Identification and Quantitative GLC Determination of Iproniazid in Human Urine

R. M. de SAGHER, A. P. De LEENHEER ˣ, and A. E. CLAEYS

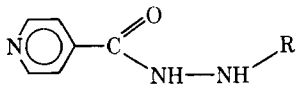
Abstract □ A GLC method for the specific identification and quantitation of iproniazid, a potent monoamine oxidase inhibitor drug, is described. The free drug is isolated from urine samples by two-phase extraction reinforced with salting out. Identification and quantitative determination at the microgram level are done on aliquots of the chloroform extract by GLC, using the 2-butyl analog as an internal standard. Iproniazid and potentially interfering compounds present in the extracts are also identified by GLC–mass spectrometry and TLC for supporting evidence of the GLC method's specificity.

Keyphrases □ Iproniazid—GLC analysis, urine □ GLC—analysis, iproniazid in urine □ Monoamine oxidase inhibitors—iproniazid, GLC analysis, urine □ CNS stimulants—iproniazid, GLC analysis, urine

Iproniazid (I) is a monoamine oxidase inhibitor drug used in Europe as a central stimulant in the treatment of mental depression. This drug is extensively metabolized, principally by hydrolysis into isonicotinic acid and isopropylhydrazine and to a lesser extent by dealkylation into isoniazid and acetone. Iproniazid, ^{14}C-labeled on the carbonyl group, was injected into rats; 98% of the label was recovered in the first 24-hr urine collection (1). Iproniazid, ^{14}C-labeled on the 2-carbon of the isopropyl group, was administered to rats, mice, and humans (2, 3). In rats and mice (2), about 15% of the label was excreted in urine (one-third as unchanged drug), but the greatest portion was expired as $^{14}CO_2$. Oral administration to two human patients (3) resulted in urinary excretion of the label of 10–13% totally and traces to 3% of free drug.

A quantitative method was described that used the sodium nitropentacyanoferroate reagent to determine the urinary concentration of iproniazid in patients receiving therapeutic doses (75–100 mg) (4). Of the iproniazid administered orally, about 10% was found as unchanged drug and a much larger quantity was detected as isonicotinic acid. The cyanogen bromide–ammonia reaction was used to examine the urinary excretion in nine normal persons dosed with iproniazid (5). Within 48 hr following administration of a 250-mg dose, about 16% was excreted as unchanged drug and 50% was excreted as isonicotinic acid. Many questions arise, however, as to the specificity of both spectrophotometric methods based on the sodium nitropentacyanoferroate or the cyanogen bromide–ammonia reaction.

The present report concerns the development of a specific and sensitive GLC determination of iproniazid in urine samples of patients receiving therapeutic doses.



I: R = $CH(CH_3)_2$
II: R = $CH(CH_3)(CH_2CH_3)$

Table I—Case Histories of Major Iproniazid Therapy

Patient	Sex and Age (years)	Body Weight, kg	Daily Dose of Orally Administered Drugs
1	Male, 28	77	Iproniazid, 50 mg
2	Female, 42	73	Iproniazid, 75 mg Methotrimeprazine[a], 75 mg
3	Male, 54	86	Iproniazid, 50 mg Amobarbital[b], 150 mg Diazepam[c], 7.5 mg Thioridazine[d], 37.5 mg
4	Male, 29	79	Iproniazid, 50 mg Diazepam[c], 5 mg Fluphenazine hydrochloride[e], 1 mg
5	Female, 58	86	Iproniazid, 25 mg Fluphenazine hydrochloride[e], 1 mg

[a] Nozinan. [b] Amytal. [c] Valium. [d] Mellaril. [e] Sevinol.

EXPERIMENTAL

Reagents—Iproniazid[1] was obtained from iproniazid phosphate as reported previously (6). The internal standard, 1-isonicotinoyl-2-(2-butyl)hydrazine (II), was synthesized according to Yale *et al.* (7) and by the method described earlier (6). All inorganic and organic chemicals used were reagent grade. High purity nitrogen was employed for evaporation of extracts.

