CHROM. 6101

GAS CHROMATOGRAPHIC BEHAVIOR OF BUFORMIN HYDROCHLORIDE, PHENFORMIN HYDROCHLORIDE AND PHENYLBIGUANIDE

THE PYROLYTIC FORMATION OF SUBSTITUTED 2,4,6-TRIAMINO-1,3,5-TRIAZINES FROM BIGUANIDES

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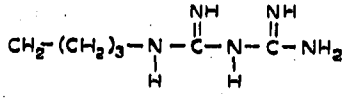
SUMMARY

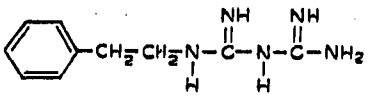
The gas chromatographic behavior of the biguanides buformin hydrochloride, phenformin hydrochloride and phenylbiguanide has been studied .Under the thermal conditions of gas chromatography or mass spectrometry, the formation of substituted *s*-triazines (melamines) from these biguanides has been demonstrated. A gas chromatographic method for the quantitation of buformin hydrochloride is described. The lower limit of detection sensitivity was 0.1 μg buformin hydrochloride "on column".

INTRODUCTION

The biguanide compounds buformin (a) and phenformin (b) are widely used as oral hypoglycemic agents in the treatment of diabetes mellitus. These biguanides



(a)



(b)

have been recognized to differ from the orally active hypoglycemic sulfonylurea drugs in their mechanism of action.

The precise quantitation of these biguanides in the body fluids of humans and experimental animals is of great importance when investigating their mechanisms of action, metabolism, interactions with other drugs, etc. No satisfactory analytical method has been published for the measurement of non-radioactive buformin. Most of the methods described in the literature pertain to phenformin[1–5]. All these reported methods, however, lack high sensitivity and/or specificity. In a majority of the publications, the utility of the method has not been demonstrated in biological systems. The present communication describes the investigation of the gas chromatographic (GC) behavior of the biguanides buformin hydrochloride, phenformin hydrochloride and phenyl biguanide which led to the development of a GC method for the determination of buformin, which could be utilized for the quantitation of the drug in pharmaceutical preparations.

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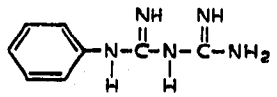
EXPERIMENTAL

Gas chromatography

GC was carried out on an F & M Model 402 gas chromatograph equipped with a flame ionization detector. Glass columns (U-shaped; 70 cm long × 3 mm I.D.) packed with 0.5 % Carbowax 20M on 80–100 mesh glass beads were utilized, unless specified otherwise. Helium was used as the carrier gas at a flow rate of about 80 ml/min. Oxygen and hydrogen flow rates were adjusted to give maximum response.

The column oven was operated isothermally at 225°; the flash heater at 276° and detector at 235°. Under these conditions the GC peak resulting from the injection of buformin hydrochloride had a retention time of 2.4 min; that from the internal standard phenylbiguanide (c) had a retention time of 1.1 min.



(c)

Internal standard. Phenylbiguanide was used as the internal standard (GC retention time quoted above). The thin-layer chromatographic (TLC) mobility of phenylbiguanide was identical with that of buformin hydrochloride in the solvent system isopropanol–methanol–formic acid (2:14:1).

Standard curve

Aliquots of a standard solution of 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 10 μg of buformin hydrochloride in methanol were placed in glass-stoppered 15-ml centrifuge tubes. Aliquots of a solution of the internal standard in methanol containing 125.8 μg of phenyl biguanide were added to each tube. All the samples were evaporated to dryness under nitrogen. Each sample was then reconstituted in 20 μl of pyridine and samples (1 μl size) of the latter were analyzed by GC. Peak height ratios were calculated by dividing the height of the peak at 2.4 min (resulting from buformin hydrochloride) by the height of the peak at 1.1 min (resulting from phenylbiguanide). The ratios of the peak heights have been expressed as a function of the concentrations of buformin hydrochloride in Fig. 1.

Gas chromatographic mass spectrometry

Gas chromatographic mass spectra were recorded on an LKB-9000 gas chromatograph–mass spectrometer. The column packing utilized for the GC analysis was 0.5 % Carbowax 20M on glass beads (80–100 mesh). The column was maintained at a temperature of 230°, and the flash heater at *ca.* 250°. Helium was used as the carrier gas at a flow rate of *ca.* 45 ml/min. Although the column had been preconditioned, "column-bleed" on the gas chromatograph–mass spectrometer could not be completely eliminated. Hence, assignment of peaks was made by comparison with spectra of the background "column-bleed".

