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GAS CHROMATOGRAPHIC DETERMINATION OF β-PHENETHYLBIGUANIDE AND ITS METABOLITE *p*-HYDROXY-β-PHENETHYLBIGUANIDE IN SERUM AND URINE*

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SUMMARY

A gas chromatographic method is presented for the detection of β-phenethylbiguanide (PEBG) and its metabolite, *p*-hydroxy-β-phenethylbiguanide (*p*-OH PEBG). The procedure is applicable for the determination of the drug and its metabolite in the serum and urine of rats. The detection limit is 0.2 μg PEBG and 0.5 μg *p*-OH PEBG per ml of serum or urine.

A time course study of blood concentration and elimination rate following intraperitoneal injection of 100 mg/kg of PEBG to normal rats was performed. β-PEBG was found to be present in the blood and the urine, *p*-OH PEBG was only detected in the urine. Twenty-four hours following intraperitoneal injection, the urine contained 32% of the administered dose, 20% as unaltered PEBG and 12% as *p*-OH PEBG.

INTRODUCTION

The biguanides, either alone or in conjunction with the sulfonylureas or with insulin, are currently used as therapeutic agents for the treatment of diabetes mellitus. The exact mechanism by which the biguanides exert their hypoglycemic effect is unknown[1].

The problems encountered in formulating a hypothesis for the mode of action of β-phenethylbiguanide (PEBG, phenformin or DBI) might be alleviated if a sensitive method, suitable for use with biological fluids, were to be developed for the assay of PEBG and its major urinary metabolite *p*-hydroxy-β-phenethylbiguanide (*p*-OH PEBG)[2,3]. A spectrophotometric method has been reported for the detection of PEBG in serum, urine and in the liver[4]. A gas chromatographic (GC) procedure for the detection of PEBG and buformin that was not applicable to serum or urine samples has

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also been reported[5]. To the best of our knowledge, no method has been reported for the quantitative detection of *p*-OH PEBG in urine or serum.

We present in the following study a GC procedure that is applicable for the quantitative detection of both PEBG and *p*-OH PEBG in serum and urine of laboratory rats. Extraction of the serum or the urine with ethanol quantitatively removes PEBG and *p*-OH PEBG. Removal of the ethanol *in vacuo*, followed by approximately 5-sec exposure to trifluoroacetic anhydride, forms derivatives that are quantitatively detectable on a GC apparatus equipped with a flame ionization detector (FID).

EXPERIMENTAL

Materials

Trifluoroacetic anhydride (M.C.B., Norwood, Ohio, U.S.A.) was redistilled and stored under vacuum in a desiccator. Analytical grade acetone was stored over molecular sieves and placed over sodium sulfate prior to use. Absolute ethanol was used as obtained. The drug PEBG and its metabolite *p*-OH PEBG were a generous gift of the U.S. Vitamin Pharmaceutical Corporation (Yonkers, N.Y., U.S.A.).

A Beckman Model 45 gas chromatograph, equipped with dual flame ionization detectors, and U-shaped 6-ft. × 1/8-in.-O.D. stainless-steel columns packed with 10% SE-30 coated on 80–100 mesh Chromosorb W HPT solid support (Beckman, Fullerton, Calif., U.S.A.) was used.

The following are the chromatographic conditions: injector heater, detector heater, detector line and column temperatures, 275°, 280°, 300°, and 208°, respectively; chart speed, 1/2 in./min; flow rates of hydrogen, helium and air, 40, 25 and 290 ml/min, respectively.

Internal standard solution

A solution of 2.0 mg/ml naphthylamine (Eastman) in absolute ethanol was prepared.

Substrate standard solutions

Absolute ethanolic solutions of PEBG, 0.2 and 2.0 mg/ml, and *p*-OH PEBG, 0.1 and 1.0 mg/ml, were prepared.

Preparation of the PEBG and the p-OH PEBG derivatives

PEBG, 500 mg, was placed in a 12-ml conical centrifuge tube followed by addition of trifluoroacetic anhydride, 1.5 ml. The tube was shaken on a vortex mixer until solution had been attained. Excess reagent was removed *in vacuo* on a Buchler Rotary Evapo-mix. The residue was re-crystallized twice from absolute ethanol, yielding a white solid, m.p. 156.2–157.5°.