Collection of Urine Samples—Urine samples were collected without preservative on successive days from a volunteer (Subject 1) and four psychiatric patients (Subjects 2–5) to whom known amounts of iproniazid were administered orally. The urine outputs of Subject 1 were assembled and analyzed separately after administration of a single 50-mg dose of iproniazid. The four psychiatric patients were under a daily combined drug therapy for at least 3 months (Table I) including iproniazid (75, 50, 50, and 25 mg, respectively). Their successive 24-hr urine outputs were collected, homogenized, and stored at −7° until analyzed.

Extraction Procedure—To each 200-ml sample, or the total volume if less was available, of urine in a 1-liter separator were added 84 g of ammonium sulfate and 2 ml of 12 *N* HCl. The solution was extracted three times with 100 ml of peroxide-free ether (freshly distilled over hydroquinone, reagent grade), and the ether layers were discarded. The aqueous phase was made basic with 5 ml of 13 *N* NaOH and again extracted three times with 100 ml of ether. Then the ether layers were back-extracted three times with fresh 25-ml portions of 0.1 *N* HCl.

The combined acidic solutions were washed three times with 50 ml of chloroform. Then 31 g of ammonium sulfate and 1.5 ml of 13 *N* NaOH were added, and the aqueous solution was extracted three times with 50 ml of chloroform. After addition of the internal standard (II, 115 μg for Subject 1 and 216 μg for Subjects 2–5 and recovery experiments), the chloroform phase was dried over anhydrous sodium sulfate, filtered, and concentrated to a smaller volume in a rotary evaporator[2].

The solution was finally evaporated to dryness in a waterbath at 40° under a nitrogen stream in 15-ml silanized conical centrifuge tubes. The residue was dissolved with the aid of a mixer[3] in 100 μl of chloroform.

GLC—A gas chromatograph[4] equipped with dual flame-ionization

[1] Marsilid.
[2] Rotavapor Büchi, Flawil, Switzerland.
[3] Grindomat Retsch, Haan, West Germany.
[4] Varian Aerograph 2800, Walnut Creek, Calif.

Table II—Partition Coefficients of Iproniazid

Extracted Phase	Extractant: Chloroform	Extractant: Ether
0.26 *N* NH_4OH	0.35	0.02
0.26 *N* NaOH	<0.01	<0.01
0.1 *N* HCl	<0.01	<0.01
Aqueous ammonium sulfate–ammonium hydroxide[a]	13	2.0
Aqueous ammonium sulfate–hydrochloric acid[b]	<0.01	<0.01

[a] Forty grams of ammonium sulfate + 2 ml of 13 *N* NH_4OH/100 ml of aqueous phase. [b] Forty grams of ammonium sulfate + 2 ml of 12 *N* HCl/100 ml of aqueous phase.

detectors was used for single-column operation. The column consisted of 5% 50:50 cyanoethylmethyl dimethyl silicone gum[5] coated on 80–100-mesh diatomaceous earth[6] and packed in a 2.5-m × 2-mm i.d. spiral silanized glass column. Conditioning was performed for at least 12 hr at 220° with a carrier gas (nitrogen) flow of about 5 ml/min and then for a few hours at operating conditions.

Analyses were carried out isothermally at an oven temperature of 180° with the injector and detector blocks maintained a few degrees higher. Nitrogen was used as the carrier gas at a linear velocity, $\bar{u}_o$, of 14.3 cm/sec (measured with methane). The hydrogen and air flow rates were adjusted to give optimum sensitivity and good stability: hydrogen, 24–38 ml/min; and air, 250–550 ml/min.

The detectors were connected to the electrometer and, *via* an electronic integrator[7], to a 0–1-mv range recorder[8]. The attenuation settings employed were 2×10^{-11} amp/mv for the electrometer,