Buformin hydrochloride. A single GC peak with a retention time of *ca.* 2.2 min was observed. A mass spectrum of this material showed prominent peaks of *m/e* 238, 223, 209, 196, 195, 182, 153, 140, 139 and 126. This gas chromatographic mass

spectrum was very similar to a mass spectrum of buformin hydrochloride recorded on the Atlas Model CH-4 mass spectrometer. The latter spectrum showed, in addition to all the peaks listed above, peaks at *m/e* 157 (mol. wt. of buformin is 157) and 167.

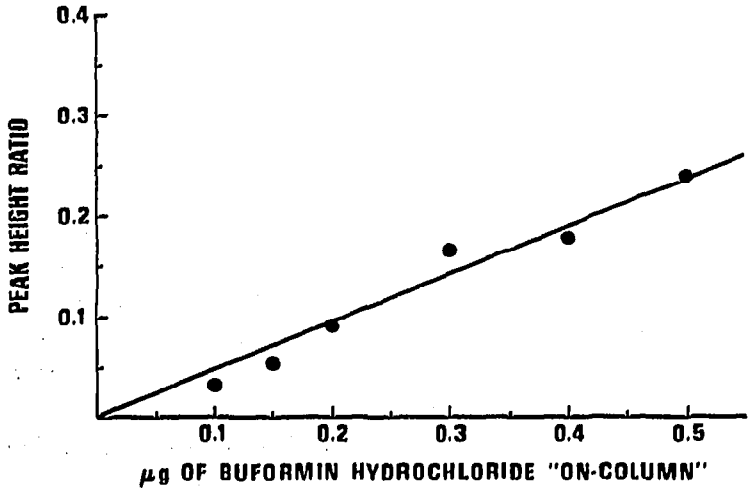
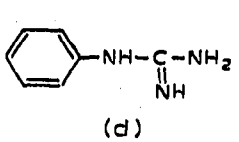
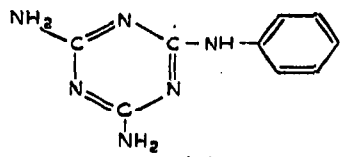


Fig. 1. Standard curve. Ratio of peak height due to buformin HCl:peak height due to phenylbiguanide (internal standard), expressed as a function of concentration of buformin hydrochloride.

Phenylbiguanide. Two GC peaks were observed. The first GC peak had a retention time of *ca.* 1 min. The corresponding mass spectrum showed an M^+ ion at *m/e* 135. This chromatographic species was judged to be phenylguanidine (d). The second GC peak had a much longer retention time (*ca.* 18 min). A mass spectrum of this material showed an M^+ ion at *m/e* 201, which indicated that it was probably the *s*-triazine (e).

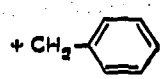


(d)

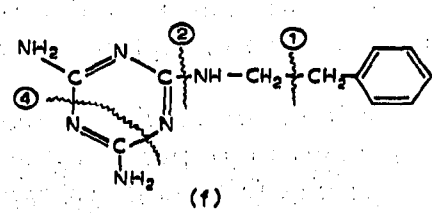


(e)

Phenformin hydrochloride. A mass spectrum of the single GC peak (retention time *ca.* 22 min) showed a M^+ ion at *m/e* 230 and prominent peaks at: (1) 139 ($M^+ - 91$); (2) 110 ($M^+ - 120$); (3) 91 ($M^+ - 139$) due to a



fragment; (4) 68 ($M^+ - 162$) due to a $N{\equiv}C{-}N{=}C^+{-}NH_2$ fragment, corresponding to the fragmentation of a thermally produced *s*-triazine in the manner indicated in (f).



(f)

Pyrolysis of buformin hydrochloride

Buformin hydrochloride (0.537 g) was heated under nitrogen for *ca.* 4 min at 230°, in a micro-distillation apparatus. Attempted distillation *in vacuo* produced a solid sublimate, which had $\lambda_{max.}^{EtOH}$ 208 mμ. The sublimate was subjected to TLC in the manner described below.

In a larger-scale pyrolysis buformin hydrochloride (1.024 g) was pyrolyzed at 235°, under nitrogen, for 5 min and the product sublimed in a sublimation apparatus.

