Simultaneous High-Performance Liquid Chromatographic Determination of Suxibuzone and Its Metabolites in Plasma and Urine

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Abstract □ A high-performance liquid chromatographic method is described for the simultaneous determination of the anti-inflammatory agent suxibuzone and its metabolites, 4-hydroxymethylphenylbutazone, phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone, in plasma and urine. Acidified plasma or urine is extracted with benzene–cyclohexane (1:1). The organic extract is reduced to dryness, and the resulting residue is redissolved in methanol. Aliquots of this solution are chromatographed on a reversed-phase column using a mobile phase of methanol–0.5 *M* KH_2PO_4 (linear gradient from 0 to 100% methanol at 8%/min with a flow rate of 2.0 ml/min) on a high-performance liquid chromatograph equipped with a UV absorbance detector (254 nm). Detection is limited to 0.10 μg/ml for suxibuzone and 4-hydroxymethylphenylbutazone and to 0.05 μg/ml for the other metabolites.

Keyphrases □ Suxibuzone—simultaneous high-performance liquid chromatographic determination with metabolites in plasma and urine □ Metabolites—suxibuzone, simultaneous high-performance liquid chromatographic determination with parent drug in plasma and urine □ High-performance liquid chromatography—analysis, suxibuzone and metabolites in plasma and urine

Suxibuzone [4-butyl-4-(hydroxymethyl)-1,2-diphenyl-3,5-pyrazolidinedione hydrogen succinate] is a new derivative of phenylbutazone that was developed as an anti-inflammatory agent. It was reported that suxibuzone was equipotent to phenylbutazone in pharmacological activity and that its toxicity was one-third to one-fifth of that of phenylbutazone in experiments with animals, indicating greater safety (1–3).

Suxibuzone is metabolized in the body mainly to phenylbutazone through 4-hydroxymethylphenylbutazone as an intermediate, with the phenylbutazone then proceeding to oxyphenbutazone and γ-hydroxyphenylbutazone (Scheme I) (4).

BACKGROUND

An assay for suxibuzone and its metabolites in plasma and urine was reported (4) in which suxibuzone and its metabolites are determined by GLC as their trimethylsilyl derivatives.

For the assay of phenylbutazone and its metabolites in plasma and urine after phenylbutazone administration, several UV (5–13), GLC (14–19), and high-performance liquid chromatographic (HPLC) (20, 21) methods have been reported. The UV method reported by Burns *et al.* (5) has many sensitivity and specificity problems. With the GLC methods, phenylbutazone and oxyphenbutazone were determined as their methyl derivatives by Midha *et al.* (16), phenylbutazone and the heptafluorobutyryl derivative of oxyphenbutazone were determined by Bruce *et al.* (17), and Tanimura *et al.* (18) assayed phenylbutazone and the trimethylsilyl derivatives of oxyphenbutazone and γ-hydroxyphenylbutazone.

An HPLC method using an adsorption chromatographic column (SIL-X) was used to determine phenylbutazone and oxyphenbutazone (20, 21).

In the present study, an HPLC method using a reversed-phase column for determination of suxibuzone and its metabolites, 4-hydroxymethylphenylbutazone, phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone, in plasma and urine after administration of suxibuzone was examined; an accurate and sensitive assay for their measurements was established.

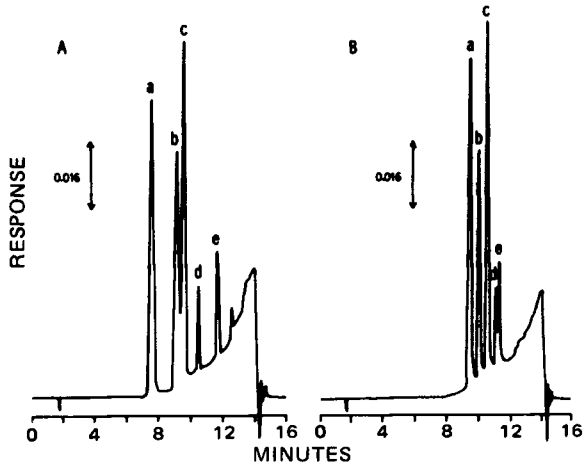

Figure 1—*High-performance liquid chromatograms of authentic samples using a mobile phase of methanol–water (A) and methanol–0.05 M KH_2PO_4 (B) with a linear gradient (0–100% methanol at 8%/min with a flow rate of 2.0 ml/min). Key: a, γ-hydroxyphenylbutazone; b, oxyphenbutazone; c, phenylbutazone; d, suxibuzone; and e, 4-hydroxymethylphenylbutazone.*

EXPERIMENTAL

Materials—Suxibuzone[1], phenylbutazone[1], oxyphenbutazone[1], and γ-hydroxyphenylbutazone[1] were used as received. 4-Hydroxymethylphenylbutazone[2] was synthesized and purified. Benzene, cyclohexane, and methanol were liquid chromatographic grade[3], and the other chemicals used were analytical grade[3].