A similar procedure was carried out with *p*-OH PEBG, yielding a white product, m.p. 138–140°.

Standardization of the GC apparatus and blank control

Aliquots of standard solutions of PEBG and *p*-OH PEBG, containing from 0.2–90 μg, were placed in a 12-ml conical centrifuge tube. Standard naphthylamine solution, 50 μl, was added and the resultant mixture was taken to dryness *in vacuo*.

Trifluoroacetic anhydride, 100 μl, was added to the solid residue and the solution was shaken with a vortex mixer for 5 sec. Excess reagent was then removed *in vacuo*, and the sample reconstituted in 100 μl of anhydrous acetone. Subsequently 1-μl aliquots were introduced into the gas chromatograph for analysis.

Blood and urine sample collection

Phenformin was administered to normal male Holtzman albino rats at the dosage rate of 100 mg/kg. The drug was administered by intraperitoneal (i.p.) injection for both the serum and urine studies. The appropriate amount of PEBG was dissolved in 0.5 ml of 0.9% saline solution.

For studies involving the serum, the animals, following i.p. injection, were etherized at appropriate time intervals and whole blood, via arterial puncture, was collected in a 12-ml conical centrifuge tube. The blood was centrifuged for 10 min at 400 *g* and the clear serum was collected in 5-ml vials, capped with PTFE, and refrigerated until analyzed.

Urine was collected directly from the bladders of the animals sacrificed for the serum during the first 30 min following drug administration. If urine was found to be present, it was withdrawn into a hypodermic syringe by inserting a 1/2-in. needle into the bladder.

The latter points for the urine study were collected at appropriate intervals in suitable metabolic cages.

Serum preparation

A solution containing 20 μl of 0.5 *N* acetic acid and 2 ml of absolute ethanol is added to 0.2 ml of serum in a 12-ml conical centrifuge tube. The mixture was shaken with a vortex mixer for 5 min to insure extraction of PEBG or possible metabolites, centrifuged for 10 min at 400 *g* and the precipitated material discarded. The supernatant solution was collected in a clean conical centrifuge tube and taken to dryness *in vacuo*, at 30°. Standard naphthylamine solution, 50 μl, was then added and the contents of the tube were again taken to complete dryness on the Buchler Rotary Evapo-mix. Trifluoroacetic anhydride, 100 μl, was added to the dried residue. The centrifuge was covered with parafilm and then shaken with a vortex mixer for approximately 5 sec. The excess reagent was evaporated under vacuum. The residue was dissolved in 100 μl of anhydrous acetone; 1 μl of this solution was then analyzed for the presence of PEBG or *p*-OH PEBG by GC.

Urine preparation

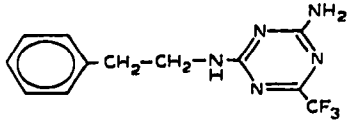
Urine, 0.2 ml, was pipetted into a 12-ml conical centrifuge tube and taken to dryness *in vacuo* at 30°. Standard naphthylamine solution, 50 μl, was added and the drying step repeated. Ethanol, 2 ml, was added and the procedure continued as described for the serum preparation.

RESULTS

Identification of the derivatives

Nuclear magnetic resonance (NMR), infrared (IR), and mass spectrometric (MS) data[6] indicate that the PEBG derivative is the substituted triazine, 2-amino-4-

trifluoromethyl-6-(2-phenylethyl)-amino-1,3,5-triazine (structure I). Such a structure is consistent with previous studies on similar reactions of biguanides by Wickramashinge and Shaw[5], Elpern[7] and Hendry and Rose[8].



