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Rapid GLC Determination of Fusaric Acid in Biological Fluids

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Abstract □ A simple, sensitive GLC assay was developed for fusaric acid, the active metabolite of bupicomide, to follow the disposition of this investigational antihypertensive agent in patients undergoing therapy. Fusaric acid is efficiently extracted from biological samples, derivatized by on-column methylation, and chromatographed using flame-ionization detection. An internal standard is utilized to quantitate results. The procedure is rapid and specific for fusaric acid, and has a lower limit of sensitivity of 0.1 μg/ml. The method is suitable for supporting pharmacokinetic studies of bupicomide following therapeutic doses in animals and humans.

Keyphrases □ Fusaric acid—active metabolite of bupicomide, GLC analysis, biological fluids □ GLC—analysis, fusaric acid (active metabolite of bupicomide), biological fluids □ Bupicomide—pharmacokinetic study by GLC analysis of fusaric acid metabolite, biological fluids

Fusaric acid (5-*n*-butylpicolinic acid) isolated from fungus culture filtrates was found to be a potent inhibitor of dopamine β-hydroxylase under both *in vitro* and *in vivo* (1) conditions and demonstrated significant hypotensive effects in animals (2–4) as well as in humans (5–6). Bupicomide[1], the amide derivative of fusaric acid, was reported to possess pharmacological activities similar to the parent compound (7).

Bupicomide is presently undergoing clinical trial as an antihypertensive agent. Studies using radiolabeled drug indicated that bupicomide underwent extensive metabolism rapidly when administered orally to animals (8). No unchanged drug was found by following the plasma time course of bupicomide. Only fusaric acid, a major metabolite, was identified.

Similar findings were reported in the same studies (8) after administration of a 15-mg/kg oral dose of bupicomide to humans. The biological half-life of fusaric acid was reported to be 8.7 hr in humans, utilizing a UV spectrophotometric procedure. However, for the analysis of fusaric acid in plasma, direct application of the UV assay to clinical studies involving therapeutic doses is limited by the lack of sensitivity and specificity.

The present work describes an analytical method in which fusaric acid is extracted from a biological sample along with an added internal standard into an organic solvent and subsequently is derivatized by on-column methylation for GLC determination. The procedure is applicable to blood and urine and is rapid enough to be performed routinely for clinical studies of patients on bupicomide therapy.

[1] Sch 10595.

EXPERIMENTAL

Reagents—All chemicals and solvents used were analytical reagent grade. The internal standard, *N*-*n*-butylphthalimide, was synthesized according to standard methods (9) by refluxing *n*-butylamine and phthalic anhydride in acetic acid. The crude product was recrystallized from alcohol–water before use, mp 32 ± 0.5° (10).

Procedure for Biological Samples—Blood samples were collected[2] and allowed to clot. The serum was separated by centrifugation and frozen until analysis. Spiked standards of fusaric acid in pooled serum were treated similarly. Urine samples also were collected, frozen, and stored until analysis.

A biological fluid, 0.5 ml, was transferred to a screw-capped[3] tube containing 2 μg of *N*-*n*-butylphthalimide, 0.5 ml of 0.1 *M* pH 4 citrate buffer that had been presaturated with sodium chloride, and 4.0 ml of methylene chloride. The mixture was mechanically shaken for 5 min and then centrifuged at 2200 rpm for 5 min to allow separation of the phases. The top layer was aspirated off, and the organic layer was decanted into a 5-ml vial[4] and evaporated to dryness under vacuum.

About 0.3 ml of acetone was used to wash the sides of the vial with intermittent vortex mixing. The acetone was removed under vacuum, and 10–15 μl of 0.1 *M* trimethylphenylammonium hydroxide[5] was added to the evaporated sample, followed by 1 min of vigorous vortex mixing. Then 1–2 μl of the mixture was injected into the gas chromatograph.

