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DETERMINATION OF SERUM TOLBUTAMIDE BY GAS CHROMATOGRAPHY

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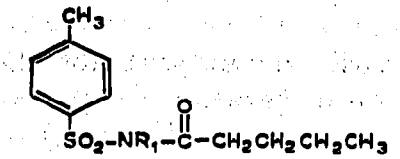
SUMMARY

A gas chromatographic method utilizing a flame ionization detector is described for the determination of tolbutamide in serum. The drug is extracted from acidified serum (pH 5.4), methylated with dimethyl sulfate and the resulting N-methyltolbutamide is determined by gas chromatography against an internal reference standard, N-propyl-*p*-chlorobenzenesulfonamide.

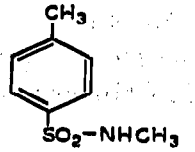
Under suitable conditions, N-methyltolbutamide is quantitatively pyrolyzed to N-methyl-*p*-toluenesulfonamide. Peak areas for equimolar concentrations of these two compounds were similar and linear with respect to the internal standard over a range of 10 to 50 μg of tolbutamide equivalents per 100 μl. Overall recoveries from human and beagle serum were 80.8 $\pm$ 3.5% and 82.0 $\pm$ 3%, respectively, based on the N-methyltolbutamide external standard curve.

INTRODUCTION

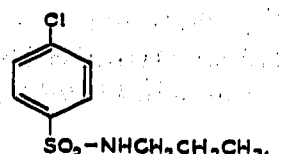
In a recent publication, SABIH AND SABIH[1] described a gas chromatographic (GC) method for the determination of tolbutamide(I) in biological fluids. This method involved the conversion of tolbutamide to its N-methyl derivative(II) by treatment with dimethyl sulfate. In general, the method appeared to offer definite advantages over existing procedures such as spectrophotometry and colorimetry, in which blank contributions were appreciable and variable.



(I) $R_1 = H$
(II) $R_1 = CH_3$



(III)



(IV)

Several unsuccessful attempts were made in these laboratories to reproduce

this method. In the first place, the methylation procedure gave an impure semi-solid product that melted at 33°, but a modification produced a pure crystalline product that melted at 50°. Elemental analysis and infrared (IR) and nuclear magnetic resonance (NMR) spectra established the integrity of II.

Contrary to claims made by SABIH AND SABIH[1], methylation of tolbutamide does not stabilize the molecule and prevent its decomposition on the column under high inlet temperature conditions. By employing a suitable column and appropriate inlet-column temperatures, II was quantitatively pyrolyzed to the corresponding sulfonamide(III). Single peaks with identical retention times (R_t 4.5 min) were obtained following injection of both compounds. Thermal decomposition under GC conditions of structurally similar *p*-tosyl carbamates to the corresponding sulfonamides was reported by FISHBEIN *et al.*[2]. In order to quantitate the procedure by the internal standard technique, a similar sulfonamide, N-*n*-propyl-4-chlorobenzenesulfonamide (IV), was selected with an R_t of 6–7 min. Peak areas of equimolar concentrations of II and III corresponding to 10–50 μg of tolbutamide equivalents were identical and linear with respect to the internal standard.

Tolbutamide was extracted from acidified human and beagle serum (pH 5.4) with chloroform and partitioned between aqueous methanol and light petroleum in order to minimize lipid interference in the chromatogram. By spiking serum samples with tolbutamide and utilizing the extraction, methylation and GC conditions described herein, overall recoveries of 80.8 and 82.0% from human and beagle serum, respectively, were obtained based on an external N-methyltolbutamide standard curve. The method was utilized to determine serum levels in three beagles following peroral administration of capsules containing 10 mg of tolbutamide/kg of body weight. Peak levels of 50–53 μg/ml were obtained between 2 and 4 h.

EXPERIMENTAL AND RESULTS

Preparation of N-methyltolbutamide (II)

Tolbutamide U.S.P. (13.5 g) was dissolved in dry acetone (50 ml) and refluxed with stirring for 1 h in the presence of anhydrous potassium carbonate (6.9 g) and dimethyl sulfate (5.5 ml). This reaction mixture was cooled and filtered, and the filtrate was evaporated under reduced pressure. The resulting oil was dissolved in diethyl ether (50 ml) and washed with 70 ml of 2% potassium hydroxide solution and water (70 ml). The ethereal solution was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to yield a semi-solid material (9.6 g), which readily crystallized on trituration with hexane, m.p. 42–47°. Analytical sample II was crystallized from diethyl ether–hexane and melted at 49–50°.