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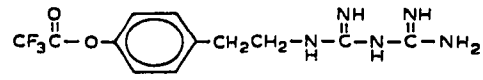
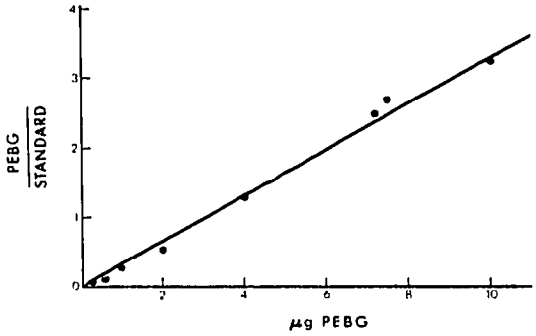


II

Likewise, NMR, IR and MS data suggest that the *p*-OH PEBG derivative is the trifluoroacetyl ester of *p*-OH PEBG (structure II).

Detector response

Figs. 1–4 show the relative response of the GC detector to the various concentrations of the PEBG or *p*-OH PEBG injected. Areas of the peaks corresponding to the response of PEBG and *p*-OH PEBG were calculated using disc integrator or a Gilman planimeter. Concentrations of PEBG and *p*-OH PEBG were expressed as ratios of peak area to peak height of the naphthylamine standard.



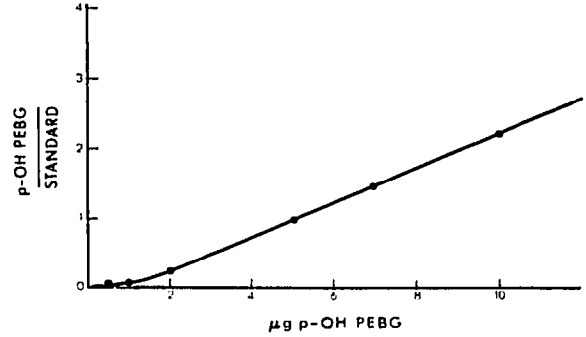


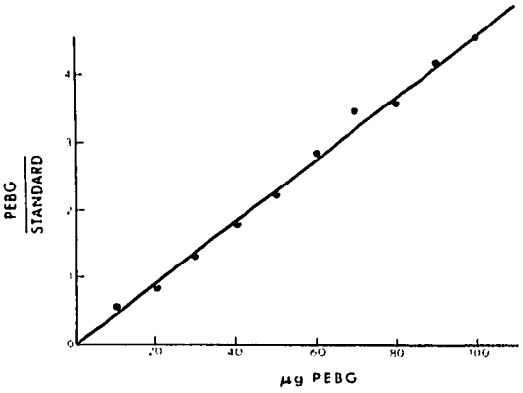
Fig. 1. Standard curve for PEBG from 0.2 to 10 μg. The ratio is PEBG peak area (range 1, attenuator 32) to naphthylamine standard peak height (range 10, attenuator 512).

Fig. 2. Standard curve for *p*-OH PEBG from 0.2 to 10 μg. The ratio is *p*-OH PEBG peak area (range 1, attenuator 8) to naphthylamine standard peak height (range 10, attenuator 512).

The purified triazine derivative of PEBG appeared as a single peak with a retention time of 4 min 50 sec. Frequently, however, PEBG in serum, urine, or standard curve samples gave rise to a second minor peak with a retention time of 3 min 10 sec. When this occurred, the areas of the major and minor peaks were added together in calculating the PEBG response. The purity of the trifluoroacetic anhydride and length of PEBG exposure time to this reagent were determined to be the causative factors in producing the second minor peak.

The metabolite, *p*-OH PEBG, gave rise to a major peak eluting at a retention time of 7 min 50 sec. The standard naphthylamine elutes at 1-min retention time.

The gas chromatograms of blank serum and urine samples, carried through the entire extraction and derivatization procedure, were free of any interfering peaks.



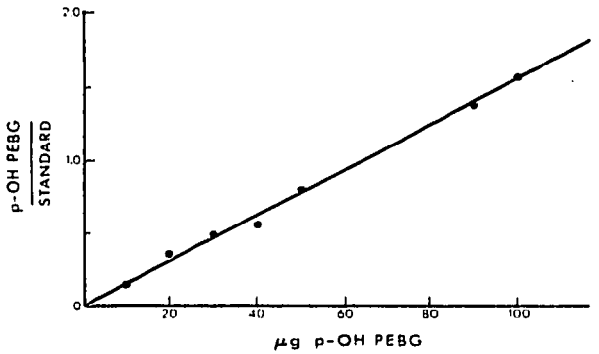


Fig. 3. Standard curve for PEBG from 10 to 100 μg. The ratio is total PEBG area (major and minor peaks, range 1, attenuator 256) to naphthylamine standard peak height (range 10, attenuator 512).