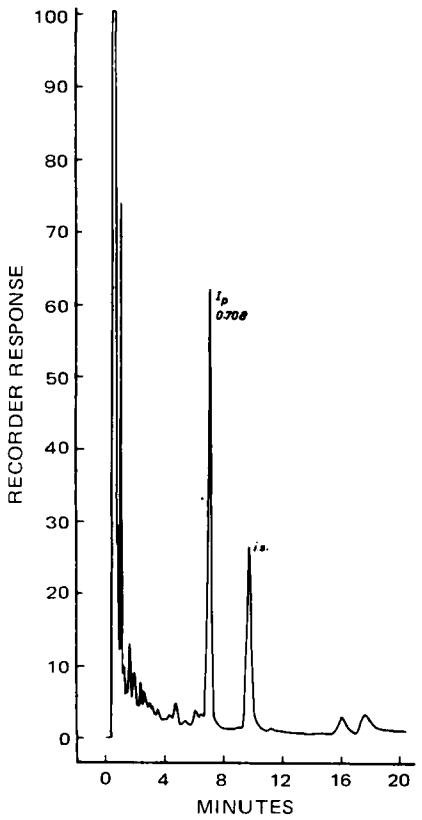


Figure 1—*Gas chromatogram of a urine extract from Subject 1. The column was 5% 50:50 cyanoethylmethyl dimethyl silicone gum* (T = *180°*, $\bar{u}_o$ = *14.3 cm/sec*); Ip = *iproniazid, and* is = *internal standard.*

[5] XE-60.
[6] Gas Chrom Q.
[7] Infotronics CRS 104, Houston, Tex.
[8] Varian Aerograph A25, Walnut Creek, Calif.

Table III—Recoveries of Iproniazid Added to Human Urine Samples

Iproniazid Added to 200 ml of Urine, μg	Iproniazid Recovered, μg	Recoveries, %
50	37.5	75.1
100	81.1	81.1
150	116.0	77.3
200	154.1	77.1
300	242.2	80.7
400	312.0	78.0
	Mean value ± *SD*, %	78.2 ± 2.3

whereas the electronic integrator input/output attenuator was used at ×5, ×10, or ×20.

All injections were made on-column with a 10-μl syringe[9], using an injection volume of approximately 1 μl.

Calculations—Calibration factors, k_i, were determined by measuring corresponding peak areas on chromatograms from the calibration series consisting of five different solutions of equal weight increments:

$$k_i = A_{Ip}w_{is}/A_{is}w_{Ip} \qquad \text{(Eq. 1)}$$

where A_{Ip} and A_{is} and w_{Ip} and w_{is} are peak areas and weights of iproniazid (*Ip*) and internal standard (*is*), respectively. For each calibration series, the mean calibration factor, $\bar{k}$, was calculated.

Results for urine samples, C_u (micrograms per 100 ml), were found by:

$$C_u = \frac{100}{0.78v}\,\frac{1}{\bar{k}}\,\frac{A_{Ip}'}{A_{is}'}\,w_{is} \qquad \text{(Eq. 2)}$$

where 100/0.78*v* is the conversion factor for an overall recovery of 78% and an extracted volume, *v* (milliliters), of urine; $\bar{k}$ is the mean calibration factor; A_{Ip}' and A_{is}' are peak areas of iproniazid as present in the chromatogram of the urine extract and the internal standard added to the chloroform phase, respectively; and w_{is} is the weight (micrograms) of the internal standard added to the chloroform extract prior to evaporation.

The urinary excretion, E_u, or percent of administered dose of iproniazid per 24 hr, recovered as unchanged drug, was calculated as follows:

$$E_u = C_uV/D \qquad \text{(Eq. 3)}$$

where C_u (micrograms per 100 ml) is the urinary concentration of iproniazid, *V* (milliliters) is the total 24-hr urinary output, and *D* (micrograms) is the amount of iproniazid administered orally in a 24-hr period.

GLC–Mass Spectrometry—All mass spectra were obtained on a gas chromatograph–mass spectrometer[10] using two column systems: (*a*) 1% polyamide resin[11] on diatomaceous earth[12] (80–100 mesh) in a spiral silanized glass column (2 m × 3.0 mm i.d.) at 200°, and (*b*) 5% cyanopropyl phenyl silicone gum[13] on diatomaceous earth[6] (80–100 mesh) in a spiral silanized glass column (1.9 m × 2.5 mm i.d.) at 197°. Helium, at a flow rate of 30 ml/min, was used as a carrier gas on both columns.

The chromatographic pattern was followed by recording the total ion current at an electron energy of 20 ev with the aid of a 0–5-mv recorder. Mass spectra were always scanned on the leading edge near the top of each peak at an electron energy of 70 ev. Injections were made on-column with the aid of a 10-μl syringe[9], using an injection volume of 1–4 μl.