HPLC Conditions—The high-performance liquid chromatograph[4] was equipped with a high-pressure injection port[5], gradient elution equipment[6], and a fixed-wavelength (254-nm) UV absorbance detector[7].

A reversed-phase chromatographic column[8] (30 cm × 3.9 mm i.d.) was used for the separation and was maintained at room temperature. The mobile phase was a linear gradient of methanol and 0.05 *M* KH_2PO_4 (0 to 100% methanol at 8%/min) at a flow rate of 2.0 ml/min. The mobile phase was degassed by applying a vacuum (~100 mm) to the solvent reservoir for ~2 min before use.

A computer system[9] was employed for quantitative calculation.

GLC Conditions—The gas chromatograph[10] was equipped with a

[1] Laboratory, Esteve, S. A., Barcelona, Spain.
[2] Research Laboratory, Taiho Pharmaceutical Co., Tokushima, Japan.
[3] Wako Pure Chemicals Co., Osaka, Japan.
[4] Model LC-2, Shimadzu, Kyoto, Japan.
[5] Model SIL-1A, Shimadzu.
[6] Model GRE-2, Shimadzu.
[7] Model UVD-2, Shimadzu.
[8] μBondapak C_{18}, Waters Associates, Milford, Mass.
[9] Chromatopac 1A, Shimadzu.
[10] Model GC-4CM, Shimadzu.

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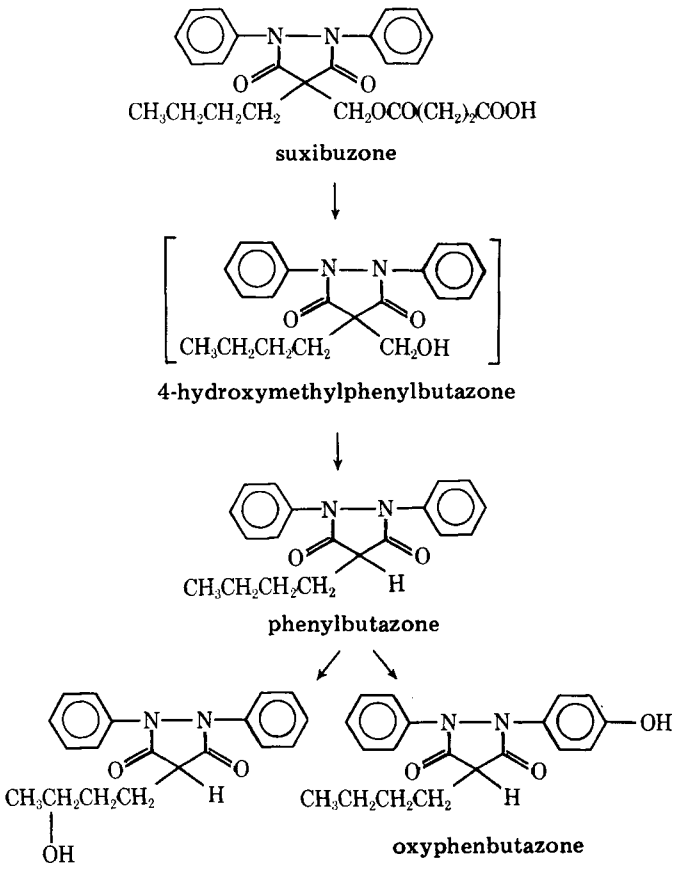

Scheme I—Main metabolic pathways of suxibuzone.

flame-ionization detector. The GLC conditions were described previously (4).

Mass Spectrometric Conditions—A mass spectrometer[11] with an electron-impact ion source was used for identification of suxibuzone and its metabolites. The mass spectrometric analysis was carried out under the following conditions: ionization energy, 75 ev; ionization current, 200 μamp; and acceleration voltage, 10 kv.