Fig. 4. Standard curve for *p*-OH PEBG from 10 to 100 μg. The ratio is *p*-OH PEBG peak area (range 1, attenuator 256) to naphthylamine peak height (range 10, attenuator 512).

Some side peaks would occasionally be found in both serum and urine blanks; however these peaks did not compromise the quantitative and qualitative responses of the gas chromatograph (Figs. 5 and 6).

Sensitivity and recovery

The practical limit of sensitivity for the assay as described and for the equipment at our disposal was about 0.2 μg for PEBG and 0.5 μg for *p*-OH PEBG per ml of serum and urine.

Recovery of the PEBG and *p*-OH PEBG was determined by comparing the absolute areas of the extracted PEBG and *p*-OH PEBG with the areas of samples directly derivatized and injected in the gas chromatograph. The actual recovery of the drug and its metabolite ranged between 90 and 105%. Nonetheless, solutions of the standard naphthylamine were consistently added to the samples to be examined and taken through the procedure to account for any losses and any variation of the GC response.

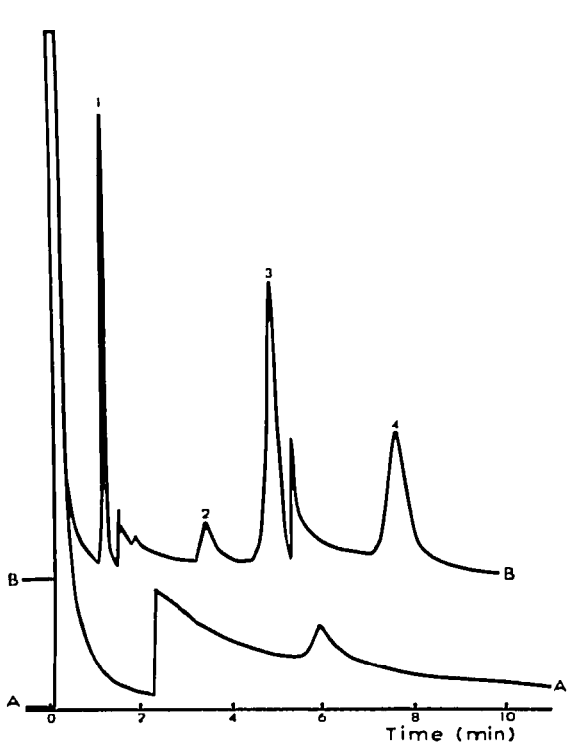
DISCUSSION

Methodology

Phenformin, upon derivatization, was frequently found to give rise to two peaks, one with a retention time of 3 min 20 sec, and the other with a retention time of 4 min 50 sec. With a purified sample of the triazine derivative dissolved in acetone only the second peak (retention time 4 min 50 sec) was observed.

The peak eluting at 3 min 20 sec (the minor peak) was found to vary in size from 0 to 20% of the area of the larger peak. The appearance of the minor peak was attributed to a side reaction of the derivatization process, which was removed when we purified the derivative for identification purposes. We were unable to characterize this minor peak.

The purity of trifluoroacetic anhydride was found to be extremely critical.



