 248°; injector temperature 240°; nitrogen at 70 ml./min.

The liquid–liquid partition chromatographic columns containing buffered ion-pair formers were 19 mm. × 300 cm. and were equipped with Teflon stopcocks. The liquid level for the mobile phase was maintained constant by using a reservoir with a distended capillary tube placed approximately 5 cm. into the column.

Tapered centrifuge tubes with a capillary tip about 2 mm. internal diameter capable of containing a volume of 15 ml. were scored at 50 μl.

Chemicals and Reagents

All chemicals and reagents were analytical reagent grade unless otherwise indicated. Polyethylene glycol[1] and diatomaceous earth[2] were obtained from Applied Sciences Labs, and Johns-Manville, respectively; chlorpheniramine maleate was supplied by Schering Corp.; *N*-desdimethylchlorpheniramine and *N*-desmonomethylchlorpheniramine were furnished by Smith, Kline & French.

General Procedure for Preparation of Liquid–Liquid Chromatographic Columns—To 10 g. of diatomaceous earth 12 ml. of buffered anion solution was added and mixed well until the diatomaceous earth appeared uniformly wet and homogeneous. Enough water-saturated chloroform was added to thoroughly wet the diatomaceous earth, and portions were added to the chromatographic column containing a small pledget of silanized glass wool and about 10 ml. of chloroform. Using a suitable length of glass dowling, the diatomaceous earth was thoroughly homogenized and tamped gently, but firmly. Subsequent to packing, about 150 ml. of water-saturated chloroform was passed through the column to remove impurities. The flow rate was maintained constant at 2 drops/sec.

Table I—Comparison of Extraction Constants for Chlorpheniramine Ion Pairs at 1 *M* $CHCl_3$ Concentration

	K_e = P.C.app./$[X^-]$ $(1 + H^+/K_a)$
Perfluorohexanoic acid	10
Trifluoroacetic acid	2.1×10^{-3}
Bromide	1.4×10^{-5}

General Procedure for Recovery Studies of Chlorpheniramine and Its Metabolites from Urine—All glassware used in this study was treated with hydrophobic monomers[3] to eliminate possible drug adsorption. The hydrophobic monomers were freely applied and the glass allowed to air dry. Following a chloroform rinse, the glass surface was rinsed with ethyl ether and allowed to dry.

To 25 ml. of urine contained in a 50-ml. glass-stoppered centrifuge tube, 25 mcg. (1 p.p.m.) of drug was added. The solution was brought to a pH of 10 with 6 *M* NaOH and extracted with three 15-ml. portions of anhydrous ethyl ether. The solution was centrifuged and the ether layer withdrawn and collected in a beaker containing 500 mg. of diatomaceous earth. The hydrochloride salt of the drug was formed by bubbling in HCl gas prior to volatilization of the ether under nitrogen gas. Five-tenths milliliter of 6 *M* NaOH was added followed by quantitative transfer to the liquid partition column. The beaker was dry rinsed with an additional 200 mg. of diatomaceous earth, the rinsings added to the column, and the column tamped.

The drug was eluted with the appropriate chloroform phase and the eluate collected in a round-bottom flask. The chloroform was slowly evaporated under nitrogen at room temperature to about 10 ml. and quantitatively transferred to a 15-ml. tapered tube. After evaporating the chloroform nearly to dryness, the residue was dissolved in dimethylformamide and diluted to the 50-μl. mark with dimethylformamide. A 2-μl. aliquot was injected onto the polyethylene glycol–KOH column and the area corresponding to the drug compared against a suitable standard solution in dimethylformamide.

RESULTS AND DISCUSSION

It has been established that chlorpheniramine formed ion pairs whose extraction constants varied depending upon the anion employed (2). Table I lists a comparison of the extraction constants for chlorpheniramine obtained in this study and by Higuchi and Kato. Because the metabolites of chlorpheniramine are secondary and primary amines, their extraction constants into chloroform should be

[1] Carbowax 20M, Applied Science Labs, State College, Pa.
[2] Celite 545, Johns-Manville, New York, N. Y.
[3] Desicote, Beckman Instruments, Inc., Fullerton, Calif.