TLC—Aliquots of 2 and 5 or 10 μl of extracts, obtained without addition of the internal standard, together with 1, 2, and 5 or 10 μg of iproniazid were spotted in a point or as a 2-cm line at 3 cm from the lower edge of 250-μm plates, prepared with equal parts of silica gel HF_{254}[14] and cellulose MN 300[15].

Two solvent systems were employed: (A) chloroform–methanol–13

[9] Hamilton 701N, Whittier, Calif.
[10] LKB 9000S, Bromma, Sweden.
[11] Versamid-930.
[12] Varaport 30.
[13] OV-225.
[14] Merck, Darmstadt, West Germany.
[15] Macherey, Nagel & Co, Düren, West Germany.

Table IV—Results for the Urinary Excretion of Iproniazid after Single-Dose Administration of 50 mg

Hours[a]	Urinary Concentration, C_u, μg/100 ml	Urinary Excretion, E_u, %	Rate of Excretion, $\Delta Ae/\Delta t$, μg/hr	Midpoint of Excretion Interval, t, hr
1.7	192	0.33	95.9	0.85
4.5	424	0.73	130.4	3.10
6.5	298	1.00	250.5	5.50
8.5	292	0.69	173.5	7.50
12.0	284	0.95	135.7	10.25
14.0	275	0.49	122.5	13.0
23.0	331	1.26	69.9	18.5
26.0	130	0.24	40.0	24.5
32.5	69	0.41	31.8	29.25
34.7	47	0.10	23.6	33.6

[a] Time in hours after oral intake of iproniazid.

N NH_4OH (90:10:1), and (B) benzene–methanol–diethylamine (90:10:1). After development, the plates were sprayed with phosphomolybdic acid reagent (8, 9) for visualization. For this reagent (8, 9), 5 g of phosphomolybdic acid[14] was dissolved in 100 ml of ethanol; after spraying, the TLC plates were exposed in a tank to ammonia vapors for 1 min.

RESULTS AND DISCUSSION

GLC of iproniazid, with its 2-butyl analog as an internal standard, was originally developed on three column systems, *i.e.*, 1% polyamide resin[11], 5% 50:50 cyanoethylmethyl dimethyl silicone gum[5], and 5% cyanopropyl phenyl silicone gum[13] (10). Separation of both compounds affords no problem, whereas quantitative measurement of iproniazid, determined on the calibration series, is feasible with a standard deviation, $\sigma_{\bar{k}}$, of about 1% or less. This excellent figure is undoubtedly due to the close structural similarity between the compound to be determined (iproniazid) and the internal standard (2-butyl analog) used.

To develop a logical extraction procedure for urine samples, partition coefficients of iproniazid between chloroform or ether and an aqueous basic or acidic phase were measured on the 5% 50:50 cyanoethylmethyl dimethyl silicone gum[5] column operated at 160° (Table II). These data indicate that iproniazid is better extracted from an aqueous ammonium sulfate–ammonium hydroxide solution with chloroform. However, the first isolation step is done with ether, because it is a more selective solvent with regard to the more apolar biological compounds and yields cleaner extracts.

Back-extraction from ether is then performed with an appropriate volume (3 × 25 ml) of 0.1 *N* HCl. Final transfer to the organic phase is achieved after alkalinization and addition of ammonium sulfate with the aid of chloroform. Six 200-ml urine samples spiked with amounts (50–400 μg) in the range anticipated for therapeutic levels of iproniazid were examined under the described conditions. Results obtained on the 5% 50:50 cyanoethylmethyl dimethyl silicone gum[5] column at 180° are presented in Table III and show a recovery relative to the internal standard of 78.2 ± 2.3%.

This performance seems acceptable, especially in view of the poor extractibility of iproniazid from an aqueous solution by double-phase extraction with organic solvents. In our experience, there is no other solution to overcome this effect than by a salting-out procedure. Although this salting-out technique increases the extraction capacity for iproniazid, it also coextracts many unwanted products as noticed in multiple analyses of blank urine samples. Small, but potentially interfering, peaks occur in all chromatographic patterns. For this reason, a careful comparison of the three GLC column systems, by injecting extracts of blank urine samples, was done at the beginning of these studies.