Analysis. Calculated for $C_{13}H_{20}N_2O_3S$: C, 54.90; H, 7.09; N, 9.85; S, 11.28%. Found: C, 55.05; H, 7.05; N, 10.16; S, 11.11%.

The NMR spectrum ($CDCl_3$) showed a characteristic singlet peak for the N-methyl group at $\lambda = 6.87$, which yielded three protons on integration.

Preparation of N-methyl-p-toluenesulfonamide (III)

Methylamine hydrochloride (3.4 g) and *p*-toluenesulfonyl chloride (9.5 g) were refluxed in pyridine (50 ml) for 3 h. The reaction mixture was then evaporated under reduced pressure and the resulting residue dissolved in diethyl ether (50 ml). After

washing it several times with water and drying it over anhydrous sodium sulfate, the ethereal solution was evaporated under reduced pressure to produce an oil that slowly crystallized from a mixture of diethyl ether–heptane (1:2). The analytical sample melted at 79–81°.

Analysis. Calculated for $C_8H_{11}NO_2S$: C, 51.90; H, 5.98; N, 7.56; S, 17.31%. Found: C, 52.30; H, 6.03; N, 7.83; S, 17.29%.

Preparation of the internal reference standard (IV)

To a stirred solution of 4-chlorobenzenesulfonyl chloride (106 g) in chloroform (300 ml), maintained below 40°, was added *n*-propylamine (36.5 g) over a 30-min period. After stirring for an additional 10 min, a 10% aqueous solution of sodium hydroxide (200 ml) was added dropwise to the reaction mixture with cooling and stirring. The chloroform layer was then separated, washed with water (2 × 100 ml), dried over anhydrous sodium sulfate and finally evaporated under reduced pressure. The resulting oil was dissolved in boiling light petroleum and allowed to crystallize. The analytical sample melted at 57–58°.

Analysis. Calculated for $C_9H_{12}ClNO_2S$: C, 46.25; H, 5.18; Cl, 15.17; N, 6.00; S, 13.72%. Found: C, 45.83; H, 5.01; Cl, 15.03; N, 5.95; S, 14.23%.

Gas chromatography

Only analytical or spectrograde reagents were used. Acetone was redistilled over anhydrous potassium carbonate prior to use. Potassium carbonate was finely powdered with a mortar and pestle, heated in an oven at 125° for 3 h and stored in a vacuum desiccator.

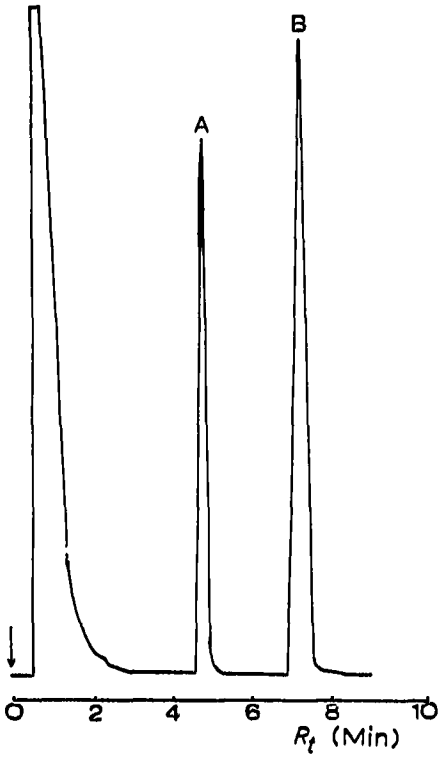
A Mikro-Tek gas chromatograph, Model MT-220, equipped with a flame ionization detector was used for the analysis. The column was U-shaped, made from borosilicate glass (6 × 1/4 ft. O.D.) packed with 3% OV-17 on 100–120 mesh Chromosorb W-HP supplied by Chromatographic Specialities, Brockville, Ontario, Canada. In order to ensure complete pyrolysis of N-methyltolbutamide, it was necessary to fill with glass-wool that portion (*ca.* 10 cm) of the column that was surrounded by the inlet heating block. This finding was discovered accidentally when a new column replacement failed to function correctly because of excess packing in the column head. Prior to use, the column was conditioned at 300° for 24 h with a nitrogen carrier gas flow-rate of 20 ml/min.