Analytical Procedure—Samples of 1.0 ml of plasma or 2.0 ml of urine

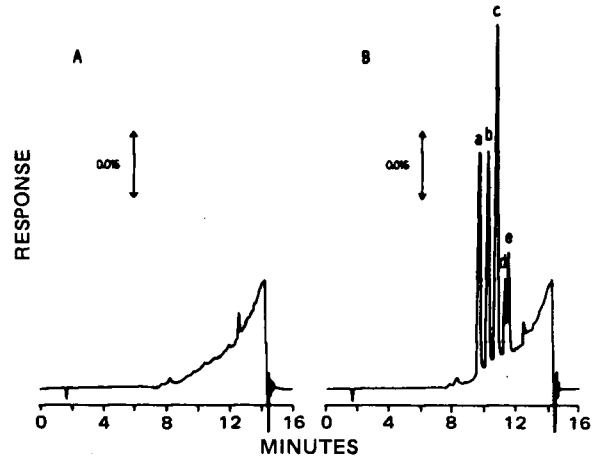

Figure 2—*High-performance liquid chromatograms of beagle dog control plasma (A) and beagle dog plasma extracts following addition of authentic samples at 5.0 μg/ml for each compound (B). Key: a, γ-hydroxyphenylbutazone; b, oxyphenbutazone; c, phenylbutazone; d, suxibuzone; and e, 4-hydroxymethylphenylbutazone.*

[11] Model JMS-01SG02, JEOL, Tokyo, Japan.

Table I—Recoveries on Extraction of Suxibuzone and Its Metabolites from Plasma and Urine of a Beagle Dog

Compound	Added, μg/ml	Recovery[a], %
Suxibuzone	50	99.4
	25	99.6
	5	99.5
Mean ± *SD*		99.5 ± 1.1
4-Hydroxymethylphenylbutazone	50	98.2
	25	97.8
	5	97.9
Mean ± *SD*		97.9 ± 1.4
Phenylbutazone	50	96.5
	25	96.4
	5	97.3
Mean ± *SD*		96.7 ± 1.7
Oxyphenbutazone	50	92.9
	25	92.5
	5	94.0
Mean ± *SD*		93.1 ± 3.7
γ-Hydroxyphenylbutazone	50	92.1
	25	93.4
	5	91.8
Mean ± *SD*		92.4 ± 4.2

[a] Each value is the mean of three HPLC determinations.

were diluted with distilled water, adjusted to pH 2.0 with 5 *N* HCl, and extracted with benzene–cyclohexane (1:1 v/v) (20 ml for plasma and 30 ml for urine) for 20 min. The organic layer containing suxibuzone and its metabolites was separated by centrifugation at 2000×*g*. This extraction was repeated once. The combined organic layer was evaporated to a suitable volume under nitrogen at 30°, transferred to a test tube (10-ml capacity), and dried under nitrogen. The residue was dissolved in 100 μl of methanol, and 2–10 μl of this solution was injected into the liquid chromatograph with a 10-μl syringe[12].

Calibration curves for the determination of suxibuzone, 4-hydroxymethylphenylbutazone, phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone by HPLC were prepared by plotting the peak area against the concentration of each compound. All of these calibration curves were linear.

The procedure for GLC determination was the same as that reported previously (4).

RESULTS AND DISCUSSION

An efficient solvent system (4), benzene–cyclohexane (1:1 v/v) acidified

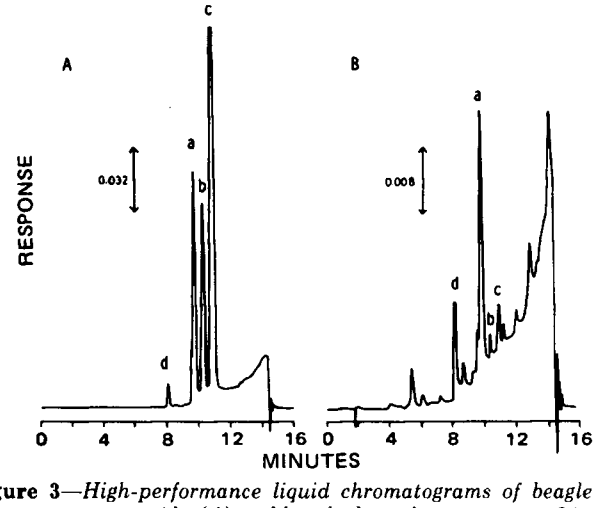

Figure 3—*High-performance liquid chromatograms of beagle dog plasma extracts at 4 hr (A) and beagle dog urine extracts at 24–48 hr (B) following a single oral dose of 142 mg of suxibuzone/kg. Key: a, γ-hydroxyphenylbutazone; b, oxyphenbutazone; c, phenylbutazone; and d, unknown metabolite.*

[12] Model 10A-RN-GP, Scientific Glass Engineering Ltd., North Melbourne, Australia.