Thin-layer chromatography

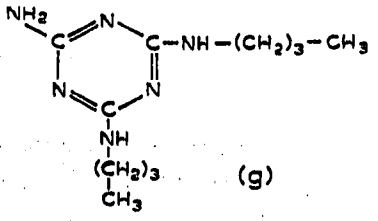
TLC was carried out on Merck pre-coated TLC plates of Silica Gel F-254 of 250-μ thickness (purchased from Brinkmann Instruments, Westbury, N.Y.).

An aliquot (10.35 mg) of the sublimate (see above) was thin-layer chromatographed in chloroform–methanol (19:1). Examination of the chromatograms under ultraviolet light (254 mμ) showed two major spots corresponding to two major pyrolysis products designated A (R_F = 0.59) and B (R_F = 0.28). Minor spots were observed at R_F = 0.83, 0.08, and 0. The silica gel zones corresponding to products A and B were removed and extracted with methanol (2 × 3 ml). Evaporation of the extracts under nitrogen yielded solid, chromatographically homogeneous pyrolysis products A (3.04 mg) and B (3.32 mg).

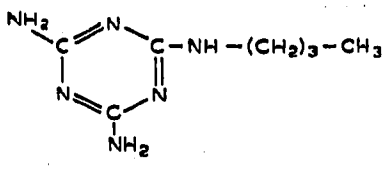
Larger scale separations were carried out on TLC plates of 2 mm silica gel layer thickness (*ca.* 58 mg per plate). These plates were developed in the solvent system chloroform–methanol (23:2). The zones were located and recovered in the manner described above. The product B was crystallized from methanol–ether, m.p. *ca.* 160° (dec. 230°). Product A resisted attempts to crystallize it.

Identification of the two major pyrolysis products

The two major pyrolysis products A and B isolated were identified as 2,4-di(*n*-butylamino)-6-amino-1,3,5-triazine (g) and 2-*n*-butylamino-4,6-diamino-1,3,5-



(g)



(h)

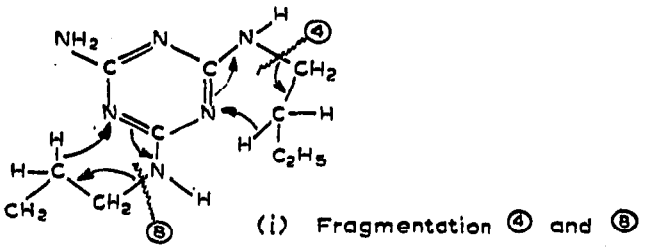
triazine (h), respectively, by mass spectrometry (MS), ultraviolet (UV) spectroscopy, infrared (IR) spectroscopy and NMR spectroscopy.

Mass spectra

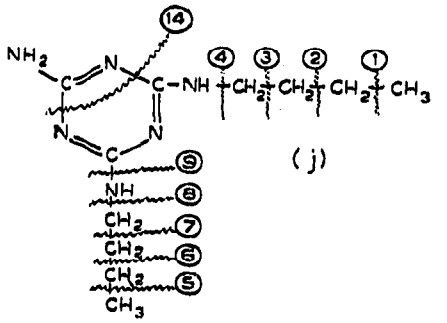
Mass spectra were recorded on a Consolidated Electrodynamics Corp., Model 21-110 high-resolution mass spectrometer. The empirical formulae were determined by high-resolution MS. The spectra were recorded on photographic plates for determination of molecular weights.

Pyrolysis product A. The experimentally determined molecular weight of 238.1924, calculated for $C_{11}H_{22}N_6$ (238.1906). The mass spectrum of product A showed a molecular ion at *m/e* 238 and prominent peaks at: (1) 223 (M^+-15); (2) 209 (M^+

−29); (3) 195 (M^+-43); (4) 182 [M^+-56, due to loss of C_4H_8 fragment probably from a rearrangement of the type indicated in (i)]; (5) 167 (M^+-71; (6) 153 (M^+-85); (7) 139 (M^+-99); (8) 126 (M^+-112) [see (i)]; (9) 111 (M^+-127); (10) 97 (M^+-141); (11) 85 (M^+-153); (12) 76 (M^+-162); (13) 72 (M^+-166); and (14) 68 (M^+-170), corresponding to a fragmentation of the type indicated in (j).