GLC—The assay was performed on a gas chromatograph[6] equipped with a flame-ionization detector. A 183-cm (6-ft) × 2-mm i.d. glass coiled column packed with 3% OV-1 on 100–120-mesh Gas Chrom Q[7] was used. GLC conditions were: column temperature, 150°; and detector and injector temperatures, 250°. The nitrogen carrier gas was maintained at 40 ml/min, while air and hydrogen flow rates were 250 and 30 ml/min, respectively. Methylation of the samples occurred in the injection port, with the derivatized product eluting as a sharp peak at ~6 min followed by the internal standard peak at ~7 min.

Injection could be resumed at ~12 min when the impurities had

[2] Vacutainers, no anticoagulant, Beckton, Dickinson and Co., Rutherford, N.J.
[3] Lined with Teflon (du Pont).
[4] Reacti-vial, Pierce Chemical Co., Rockford, Ill.
[5] Eastman Organic Chemicals, Rochester, N.Y.
[6] Varian model 2700 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Hitachi Perkin-Elmer recorder, model 156.
[7] Applied Science Laboratories, State College, Pa.

Table I—Variation of Extraction of Fusaric Acid with pH

pH	Extraction of Fusaric Acid[a,b], %	
	0.1 *M* Citrate Buffer	0.1 *M* Citrate Buffer with 2 *M* NaCl
1.65	71.8	75.3
2.10	76.4	80.5
2.60	77.4	82.8
3.05	78.0	84.9
4.05	78.0	84.9
4.55	77.5	84.9
5.05	75.4	83.9
6.08	55.7	76.6

[a] Average of two determinations. [b] Based on equal volumes of methylene chloride and buffer solution.

been eluted from the column. At the end of the day, the column was reconditioned at 220° and periodically treated with a mixture of trimethylsilyl donors[8]. The detector was cleaned by sonication in acetone after several days of use. These steps ensured optimum efficiency and sensitivity of the system.

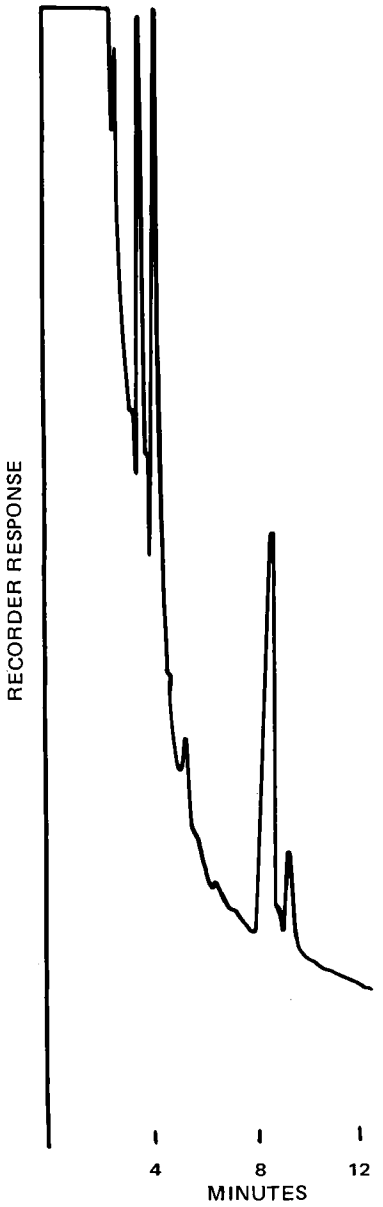

Figure 1—*Gas chromatogram of a serum control.*

[8] Silyl 8, Pierce Chemical Co., Rockford, Ill.