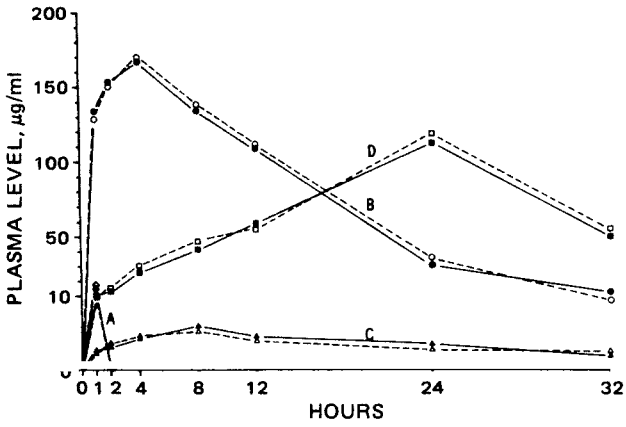

Figure 4—*Comparison of plasma levels of suxibuzone and its metabolites determined by HPLC (closed symbols) and GLC (open symbols) methods following a single oral dose of 142 mg of suxibuzone/kg to a beagle dog. Key: A (◆ and ◇), suxibuzone; B (● and ○), phenylbutazone; C (▲ and △), oxyphenbutazone; and D (■ and □), γ-hydroxyphenylbutazone.*

with hydrochloric acid, was used for the extraction of suxibuzone and its metabolites from plasma and urine. Chloroform, methylene chloride, and ethylene dichloride could not be used as the solvent because of the decomposition, such as oxidation, of the extract during the extraction and evaporation.

A reversed-phase column[8] was used for the HPLC separation. Chromatographic separation of suxibuzone, 4-hydroxymethylphenylbutazone, phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone was first examined using authentic samples. When a linear gradient system with methanol–0.01 *M* sodium acetate buffer (pH 4.0) was used as the mobile phase, suxibuzone and 4-hydroxymethylphenylbutazone were not separated, even when the initial methanol concentration was varied. As shown in Fig. 1, a linear gradient system with methanol–water or methanol–0.05 *M* KH_2PO_4 (0–100% methanol at 8%/min) gave good separation of suxibuzone and the four metabolites. Each compound gave only one chromatographic peak under this condition. Furthermore, no interfering peaks of biological extracts from the plasma and urine of a beagle dog were observed in either of these mobile phases.

On the basis of these results and the knowledge that phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone are major metabolites of suxibuzone (4), the linear gradient system with methanol–0.05 *M* KH_2PO_4 as the mobile phase, which had given the best separation of these compounds, was employed for subsequent experiments.

Methanol was used as a solvent for redissolving the residue obtained by the evaporation to dryness of the benzene–cyclohexane extract from plasma and urine since suxibuzone and its metabolites all are readily soluble in this solvent. The dissolution was made immediately before the chromatographic assay. No decomposition of suxibuzone and its metabolites in methanol was observed. Similar assay results also were obtained when water was used as a solvent for the sample residue.

The chromatogram of suxibuzone, 4-hydroxymethylphenylbutazone, phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone extracted from beagle dog plasma following addition of 5.0 μg/ml of each compound and the chromatogram of a control plasma extract are shown in Fig. 2. The chromatogram of the beagle dog urine extract was similar to that of the plasma extract. The retention times of suxibuzone, 4-hydroxymethylphenylbutazone, phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone were 11.0, 11.2, 10.5, 10.0, and 9.4 min, respectively, and the time required for the assay was 15 min. Each fraction eluted from the HPLC column was collected separately, and the substances were confirmed by mass spectrometric analysis; suxibuzone: *m/e* 438 [M^+], 308, 264, and 183; 4-hydroxymethylphenylbutazone: *m/e* 338 [M^+], 308, 264, and 183; phenylbutazone: *m/e* 308 [M^+], 252, and 183; oxyphenbutazone: *m/e* 324 [M^+], 268, and 199; and γ-hydroxyphenylbutazone: *m/e* 324 [M^+], 309, 280, 266, and 183.