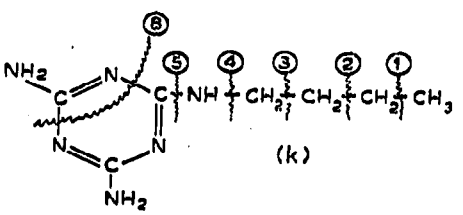


(i) Fragmentation ④ and ⑧



(j)

Pyrolysis product B. The experimentally determined molecular weight was 182.1267, calculated for $C_7H_{14}N_6$ (182.1820). The mass spectrum of the product B showed a molecular ion at *m/e* 182 and prominent peaks at: (1) 167 (M^+-15); (2) 153 (M^+-29); (3) 139 (M^+-43); (4) 126 (M^+-56) due to the loss of a C_4H_8 fragment by a rearrangement analogous to that shown in (i); (5) 111 (M^+-127); (6) 97 (M^+-85); (7) 85 (M^+-97); (8) 68 (M^+-114), corresponding to a fragmentation of the type indicated in (k).



(k)

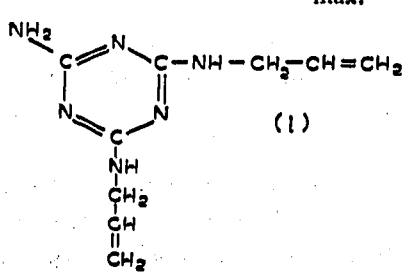
UV spectra

UV spectra were recorded in ethanol, on a Cary recording spectrophotometer Model 15.

Pyrolysis product A. The UV spectrum of the product A showed $\lambda_{max.}^{EtOH}$ 216 mμ.

Pyrolysis product B. The UV spectrum of the product B showed $\lambda_{max.}^{EtOH}$ 208 mμ.

Authentic 2,4-di(allylamino)-6-amino-1,3,5-triazine (1). The UV spectrum of this model triazine showed $\lambda_{max.}^{EtOH}$ 216 mμ.



(1)

Buformin hydrochloride. The UV spectrum of buformin hydrochloride showed $\lambda_{max.}^{EtOH}$ 235 mμ.

Infrared spectra

IR spectra were recorded from micro KBr pellets on a Perkin-Elmer Model 421 spectrometer.

Pyrolysis product A. The IR spectrum of product A showed $\nu_{max.}$3320 (−NH); 2960, 2930, 2860 (−CH); 1555, 1520 (shoulder) C=N/−NH deformation); 1460, 1380, 1355, 1225, 1155, 1110, 810 cm^{-1} (C−H/C−N, etc.).

Pyrolysis product B. The IR spectrum of product B showed $\nu_{max.}$ 3480, 3320, 3160 (−NH); 2950, 2920, 2860 (C−H); 1675, 1625, 1550 (C=N/−NH deformation); 1485, 1455, 1380, 1355, 1310, 1220, 1190, 1145, 1110, 1085, 1060, 1000, 980, 810 cm^{-1} (C–H/C=N, etc.).

NMR spectra

NMR spectra were recorded on a Varian HA 100 NMR spectrometer at 100 MHz using CD_3OD solutions of the compounds.

Pyrolysis product A. The NMR spectrum of product A showed the following signals: (1) triplet based at 0.94δ, due to $-CH_3$ of the two *n*-butylamino side-chains; (2) multiplet centred at 1.47δ, due to $-CH_2-CH_2-CH_3$ of the two *n*-butylamino side-chains; (3) multiplet centered at 3.3δ, due to $-NH-CH_2$ of the two side-chains; and (4) deuterated −NH and $-NH_2$ at 4.82δ.

Pyrolysis product B. The NMR spectrum of the product B was analogous to that of A.

RESULTS AND DISCUSSION

The GC behavior of buformin hydrochloride was explored using a chromatographic column packed with 0.5% Carbowax 20M on 80–100 mesh glass beads (column temp. 225°; flash heater temp. 276°). Such a column packing and similar chromatographic conditions have been recommended for thermally unstable alkyl substituted ureas[6]. GC of buformin hydrochloride under these conditions yielded a single GC peak (of *ca.* 2.4 min retention time). The nature of the chromatographic species was investigated on a gas chromatograph–mass spectrometer analyzer unit, using the same column packing as used on the gas chromatograph. The column was maintained at a temperature of 230° (flash heater temp. 250°). A mass spectrum of the GC peak showed prominent peaks at *m/e* 238, 223, 209, 196, 195, 182, 153, 140, 139, and 126. Since buformin had a molecular weight of 157, the peaks above *m/e* 157 appeared to be anomalous. It was observed, however, that a mass spectrum of buformin hydrochloride recorded on the Atlas Model CH-4 mass spectrometer was very similar. This spectrum showed all the peaks recorded above, together with additional peaks at *m/e* 157 and 167. These observations suggested the probable incidence of a thermal intermolecular rearrangement of buformin leading to a higher molecular weight product (or products) under the pyrolytic conditions of GC or MS.