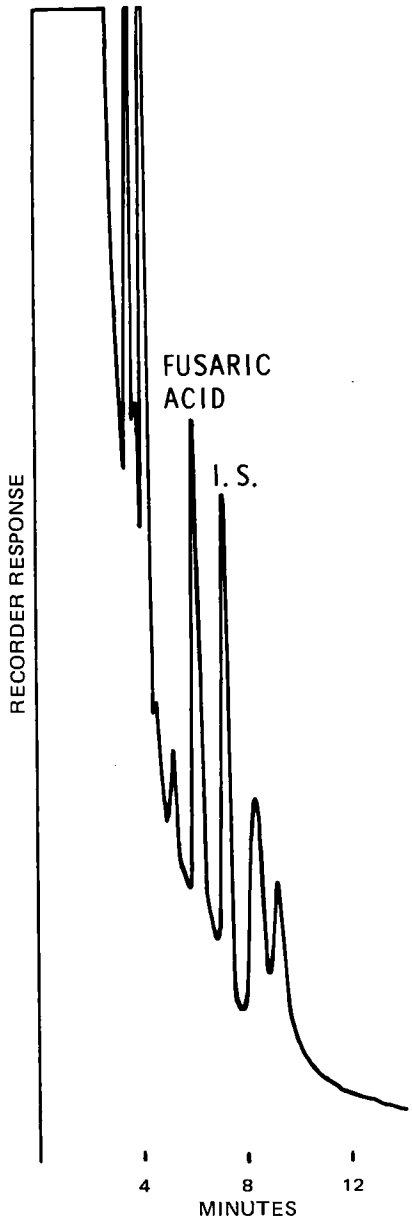

Figure 2—*Gas chromatogram of a serum sample of fusaric acid with the addition of* N-n-*butylphthalimide (I.S.) (fusaric acid concentration = 5.00 μg/ml;* N-n-*butylphthalimide concentration = 0.13 μg/μl injected).*

RESULTS AND DISCUSSION

The amphoteric nature of the fusaric acid molecule requires careful consideration of the extraction system to assure maximal extraction efficiency and minimum interference from biological samples. Methylene chloride was the solvent of choice. The variation in the percent recovery with pH was systematically examined (Table I). It was apparent that pH 4 was optimal and that the addition of salt not only enhanced the extraction but also made the percent recovery less dependent on the pH. Under the assay conditions, the recovery of fusaric acid from biological samples was better than 90%.

The addition of an internal standard to the samples prior to extraction eliminated the necessity of pipetting and allowed almost quantitative transfer of the extract. *N-n*-Butylphthalimide was selected because of its stability, purity, and extraction properties as well as its convenient retention time under the chromatographic conditions. Besides methylation, other alkylations of fusaric acid with triethyl-, propyl-, and butylammonium hydroxides were attempted to determine optimal sensitivity and specificity. However, after carrying through the procedure with plasma, serum, and

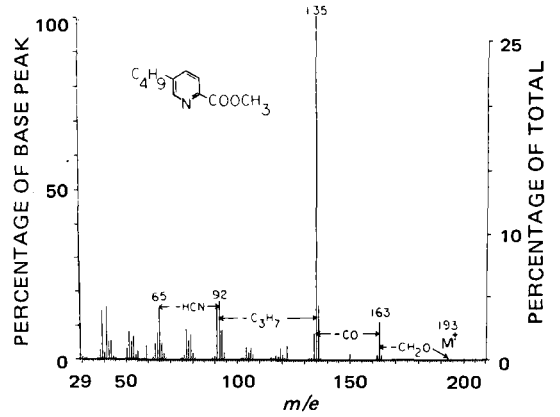

Figure 3—*GLC–mass spectrum of the methyl ester of fusaric acid.*

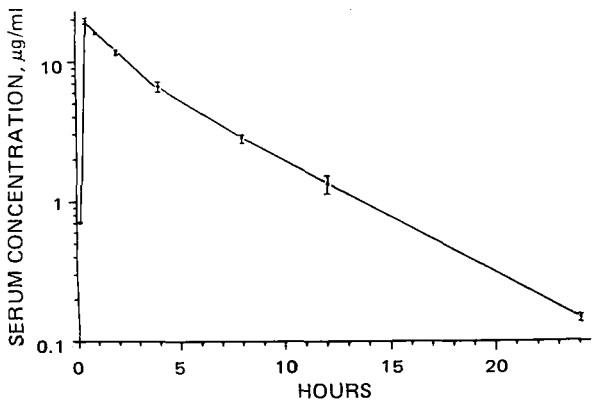

Figure 4—*Serum concentration of fusaric acid in a fasted normal volunteer following a single oral dose of 200 mg of bupicomide.*

urine blanks, only the methylated product eluted without interference.