The injection and detector temperatures were 285° and the column temperature was 190°. Oxygen and hydrogen flow-rates were adjusted to give maximum response. Nitrogen was used as the carrier gas at a flow-rate of 45–55 ml/min. The conditions of flow-rate and oven temperature must be adjusted to obtain an R_t of 4.5 min for II and III and of 6.8 min for IV. The solid-state electrometer input was set at 10, output attenuation at 32 and bucking range selector at 2×10^{-9}. Peak areas were recorded by means of a Model LD 11 B instrument made by Westronics Inc., Fort Worth, Texas, U.S.A., and integrated electronically (Model 3370 A, Hewlett-Packard, Pointe Claire, Quebec, Canada).

Comparison of standard curves for II and III

Standard solutions of II and III were prepared in chloroform at molar concentrations equivalent to 1 mg of tolbutamide per millilitre. Appropriate aliquots (10–50

μl) of these solutions were transferred by pipette with a 50-μl syringe into individual glass-stoppered centrifuge tubes (15 ml). The solvent was evaporated under a stream of nitrogen and the tubes were dried *in vacuo* for 5 min. Each sample was then dissolved in 100 μl of internal standard solution prepared by dissolving compound IV in chloroform (0.6 mg/ml). Approximately 2.5 μl of the sample solutions were injected into the GC apparatus. A typical chromatogram is shown in Fig. 1. Peak area ratios of tolbutamide equivalents of II and III to the internal standard are linear and identical (Fig. 2).



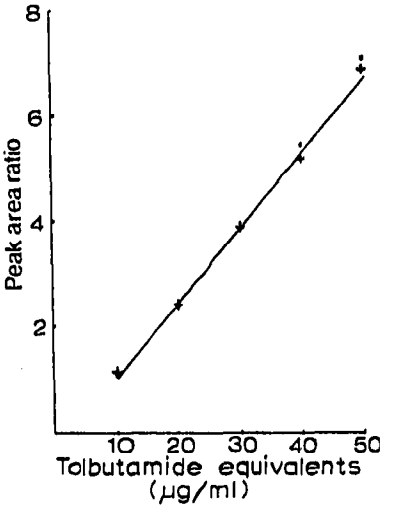


Fig. 1. A typical chromatogram for either II or III. Peak A = II or III; peak B = internal standard (IV).

Fig. 2. Peak area ratios of tolbutamide equivalents of II (+) or III (●) to internal standard (IV).

Preparation of the sample for GC analysis

Recovery of tolbutamide from serum. Ten human and beagle serum samples (1 ml) were transferred into micro-separating funnels (30 ml) and spiked with tolbutamide (25 μg) from a methanolic stock solution (0.2 mg/ml). After mixing, the samples were acidified with $M/30$ phosphoric acid (2 ml) and extracted fairly vigorously with chloroform (3 × 10 ml). A lower emulsified chloroform layer separated, which was filtered immediately through a funnel containing a cotton plug and anhydrous sodium sulfate (15 g) into glass-stoppered centrifuge tubes (40 ml). The sulfate was washed with chloroform (8 ml) and the washings combined with the chloroform extracts. After concentration of the solvent (2 ml) under a stream of nitrogen on a water-bath and final evaporation at room temperature, the resulting residue was dissolved and partitioned between 90:10 methanol–water (2 ml) and light petroleum (3 ml) with

vortex mixing. After removing and discarding the upper light petroleum layer by means of a disposable Pasteur pipette, the washing was repeated with fresh solvent (2 × 3 ml). No detectable loss of tolbutamide was observed following this clean-up procedure and chromatograms demonstrating the usefulness of this procedure are shown in Fig. 3. The methanol–water layer was evaporated to dryness on a water-bath under nitrogen and the resulting residue dried *in vacuo* (0.1 mm) for 5 min. Dried samples were then ready for the following methylation procedure.