Table II—Comparison of Urine Levels of Suxibuzone and Its Metabolites Determined by HPLC and GLC Methods following a Single Oral Dose of 142 mg of Suxibuzone/kg to a Beagle Dog

Compound	Mole Percent of Dose					
	0–12 hr		12–24 hr		24–48 hr	
	HPLC	GLC	HPLC	GLC	HPLC	GLC
Suxibuzone	ND[a]	ND	ND	ND	ND	ND
4-Hydroxymethylphenylbutazone	ND	ND	ND	ND	ND	ND
Phenylbutazone	0.04	0.05	0.98	1.04	1.23	1.27
Oxyphenbutazone	ND	ND	0.11	0.09	0.31	0.33
γ-Hydroxyphenylbutazone	0.85	0.82	1.96	1.93	19.11	18.99

[a] Not detectable.

The detection limits under these HPLC conditions were 0.1 μg/ml for suxibuzone and 4-hydroxymethylphenylbutazone and 0.05 μg/ml for the other suxibuzone metabolites. The present method had an accuracy of ±2.3–3.1% and very good reproducibility.

Known amounts (5, 25, and 50 μg/ml) of suxibuzone and the four metabolites were added to the plasma and urine (1.0 ml) of beagle dogs, and the recovery of each compound was determined. The results are given in Table I.

One metabolite, *p*,γ-dihydroxyphenylbutazone (4), showed an extraction recovery of <20–30% using benzene–cyclohexane; therefore, this metabolite could not be assayed quantitatively by the present method. A GLC method (4) showed the same result. Although chloroform and ethylene dichloride were able to extract this metabolite efficiently, these solvents were unsuitable because of its decomposition. The retention time for *p*,γ-dihydroxyphenylbutazone under the present HPLC conditions was 10.2 min, and this metabolite had no effect on the separation of suxibuzone and the other metabolites.

A 142-mg/kg (0.32-mmole) dose of suxibuzone was administered orally to the beagle dog, and the concentration of suxibuzone and its metabolites in the plasma and urine was determined using the present method. The chromatogram of the plasma extract at 4 hr and that of the urine extract at 24–48 hr after administration are shown in Figs. 3A and 3B, respectively. With the beagle dog, 4-hydroxymethylphenylbutazone was not detected, as was reported previously (4). The concentrations of suxibuzone and its metabolites in plasma and urine determined by the HPLC method were compared with those reported previously for a GLC determination (4) (Fig. 4 and Table II). The overall difference between the value obtained by the two methods was 4.6%.

The present assay then was applied to the plasma and urine of other animals and humans to which suxibuzone and its metabolites were added. The results obtained for the chromatographic separation, recovery, and sensitivity were in good agreement with those obtained with beagle dog plasma and urine.

The present method is simple and rapid and has high accuracy and sensitivity. It should be useful for basic and clinical pharmacological investigations on suxibuzone.

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Kinetics of Hydrolysis of Methenamine

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Abstract □ The kinetics of degradation of methenamine were studied in citrate–phosphate buffers between pH 2.0 and 7.4 at 37.5°. GLC was used to monitor the rate of hydrolysis. The conversion of methenamine to formaldehyde was found to be pH dependent in the buffers of constant ionic strength, with the reaction half-life decreasing from 13.8 hr at pH 5.8 to 1.6 hr at pH 2.0. The kinetics of degradation also were measured at 47, 57, and 67°, and the reaction obeyed the Arrhenius relationship. At pH 2.0, the activation energy was calculated to be 23.5 kcal/mole; at pH 5.1, it was 12.0 kcal/mole.

Keyphrases □ Methenamine—hydrolysis kinetics in citrate–phosphate buffers □ Hydrolysis kinetics—methenamine in citrate–phosphate buffers □ Kinetics—hydrolysis of methenamine in citrate–phosphate buffers

Methenamine (hexamethylenetetramine) is a urinary antibacterial useful in the long-term therapy of chronic urinary tract infections. The antibacterial activity of methenamine is derived from formaldehyde produced upon hydrolysis of methenamine in the bladder. The amount of hydrolysis is a function of the acidity of the urine (1).

BACKGROUND

Methenamine was introduced for the treatment of urinary infections in 1894 (2). Although this drug has been used for over 80 years and its pharmacological activity depends on the extent and rate of hydrolysis of the parent compounds in acidic media (3), there are few reported studies on the *in vitro* hydrolysis of methenamine. In 1935, Philippi and Löbering (4) studied the hydrolysis of methenamine in buffer solutions. Their assay method was based on the formation of a silver nitrate–methenamine complex. According to Slowick and Kelley (5), this procedure produces inaccurate and undependable results, so the findings of Philippi and Löbering (4) probably are in error.