TABLE II—EFFECT OF VARIOUS ANIONS ON THE COLUMN CHROMATOGRAPHIC SEPARATION OF CHLORPHENIRAMINE AND ITS DEMETHYLATED METABOLITES

	% Eluted[a]	
	Chlorpheniramine	Desmonomethylchlorpheniramine
Perfluorohexanoic acid, 0.25 *M*		
Fraction		
1	100	100
2		
Trifluoroacetic acid, 0.25 *M*		
Fraction		
1	100	85
2	—[b]	15
3	—	—
Sodium bromide, 0.25 *M*		
Fraction		
1	80	—
2	20	—
3	—	22
4	—	60
5	—	15
6	—	3

[a] *N*-desdimethyl chlorpheniramine was not eluted under these conditions. [b] Dashes indicate no detectable drug.

TABLE III—EFFECT OF VARYING BROMIDE ANION CONCENTRATION ON THE COLUMN CHROMATOGRAPHIC SEPARATION OF CHLORPHENIRAMINE AND ITS DEMETHYLATED METABOLITES

	% Eluted[a]	
	Chlorpheniramine	Desmonomethylchlorpheniramine
Sodium bromide, 1 *M*		
Fraction		
1	100	55
2	—[b]	45
3	—	—
Sodium bromide, 0.50 *M*		
Fraction		
1		
1	99	30
2	1	65
3	—	5
4	—	—
Sodium bromide, 0.25 *M*		
Fraction		
1	80	—
2	20	—
3	—	22
4	—	60
5	—	15
6	—	3

[a] *N*-Desdimethylchlorpheniramine was not eluted under these conditions. [b] Dashes indicate no detectable drug.

different enough from chlorpheniramine to allow separation of the amines from one another.

Liquid–liquid partition columns containing 0.25 *M* perfluorohexanoic acid, trifluoroacetic acid, and sodium bromide in 0.01 *M* citrate buffer at pH 3.5 were prepared, and 0.5 mg. each of the amines was eluted from individual columns using water-saturated chloroform as eluate. Twenty-five-milliliter fractions were collected and determined spectrophotometrically from 300–250 mμ. The results of this experiment are presented in Table II. It can be seen that only the bromide column effectively separated each of the amines. An examination of Table I will reveal a difference of almost 10^6 in the extraction constants for chlorpheniramine using perfluorohexanoic anion and bromide anion as ion-pair formers. The same differences could be anticipated for ion pairs of the metabolites. However, if the partition coefficients of all three amine-ion pairs into chloroform were high, regardless of their individual differences in magnitude, then it would be difficult to separate them effectively on a partition column since all three would be eluted rapidly. To further illustrate this point, Table III exhibits the results of an experiment in which partition columns containing 1 *M*, 0.5 *M*, and 0.25 *M* bromide ion were prepared, buffered as before. It would be expected that the magnitudes of the partition coefficients for the amines from 1 *M* bromide to 0.25 *M* bromide would be considerably less than 10^6, yet only 0.25 *M* bromide effectively separated chlorpheniramine from its secondary metabolite. Consequently, anion polarizability as well as concentration are factors that contribute to separation. The primary metabolite was not eluted from any of the columns.

In an attempt to explain the lack of elution of the *N*-desdimethyl metabolite, it was suggested that the compound either did not form an ion pair or was so strongly adsorbed to the diatomaceous earth that even though an ion pair was formed, it was not eluted. Partition coefficients were determined for chlorpheniramine and its metabolites using equal volumes of mutually saturated pH 3.5, 0.25 *M* bromide in 0.01 *M* citrate buffer, and chloroform. The data, tabulated in Table IV, indicate that although the primary metabolite formed an ion pair with bromide with a partition coefficient close to that of the secondary amine, the more polar, basic primary metabolite was probably adsorbed by the diatomaceous earth during column separation.

Chloroform saturated with ammonia was used to elute *N*-desdimethylchlorpheniramine which was found between the fourth and tenth 25-ml. fractions.