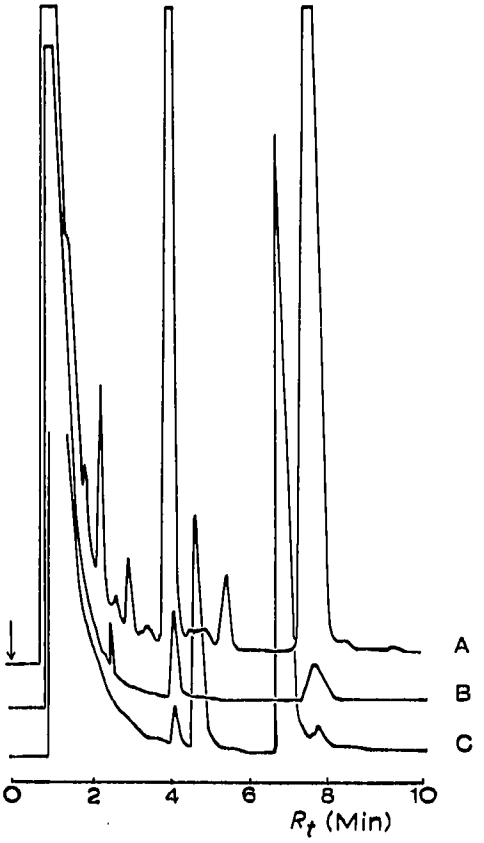


Fig. 3. Gas chromatograms of methylated extracts of (A) beagle control serum before clean-up, (B) after clean-up and (C) actual sample treated with 25 μg of tolbutamide and containing internal standard.

Methylation procedure. Samples from the above extraction procedure were dissolved in dry acetone (10 ml) and the mixture was allowed to reflux on a water-bath for 15 min in the presence of activated potassium carbonate powder (50 mg) and freshly distilled dimethyl sulfate (0.1 ml). The reaction mixtures were concentrated under nitrogen to approximately 1 ml and then diluted with saturated sodium chloride solution (5 ml). Each sample was extracted with hexane (3 × 4.5 ml) using vortex mixing. After centrifugation, the organic layer was removed by means of a Pasteur pipette and transferred to a glass-stoppered centrifuge tube (15 ml). Precautions must be taken during this step to avoid uptake of the aqueous layer, which interferes with the GC analysis. The extracts were combined and evaporated to dryness on a water-bath under nitrogen and the resulting residue was dried under a high vacuum for 5 min. The samples were then dissolved in the internal standard solution (100 μl) for

GC analysis. Overall recoveries were 80.8 ± 3.5% and 82.0 ± 3% from human and beagle serum, respectively.

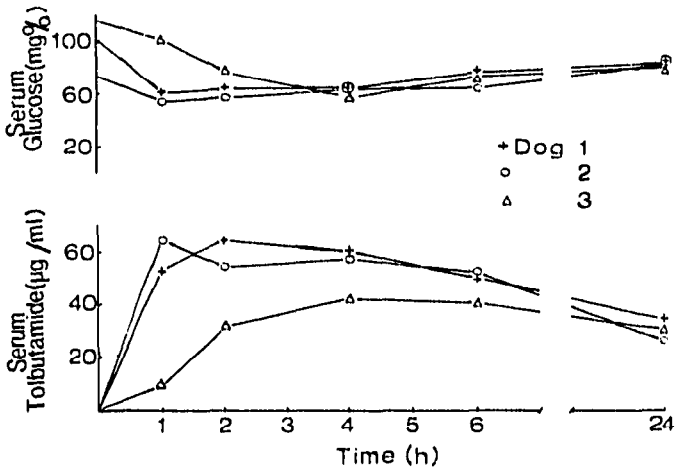


Fig. 4. Serum tolbutamide and glucose levels in three beagles following peroral administration (10 mg/kg).

Determination of serum tolbutamide in dosed beagles

Three beagles weighing approximately 10 kg were fasted for 18 h and capsules containing tolbutamide U.S.P. (< 200 mesh) were administered perorally in a single dose of 10 mg/kg. The animals were fasted for an additional 6 h and then fed. Blood samples (10 ml) were drawn by venipuncture from a radial vein at 0, 1, 2, 4, 6 and 24 h. Serum was separated and tolbutamide recovered and determined according to the above procedure. In analyzing complete sets of serum samples, it is advantageous to spike the zero-hour samples with tolbutamide (*e.g.*, 25 μg/ml) and to determine the percentage recovery for each set. This procedure provides a check on the accuracy of the extraction and methylation steps on a given day. Serum tolbutamide results for the three dogs are shown in Fig. 4. Serum glucose levels were monitored simultaneously on an AutoAnalyzer and an excellent correlation between drug effect and serum drug level was observed in beagle No. 3.

REFERENCES

1 K. Sabih and K. Sabih, *J. Pharm. Sci.*, 59 (1970) 782.
2 L. Fishbein, W. L. Zielinski, Jr., and R. O. Thomas, *J. Chromatogr.*, 30 (1967) 596.