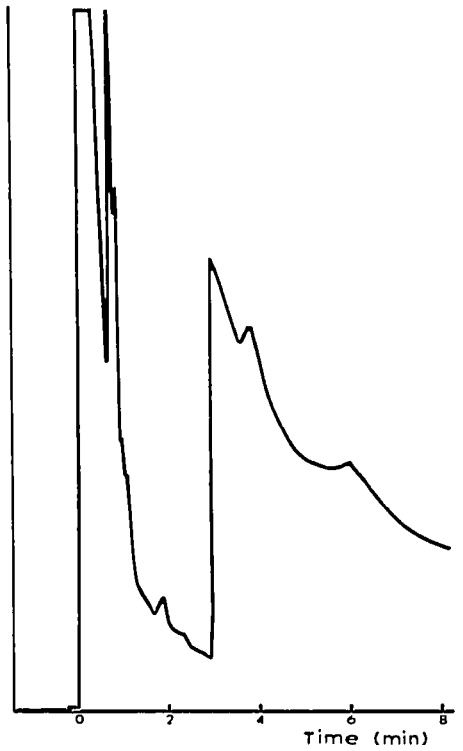


Fig. 5. Typical CG tracings. (A) Serum control; (B) chromatogram of naphthylamine standard (1), PEBG (2 and 3) and *p*-OH PEBG (4).

Fig. 6. Sample chromatogram of urine control. Attenuator change, 512 × 10 to 128 × 1.

Only freshly distilled reagent produced the single peak eluting at 4 min 50 sec. On storing the freshly distilled reagent overnight, we found the minor peak to be produced, even with 5-sec exposure. Only the combination of freshly distilled, unstored reagent, and approximately 5-sec reaction time, produced the major (triazine) peak eluting at 4 min 50 sec. Using control samples, we determined the area of the major and minor peaks to be additive and to produce quantitative and qualitative recoveries.

The duration of exposure to the reagent was investigated using time intervals from approximately 5 sec to 24 h. With increasing length of exposure the relationship between peak areas is altered. The minor peak becomes increasingly larger and the major peak diminishes in size but the latter does not disappear completely even after 24 h. An approximate 5-sec exposure time was found to maximize the formation of the major triazine peak.

A similar behavior was also observed with *p*-OH PEBG. A 24-h exposure time to the reagent produced a new peak with a retention time of 5 min 5 sec. From its retention time and elution characteristics we believe that this new derivative of *p*-OH PEBG may be the esterified triazine product. However, it was not characterized since we had established approximately 5 sec as optimum treatment time for PEBG. With this exposure time the *p*-OH PEBG produced a satisfactory response on the GC apparatus.

Metabolic studies

Samples of urine and serum were analyzed for the presence of PEBG and *p*-OH PEBG as described under Experimental. Following i.p. injection of PEBG, a short time course study was performed showing the time dependent concentration of PEBG present in the serum. (Fig. 7)

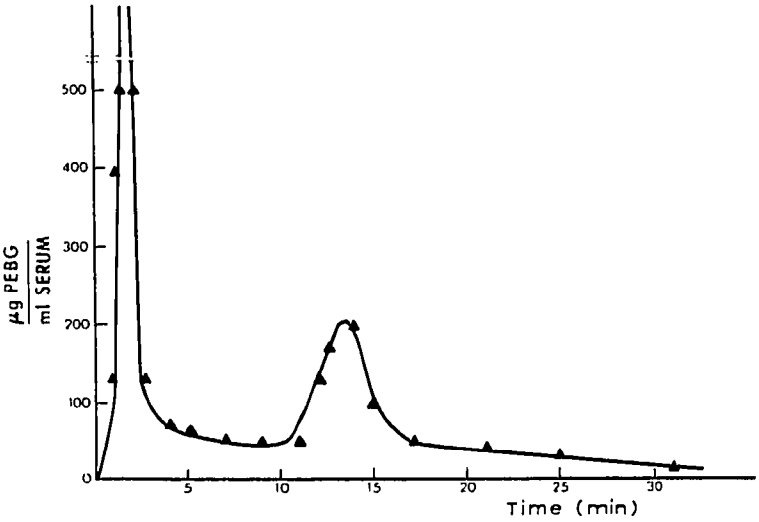


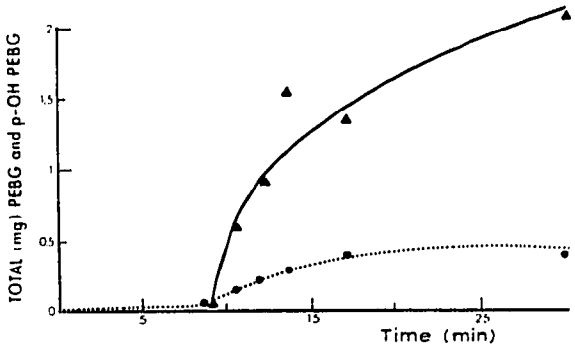
Fig. 7. Concentration of PEBG present in the serum following PEBG injection.