Table V illustrates the complete separation as well as quantitative and reproducible results obtained for elution of 500 mcg. each of the amines from the 0.25 *M* bromide partition column. Eluting with water-saturated chloroform, the first 35 ml. containing only chlorpheniramine was determined spectrophotometrically and the percent recovery of chlorpheniramine calculated. In a similar manner, the next 150 ml. of eluate containing only the secondary metabolite was collected in 25-ml. fractions, and the recovery of *N*-desmonomethylchlorpheniramine calculated.

TABLE IV—PARTITION COEFFICIENTS USING 0.25 *M* BROMIDE ION IN 0.01 *M* CITRATE, pH 3.5 OF ION PAIRS OF CHLORPHENIRAMINE AND ITS METABOLITES

Tertiary Amine	Secondary Amine	Primary Amine
1.500[a]	0.280[a]	0.193[a]

[a] Corrected for free base extraction.

TABLE V—RECOVERIES OF AMINES FROM BROMIDE-PARTITION COLUMN

Trial	3° Amine, %	2° Amine, %	1° Amine, %
1	100.0	99.7	100.0
2	100.0	99.7	100.0
3	100.1	100.1	100.1
4	100.0	100.0	99.7
5	100.1	100.1	99.9

The eluent was changed to ammonia-saturated chloroform, the first 75 ml. discarded, and the following 175 ml. containing only *N*-desdimethylchlorpheniramine collected in 25-ml. fractions. The percent recovery of the primary metabolite was calculated from spectrophotometric data.

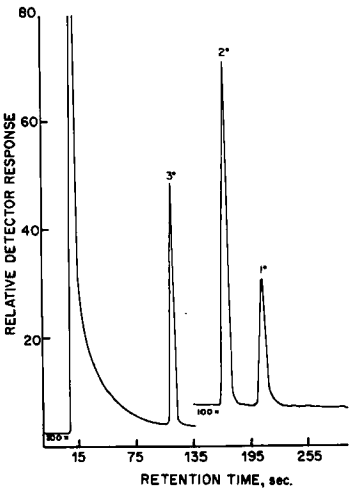

Fig. 1—Chlorpheniramine and its demethylated metabolites, 2 mcg. in dimethylformamide. Column: Gas-Chrom Q with 2.5% KOH, 2% polyethylene glycol. Nitrogen flow rate 70 ml./min. Injector 240°, column 235°, and detector 248°. Attenuation as noted on plot.

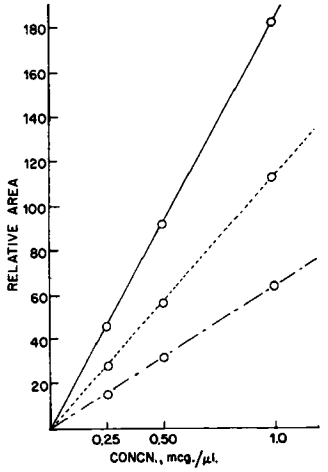

Fig. 2—Detector response for chlorpheniramine and its metabolites. Key: ——, chlorpheniramine maleate; - - -, N-desmonomethylchlorpheniramine; — - —, N-desdimethylchlorpheniramine.

TABLE VI—RECOVERIES OF CHLORPHENIRAMINE AND ITS METABOLITES AT 1 p.p.m. FROM URINE

Chlorpheniramine, %	Secondary Metabolite, %	Primary Metabolite, %
97.6	96.3	97.6
101.1	98.3	99.1
98.6	98.2	96.9
102.2	99.7	98.4
Av. 100.0	98.1	98.0

Recovery of microgram quantities of the drug and its metabolites from urine necessitated the need for a sensitive method of detection. Gas chromatography was chosen. However, since the analytical method itself is specific, any sensitive, nonselective means of determination would be suitable. Figure 1 illustrates the symmetrical peaks obtained for 2 mcg. of chlorpheniramine and its metabolites in dimethylformamide on the polyethylene glycol –KOH column. For chlorpheniramine, 2,500 theoretical plates were calculated, 3,010 for the secondary metabolite, and 2,580 for the primary metabolite. The linear flame-ionization response for the amines is illustrated in Fig. 2.