Quantitative determination on the 1% polyamide resin[11] column was given up because of interference from cotinine present in the extracts of urine of patients (smokers). Cotinine might be separated successfully from iproniazid by lowering the oven temperature to 150°, but this change results in lengthy analysis times. On the 5% cyanopropyl silicone gum[13] column, dibutyl phthalate was an artifact that overlapped with the internal standard under the experimental conditions. Therefore, all further analyses were carried out using the 5% 50:50 cyanoethylmethyl dimethyl silicone gum[5] column at 180°. It separated entirely unwanted products from the compound to be determined (iproniazid) and the internal standard.

Identification of the unwanted products by GLC–mass spectrometry revealed the presence of cotinine and hydroxycotinine (both normal metabolites of nicotine ingested by smokers), dibutyl phthalate (an artifact of organic solvents), and the xanthine derivatives (normal metabolites for coffee drinkers from caffeine) theobromine (3,7-dimethylxanthine) and paraxanthine (1,7-dimethyl-

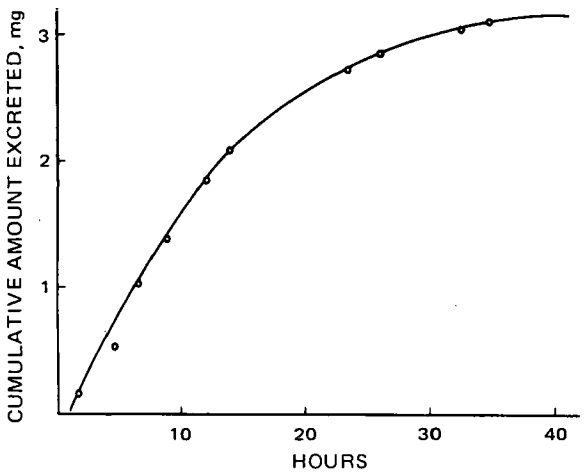


Figure 2—*Cumulative amount of iproniazid excreted in urine as a function of time for single-dose administration of 50 mg.*

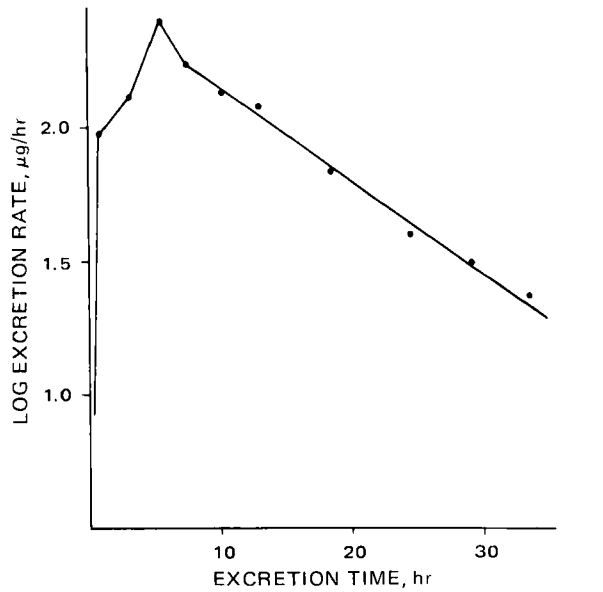


Figure 3—*Plot of the logarithm of the urinary excretion rate,* ΔAe/Δt, versus *the excretion time,* t.

Table V—Quantitative Results of GLC Determination of Iproniazid in Urine Samples of Psychiatric Patients Receiving Drug Therapy

Subject	Sample	Urinary Concentration, C_u, μg/100 ml	Urinary Excretion, E_u, %
2	1	178	3.89
	2	7	0.28
	3	120	3.29
	4	113	3.72
	5	165	4.02
3	1	125	2.42
	2	90	3.26
	3	107	2.80
	4	132	2.48
	5	193	3.85
4	1	156	7.37
	2	345	8.61
	3	140	3.41
	4	193	5.76
	5	264	6.07
5	1	46	2.72
	2	19	0.67
	3	22	0.34
	4	77	3.72
	5	60	3.14
	6	14	0.59

xanthine). With the exception of paraxanthine, which was detected only by comparison of its mass spectral data with those reported in the literature (11, 12), these compounds had identical retention characteristics on GLC analysis with authentic samples. No interference of the other drugs used or their metabolites was noticed in the chromatographic region where iproniazid and the internal standard eluted.