A paper published by Tada (6) summarized work done from 1951 to 1958 (7–10) in Japan and listed the rates of decomposition of methenamine in hydrochloric acid at pH ≤1.6. Decomposition also was measured in acetic acid–sodium acetate buffer solutions in the pH range of ~4–6. Tada's (6) assay involved the titration of excess acid not consumed in the degradation reaction. Titration procedures normally are tedious and time consuming.

Investigations into the pharmacology of methenamine have been difficult because all of the methods are based on the measurement of formaldehyde liberated from methenamine. Early studies used methods that were relatively insensitive. Many of the analytical procedures are not applicable in the presence of intact methenamine (11). Most studies on urinary formaldehyde concentrations used the colorimetric method of Tanenbaum and Bricker (12). The acidity of the reagents used in this method recently was found to convert 20–30% of the residual methenamine to formaldehyde (13).

Useful scientific data for methenamine compounds generally are not available. The pharmacokinetics and mechanisms of action of this drug have not been well elucidated. Because of the use of other urinary tract antibacterials like nitrofurantoin and the sulfonamides, few recent studies on methenamine have been reported.

This report presents a study of the hydrolysis of methenamine between pH 2.0 and 7.4 in aqueous buffers. The rate of hydrolysis of methenamine was followed by measuring the change in the concentration of intact methenamine determined by the specific and accurate GLC procedure of Strom and Jun (14).

EXPERIMENTAL

Apparatus—The gas chromatograph[1] was equipped with a dual flame-ionization detector. A 1.8-m × 6-mm i.d. glass column was packed with 3% OV-17 on 80–100-mesh Chromosorb W-HP. The operating temperatures were 245° for the injection port, 190° (isothermal) for the column oven, and 250° for the detector. Nitrogen at a flow rate of 45 ml/min was the carrier gas. The flow rates of hydrogen and compressed air were adjusted to optimum sensitivity. The electrometer range was 10^{-10} amp/mv.

Calibration Curve—Methenamine[2] was recrystallized from absolute ethanol. Methylparaben[3] (methyl *p*-hydroxybenzoate), the internal standard, was used as received. Aliquots of 0.5, 1.5, 2.5, 5.0, 7.5, and 10 ml of methenamine solution (2 mg/ml in water containing 2 mg of methylparaben/ml) were added to individual 10-ml volumetric flasks and diluted to volume with water (containing 2 mg of methylparaben/ml). Each solution (1 μl) was injected into the chromatograph. The calibration curve was obtained by plotting the known concentration of methenamine *versus* the corresponding peak height ratio (methenamine/methylparaben).

Buffer Solutions—All buffer solutions were prepared in deionized, distilled water and were adjusted to an ionic strength of 0.5 with potassium chloride. Buffer solutions in the pH range of 2.0–7.4 were prepared by combining various amounts of 0.1 *M* citric acid and 0.2 *M* dibasic sodium phosphate stock solutions (15).

Stability Studies—*Effect of pH*—Amounts of methenamine equal to 0.75 mg/ml were weighed and placed in volumetric flasks. At time zero, the methenamine was dissolved in buffer solutions of pH 2.0, 2.4, 3.4, 4.6, 5.1, 5.5, 5.8, or 7.4 previously equilibrated at 37.5°. After dilution with the respective buffers, 10.0-ml samples were removed and placed in 16 × 125-mm culture tubes with polytef-lined screw caps. These tubes were placed into a constant-temperature water bath.

At suitable time intervals, one culture tube was removed and cooled to room temperature by quenching in an ice bath. One milliliter of the internal standard solution (22 mg of methylparaben/ml in methanol) was added, and the sample was subjected immediately to the GLC analysis. The pH was measured at the beginning and end of each run, and no significant change was found.

Effect of Temperature—Methenamine at 0.75 mg/ml also was studied kinetically at 47, 57, and 67° in pH 2.0, 5.1, and 7.4 buffers.

RESULTS AND DISCUSSION

Figure 1 shows a typical gas chromatogram obtained for an aqueous solution of methenamine (t_R = 0.8 min) and methylparaben, the internal standard (t_R = 1.7 min). Both methenamine and the internal standard gave sharp, well-resolved peaks (resolution, R_s = 5.4) with no tailing. A

[1] Varian Aerograph model 1830, Varian Instruments Division, Palo Alto, CA 94304.
[2] Riker Laboratories, Northridge, CA 91324.
[3] Fisher Scientific Co., Fair Lawn, NJ 07410.

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