Recovery studies were next initiated at the 1-p.p.m. level from urine. Aqueous solutions of chlorpheniramine and its metabolites were prepared and 25 mcg. of each drug added to 25 ml. of urine. During evaporation of the ether extract under nitrogen gas, an all-glass system was used to eliminate contamination of the sample with plasticizer. Loss of the secondary and primary amines as free bases from ethereal solution was eliminated by forming hydrochloride salts. Further, since the primary metabolite was eluted from the bromide partition column as a free base, the hydrochloride salt of the drug was formed in the chloroform solution prior to volatilization. Table VI exhibits the quantitative and reproducible recoveries of chlorpheniramine and its metabolites from urine.

CONCLUSIONS

In the present study, an attempt has been made, using chlorpheniramine as a model drug undergoing *N*-demethylation, to evaluate the feasibility of using ion-pair formation as a method to selectively separate the drug from its metabolites. In this investigation the selectivity of various anions on the column chromatographic separation of chlorpheniramine and its demethylated metabolites was studied. On the basis of the studies described, a general method for selectively separating *N*-demethylated metabolized drugs and their metabolites is proposed. The method offers the advantage of complete isolation of all components prior to determination, permitting a nonselective means of analysis.

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Keyphrases

Chlorpheniramine, dealkylated metabolites—analysis
Urine—chlorpheniramine, metabolites, separation, determination
Ion-pair partition chromatography—separation
GLC—analysis
UV spectrophotometry—analysis

Comparative Effects of Chlorpromazine Hydrochloride and Quaternary Chlorpromazine Hydrochloride on the Central Nervous Systems of Rats and Mice

By N. WATZMAN*, A. A. MANIAN†, H. BARRY, III, and J. P. BUCKLEY

The effects of quaternary chlorpromazine HCl (QCPZ) and the tertiary parent compound chlorpromazine hydrochloride (CPZ) on the central nervous system were investigated in male rats and mice. CPZ was much more potent than QCPZ in inducing depressant effects on spontaneous activity of mice, forced motor activity of rats, continuous shock avoidance of rats, and potentiation of hexobarbital sleeping time in mice. Neither compound significantly altered the threshold of convulsive seizures induced by either pentylenetetrazol or strychnine. Although quaternization of the CPZ molecule at the terminal side chain nitrogen produced a decrease in central nervous system activity, the resulting compound was much more toxic than CPZ.

THE QUATERNIZATION of chlorpromazine (CPZ) by introducing a functional ^{14}C-methyl group into the terminal side chain nitrogen provides a highly sensitive and convenient method for the quantitative analysis of minute amounts of this compound and some of its metabolites in human blood plasma (1). Quaternary ammonium compound formation, in all likelihood, could also be used for determining plasma levels of other psychoactive amines employed in the treatment of the mentally ill.

Scientific literature discloses two references to biological information on this quaternary compound.[1] The first appears in a summary of an investigation by Seeman (2) on *in vitro* membrane stabilization by the phenothiazines, and the other by Fog *et al.* (3) describing the effects of quaternary phenothiazines administered *via* intracerebral injection in rats. Some quaternary phenothiazines such as 10-(α-dimethylamino-propionyl)-phenothiazine methobromide[2] (4, 5) have anticholinergic and ganglion-blocking actions while others such as 1-(10-phenothiazinylmethyl)-ethyl-2-hydroxyethyldimethyl ammonium chloride[3] (6–9) possess antihistaminic activity. Excellent review articles on the chemistry and pharmacologic actions of quaternary ammonium compounds, in general, have been presented by Cavallito and Gray (10) and D'Arcy and Taylor (11, 12).

This current study was undertaken to investigate the comparative pharmacologic activity of the quaternary chlorpromazine, 1-(2-chloro-10-phenothiazinyl)propyl - 3 - trimethylammonium chloride (QCPZ) (I) and the tertiary parent drug chlorpromazine HCl (CPZ),[4] in a battery of biological tests.

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[2] Secergan, Aktiebolaget Astra, Sodertalje, Sweden.
[3] Aprobit, Recip Co., Stockholm, Sweden.
[4] Thorazine, Smith, Kline & French, Philadelphia, Pa.