Figures 1 and 2 represent typical chromatograms from extracted human serum control and sample, respectively. The sharpness of the peaks enabled the use of peak height ratios for quantitation. A linear standard curve, corresponding to original concentrations of fusaric acid of from 0.2 to 20 μg/ml, was obtained. The sensitivity limit was 10 ng of injected compound. Standard deviation at 1 μg/ml was 1.5% for five replicate samples.

Specimens containing higher concentrations were originally diluted with pooled serum and analyzed as already described. Subsequently, water was used to adjust all samples to 0.5 ml since no differences were observed in extraction efficiency. Serum samples with fusaric acid levels as high as 100 μg/ml were quantitated in this manner.

To ascertain the specificity of the method, the biological samples were analyzed by injection into a gas–liquid chromatograph interfaced with a mass spectrometer[9]. The resulting mass spectrum of the peak eluting at 6 min is shown in Fig. 3. It corresponded to the methyl ester of fusaric acid.

The applicability of the assay to clinical studies is demonstrated by following the serum time course of fusaric acid in a fasted normal volunteer after a single oral dose of 200 mg of bupicomide. Urine specimens from the same period were collected and analyzed (Figs. 4 and 5). The serum concentration of fusaric acid rose sharply, reaching peak levels within 1 hr, and then declined rapidly, with a half-life estimated to be 3.7 hr. Over a 24-hr period, only about 3.5% of the dose was excreted as fusaric acid.

While others (8, 11) obtained similar urinary data in animals, the half-life of fusaric acid in humans was reported to be 8.7 hr (8). Such discrepancy may be due to variations in the subjects, doses, or experimental conditions or, very likely, to the nonspecificity of the analytical method. Since fusaric acid has been demonstrated to undergo side-chain oxidations in the body (11), metabolites would

[9] Varian model CH-7, Varian Mat Divisions, Springfield, N.J.

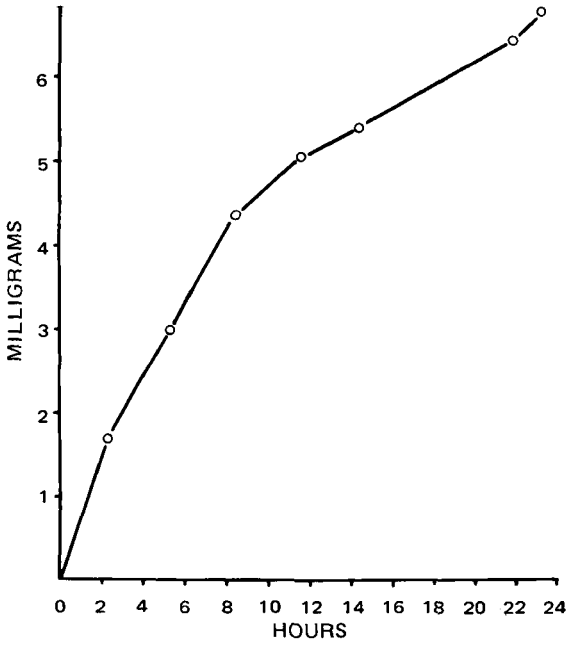

Figure 5—*Cumulative urinary excretion of fusaric acid in a fasted normal volunteer following a single oral dose of 200 mg of bupicomide.*

not be readily distinguishable from the parent compound when analyzed by the UV method. Apparent higher plasma concentration of fusaric acid would result, and the estimated half-life would appear longer.

The GLC procedure described here has been adapted for analyzing heart and brain tissue samples to delineate tissue distribution in rats. Additional studies are in progress to extend the method to other tissues and to determine the ultimate sensitivity requirements for establishing pharmacokinetic parameters in animals and humans.

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