Under our experimental conditions, the metabolite *p*-OH PEBG was not detected in the serum at 30 min, 1 h, or 2 h following i.p. injection of PEBG. We did show the metabolite to be present in the urine of animals, following injection of the drug. It appears the metabolite is rapidly excreted in the urine along with PEBG (Figs. 8 and 9). The maximum concentrations are observed at 6 h and 12 h for PEBG and *p*-OH PEBG, respectively. No other study has been reported which quantitatively determines the presence and the secretion rate of the *p*-OH PEBG in urine.

The total amount of PEBG excreted in the urine during the first 24 h was found to be approximately 20% of the injected dosage, which is in agreement with the 25% value reported by Beckman[3]. The metabolite was found to be approximately 12% of the PEBG administered. No other data were available to compare the amount of *p*-OH PEBG released in the urine.

It has been shown that close to 50% of the drug is excreted in the feces[2]. The application of our procedure to the detection of the drug and metabolite present in the feces failed to give any reliable results because of the many interfering peaks. A more suitable extraction procedure of the feces which would lead to elimination of most of the interfering peaks is being sought.

The problems incurred during the application of the above method to the detection of the compounds in the liver and in the gastric juices were similar to the ones encountered in the study of the feces. Although we have applied this method for the detection of PEBG and *p*-OH PEBG present in serum and urine of rats following



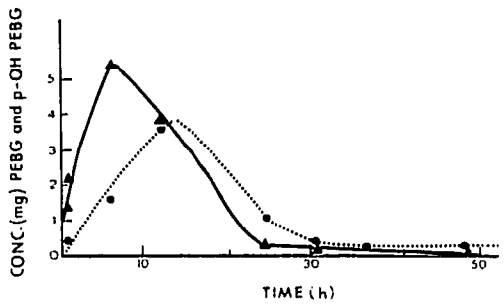


Fig. 8. Total concentration of PEBG (▲) and *p*-OH PEBG (●) present in the urine within the first 30 min following PEBG injection.

Fig. 9. Concentration of PEBG (▲) and *p*-OH PEGB (●) present in the urine following PEBG injection.

i.p. injection of 100 mg/kg of PEBG, we believe the procedure is applicable in the clinical laboratory. Normal human dosage is 1 mg/kg. Collection of a greater volume of serum and the use of an electron capture detector should increase the sensitivity to within clinical limits.

We have derivatized buformin in trifluoroacetic anhydride, and the resultant derivative, not yet characterized, is detectable on a gas chromatograph equipped with a FID.

It is hoped that this GC method will provide the basis for improvement in the detection of PEBG, its metabolite, *p*-OH PEBG, and buformin present in biological fluids and tissues. Further, it will be applied for the *in vivo* or *in vitro* studies of the drugs, which could eventually lead to a more complete knowledge of the mechanism of action of the biguanide drugs.

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REFERENCES

1 H. Mehmert, in W. J. Malaisse and J. Pirat (Editors) and J. Wallace-Owen (Co-editor), *Diabetes, Proceeding of the Eighth Congress of the International Diabetes Federation*, Excerpta Medica, Amsterdam, 1973, p. 603.
2 A. N. Wick, E. R. Larson and G. S. Serif, *J. Biol. Chem.*, 233 (1958) 296.
3 R. Beckman, *Diabetologia*, 3 (1966) 368.
4 L. Freedman, M. Blitz and S. Zak, *J. Lab. Clin. Med.*, 58 (1959) 662.
5 J. A. F. Wickramashinge and S. R. Shaw, *J. Chromatogr.*, 71 (1972) 265.
6 M. Mottale, *M.S. Thesis*, San Diego State University, San Diego, Calif., 1974.
7 B. Elpern, *Ann. N.Y. Acad. Sci.*, 148 (1968) 577.
8 J. A. Hendry and A. Rose, *J. Chem. Soc.*, 43 (1958) 1134.