The nature of the thermally produced GC peak material was further investigated by collection of a sample of the corresponding GC effluent. Aliquots (1 μl) of a solution of buformin hydrochloride in pyridine (24.4 μg/μl) were repeatedly injected on to the Carbowax column under the chromatographic conditions described earlier. The effluent corresponding to 2.4 min retention time was collected after each injection,

by condensation in dry-ice cooled capillary tubes held over the unlighted flame-jet of the flame ionization detector. The pooled condensate (73 μg) was examined by UV spectroscopy, GC and TLC. The UV spectrum of the condensate showed $\lambda_{max.}^{EtOH}$ at 210 mμ, thus indicating that the GC species was not buformin hydrochloride ($\lambda_{max.}^{EtOH}$ 235 mμ). Re-injection of an aliquot of the condensate produced the same GC peak observed with buformin hydrochloride, indicating that the pyrolytic product was thermally stable. TLC of the condensate on silica gel in chloroform–methanol (19:1) showed that it was not homogeneous but comprised two products designated A (R_F = 0.59) and B (R_F = 0.28).

In order to identify the two products A and B resulting from the GC of buformin at elevated temperatures, an independent pyrolysis of buformin hydrochloride was carried out. Buformin hydrochloride was pyrolyzed at 230° to 235° in an atmosphere of nitrogen for 4 to 5 min. The product was sublimed *in vacuo*. Examination of the solid sublimate by TLC revealed that it contained the same two products A and B observed in the GC condensate (see above) as major products. Minor products (R_F = 0.83; 0.08) were observed but were not investigated further. On the basis of UV luminescence quenching on TLC, both the product from the independent pyrolysis of buformin hydrochloride as well as the GC peak condensate, appeared to contain more of constituent A than of B.

The chromatographically homogeneous solid products A and B were analyzed by high-resolution MS and the corresponding empirical formulae were established. The molecular weight of 238.1924 determined for product A, calculated for $C_{11}H_{22}N_6$ (238.1906). This was consistent with the melamine structure (g) proposed for product A. The structure as well as methods of preparation and properties of such melamines are well documented[7]. The fragmentation pattern (see EXPERIMENTAL section for details) which indicated progressive fragmentation of the *n*-butyl side-chains followed by the fission of the triazine ring itself, supported this assignment. The molecular weight of 182.1267 determined for product B calculated for $C_7H_{14}N_6$ (182.1280) which was consistent with the proposed structure (h) for product B. The fragmentation pattern (see EXPERIMENTAL section for details) which was analogous to that of product A supported the structure assignment.

The UV spectrum of product A showed $\lambda_{max.}^{EtOH}$216 mμ and that of product B showed $\lambda_{max.}^{EtOH}$208 mμ. The observation that the model dialkyl-substituted 2,4,6-triamino-1,3,5-triazine (l) had a $\lambda_{max.}^{EtOH}$216 mμ lent weight to the structure assignment for product A. The subsequent comparison of the UV spectrum of product B with that of authentic 2-*n*-butylamino-4,6-diamino-1,3,5-triazine[8,9], which showed a $\lambda_{max.}^{EtOH}$208 mμ (see below), provided confirmatory evidence for the structure of product B. It is noteworthy that the GC peak condensate which apparently consisted of a mixture of products A and B showed $\lambda_{max.}^{EtOH}$ 210 mμ.

The IR and NMR spectra of the products A and B (see EXPERIMENTAL section for details) were compatible with the structures proposed and supported the assignments made. The evidence from IR and NMR spectroscopy, however, was not diagnostic in the establishment of structures.

Subsequent to the identification of the pyrolytic products A and B as 2,4-di(*n*-butylamino)-6-amino-1,3,5-triazine and 2-*n*-butylamino-4,6-diamino-1,3,5-triazine, an authentic sample of the latter compound[8,9] was compared with the pyrolytic product B. The two specimens were found to be identical on the basis of TLC

[on silica gel in chloroform–methanol (19:1)] and UV spectroscopic analysis. The pyrolytic product A apparently has not been reported.