For verification of the specificity of results, aliquots of some urine extracts of psychiatric patients were examined by GLC–mass spectrometry. In all cases, iproniazid and the internal standard were positively identified by their mass spectral characteristics. Furthermore, other 200-ml aliquots of all urine samples of psychiatric patients were taken through the extraction procedure without the addition of the internal standard at the chloroform phase and chromatographed in Solvent Systems A and B by TLC. Except for one case, iproniazid was easily detected in all urine extracts (solvent system and R_f value: A, 0.6; and B, 0.3). Blank urine extracts developed in the same mobile phases did not yield interfering bands (spots).

Iproniazid was administered in a single dose of 50 mg to one subject, and successive urine outputs were collected and analyzed separately. A representative chromatogram is shown in Fig. 1. Results for the urinary excretion of free drug as a function of time is presented in Table IV. It appears that 6.21% of the orally administered dose of iproniazid was excreted cumulatively in urine within 34.7 hr (Fig. 2). These data can be treated to provide some pharmacokinetic information. Therefore, if it is assumed that the rate of excretion is proportional to the plasma concentration, then the proportionality constant is renal clearance, and a rate of excretion plot will parallel the plasma concentration curve. The latter allows one to determine the half-life.

From least-squares curve fit calculations performed on the last seven data ($\Delta Ae/\Delta t$ *versus* time), the exponential function $\Delta Ae/\Delta t = 310.1\, e^{-0.0785t}$ was found with a coefficient of correlation $r = 0.996$. The plot of log rate of excretion *versus* time (Fig. 3) indicates a half-life of 8.8 hr, which agrees well with results obtained by Koechlin *et al.* (3). Indeed, these authors indicated that the fall-off of the curves of the rates of urinary excretion of radioactivity reveals the presence of at least two components, with half-lives of approximately 10 and 17–20 hr, respectively. Comparison with our results identifies the first compound as free iproniazid.

Similar percent urinary excretion results were obtained for four psychiatric patients to whom doses of 25–75 mg of iproniazid were orally administered each day. A typical gas chromatogram of a urine extract from a psychiatric patient dosed with 75 mg of iproniazid is given in Fig. 4. Results for the percent urinary excretions of iproniazid in 24 hr are given in Table V. Under these conditions, 2–8% of the daily dose ingested was excreted in the 24-hr urine outputs.

The urinary excretion rate of iproniazid occurs rather slowly, as seen from results for Subject 1. Compared to iproclozide, another monoamine oxidase inhibitor drug described previously (13), the urinary excretion rate of iproniazid is about 15–20 times higher and the excretion persists for a much longer time after drug administration.

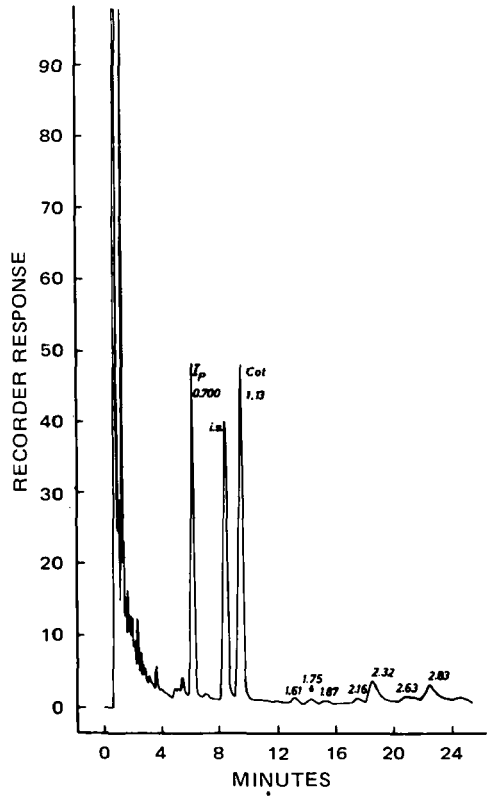


Figure 4—*Gas chromatogram of a urine extract from Subject 2. The column was 5% 50:50 cyanoethylmethyl dimethyl silicone gum (T = 180°, $\bar{u}_o$ = 14.3 cm/sec); Ip = iproniazid, is = internal standard, and Cot = cotinine).*

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Solubilization as a Method for Studying Self-Association: Solubility of Naphthalene in the Bile Salt Sodium Cholate and the Complex Pattern of Its Aggregation