Having thus identified the thermal products A and B resulting from buformin hydrochloride, the GC behavior of the pure constituents A and B were examined and compared with that of buformin. Under the conditions of GC employed (see EXPERIMENTAL section), the products A and B had retention times of 2.1 and 2.2 min, respectively; the peak resulting from buformin hydrochloride had a retention time of 2.4 min. Injection of a mixture (1:1) of product A and buformin hydrochloride yielded a single GC peak of retention time of 2.1 min. Similarly, a mixture (1:1) of product B and buformin hydrochloride yielded a single GC peak of retention time of 2.3 min. It was thus apparent that the pyrolysis products A and B have similar (if not identical) retention times and they are not resolved on GC under the conditions employed. The single unresolved peak comprising 2,4-di(*n*-butylamino)-6-amino-1,3,5-triazine and 2-*n*-butylamino-4,6-diamino-1,3,5-triazine, resulting from the GC of buformin hydrochloride, seemed amenable to use in the quantitation of buformin. With a view to examine the feasibility of this approach, a search was made for a satisfactory internal standard. Phenylbiguanide was deemed to be the most satisfactory. The TLC mobility was found to be identical with that of buformin hydrochloride on silica gel in the solvent system isopropanol–methanol–formic acid (2:14:1). GC of phenylbiguanide under the conditions employed (see EXPERIMENTAL section for details) produced a single sharp peak of retention time 1.1 min. GC–MS analysis revealed that the material corresponding to this peak was probably phenylguanidine, with a molecular ion at *m/e* 135. A second GC peak of much longer retention time (*ca.* 18 minutes) was also observed on the gas chromatograph–mass spectrometer. The mass spectrum of this material which showed a molecular ion at *m/e* 201 indicated that it was probably the *s*-triazine (e)[8,9]. This peak, however, was not apparent on the gas chromatograph.

Employing phenylbiguanide as an internal standard, the GC response due to buformin hydrochloride [as measured by the composite peak comprising the two *s*-triazines (g) and (h)] was examined over the "on-column" concentration range of *ca.* 0.05 μg to 0.8 μg of buformin hydrochloride. Aliquots of a standard solution of buformin hydrochloride in methanol were evaporated under nitrogen and reconstituted in pyridine (20 μl). Samples (1 μl size) of the pyridine solution were analyzed. The GC response was found to be linear over the concentration range examined (see Fig. 1). The "on-column" lower limit of detection sensitivity was 0.1 μg buformin hydrochloride.

Effort was then directed towards the application of this GC procedure to the determination of buformin hydrochloride in biological specimens, *e.g.*, plasma. All such attempts, however, proved to be abortive. The method was insensitive at the low concentrations of buformin hydrochloride being considered. The microgram quantities of buformin hydrochloride extracted (with chloroform–methanol 17:3) from the strongly alkalinized biological matrix (*e.g.*, plasma) under conditions analogous to those described for phenformin[2], failed to produce any reliable GC response. Traces of alkali or acid (resulting from the attempted neutralization of excess alkali) present in the organic extracts may have interfered with the formation of the *s*-triazines. The utility of the GC method, therefore, could not be demonstrated for the determination of buformin in biological specimens. The

procedure, however, could be conveniently adapted for the determination of the drug in pharmaceutical preparations. High specificity of determination may be achieved by incorporating a TLC purification step (see EXPERIMENTAL section for details).

The GC behavior of phenformin was found to be analogous to that of buformin. GC of phenformin hydrochloride [explored on a Varian Aerograph Model 1700, gas chromatograph using a spiral glass column (122 cm long) packed with 0.5 % Carbowax 20M on glass beads (80–100 mesh) under conditions analogous to those described in the EXPERIMENTAL section] produced a single GC peak of a retention time of 4 min (under the same conditions, the GC peak due to buformin hydrochloride had a retention time of 7.5 min). GC–MS analysis under the conditions described for buformin hydrochloride (see EXPERIMENTAL section) showed that the GC peak resulting from phenformin hydrochloride comprised the single monosubstituted melamine (f), *viz.*, 2-(β-phenethylamino)-4,6-diamino-1,3,5-triazine[10] (see EXPERIMENTAL section for interpretation of the mass spectrum).

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