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Abstract □ Solubilization of uncharged, slightly soluble solutes is shown to be a useful approach for investigating patterns of self-association. The solubility of naphthalene in aqueous solutions of sodium cholate was determined over the concentration range of 0–0.20 mole/liter at 25°. Bile salts such as sodium cholate have many detergent-like properties and exhibit hydrophobic self-association in aqueous solutions. It has become customary to describe this aggregation using the model of micelle formation. The naphthalene solubility data show that the CMC for sodium cholate is not well defined. Comparison with solubilization in a typical micelle-forming system, sodium decanesulfonate, shows clearly that sodium cholate does not resemble a micelle-forming system. Further examination of the solubility data in terms of mutual association of naphthalene with aggregate species shows that the self-association of sodium cholate is not consistent with the formation of (*a*) only large micelles containing 10 or more monomers, (*b*) only dimers, (*c*) dimers and large micelles, and (*d*) any unique oligomer or multimer. A complex pattern of association, including the formation of dimers and one or more higher oligomers, is indicated.

Keyphrases □ Solubilization—method for study of self-association, naphthalene in aqueous solutions of sodium cholate compared to sodium decanesulfonate □ Micelle formation—solubilization of naphthalene in aqueous sodium cholate solutions compared to sodium decanesulfonate, method for study of self-association □ Naphthalene—solubility in aqueous sodium cholate solutions, method for study of self-association □ Sodium cholate—solvent for naphthalene, method for study of self-association

Bile salts are considered to be physiological surfactants (1–3). In common with ordinary surfactants or detergents, they contain a large hydrophobic moiety, which is responsible for their ability to emulsify and solubilize fats and lipids. Like detergents, they exhibit a tendency toward hydrophobic self-association (aggregation) in aqueous solution (1–8).

It has become customary to describe this aggregation using the model of detergent micelles (1–3). As discussed recently (9), hydrophobic self-association in aqueous solution can have very different patterns, depending upon the structure of the hydrophobic solutes. Fundamental requirements of an extensive cooperativity of self-association in the early stages of growth for a micelle-forming pattern of self-association and the existence of a critical micellization concentration (CMC) are not satisfied by all molecular structures.

The purposes of the present work were to examine how well the micellar model applies to the self-association of sodium cholate and to investigate the more general problem of how the solubilization by aggregates of a slightly soluble, uncharged solute can be used to provide information about self-association of aggregating solutes.

EXPERIMENTAL

Materials—Cholic acid[1] was recrystallized according to the method of Hofmann (10). Naphthalene[2] was purified by sublimation before use.

Apparatus—Absorbance measurements were made using silica cells of 1-cm path length in a spectrophotometer[3]. The constant-temperature bath for the solubilization studies was equipped with a thermoregulator[4], and the temperature was maintained at 25 ± 0.1°.

Solubility Experiments—Stock solutions of sodium cholate were obtained by titration of weighed quantities of cholic acid to pH 8–9 with sodium hydroxide solutions. These solutions were then brought to volume with double-distilled water. The desired concentrations of sodium cholate were prepared by volumetric dilution of this stock solution.

For the solubilization studies, 5 ml of a solution of sodium cholate was placed in a 2-dram vial to which naphthalene crystals were added in amounts more than sufficient to produce saturation. The vials were covered[5], sealed with parafilm, capped, placed in a water bath, and rotated at 25 ± 0.1° for 3 days. After this time, an appropriate volume, usually 2 ml, was withdrawn with a pipet whose tip had been covered with glass wool to filter excess crystals of naphthalene remaining in the solution.

The sample of cholate solution was quickly diluted to an appropriate volume with double-distilled water. The samples were always added to a volume of water close to that needed for the final volume to prevent precipitation of solid naphthalene crystals and to reduce loss of naphthalene by evaporation. Because of the volatility of naphthalene and its low solubility in water, losses due to evaporation on extensive handling of aqueous solutions can be serious.

[1] Aldrich Chemical Co., Milwaukee, WI 53233
[2] Baker and Adamson quality, Allied Chemicals, Morristown, N.J.
[3] Cary 16.
[4] Model 20, Bronwill Scientific, Rochester, N.Y.
[5] With a liner of Teflon (du Pont).