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Short Communication

Gas chromatographic–mass spectrometric method for analysis of a pyrimido-*s*-triazine and some of its metabolites in dog urine

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ABSTRACT

3-Phenylpyrimido-[3,4-*a*]-*s*-triazines exhibit antiparasitic, antibacterial and antifungal activity. In order to study the metabolism of these heterocycles, 9,9-diethyl-3-phenyl-6,8-dioxo-2,3,4,5,6,7,8,9-octahydropyrimido[3,4-*a*]-*s*-triazine (TZ) was administered to dogs. Three potential metabolites were synthesized, and these models were identified and quantified with gas chromatography–mass spectrometry. The heterobicyclic compounds, TZ and its hydroxy derivative, underwent thermal degradation under chromatographic conditions. Dog urine spiked with the model metabolites was extracted, and the substances were quantified. The urine of dogs treated with TZ was studied, and two of the potential metabolites were recovered, identified and quantified.

INTRODUCTION

Derivatives of pyrimido-*s*-triazine (Fig. 1) exhibit antiparasitic [1], antibacterial [2] and antifungal [2] activities, which vary with the nature of the substituent R in position 3 on the heterobicycle. Substituted 3-phenyl derivatives have proved to be the most effective [2]. It is thus important to investigate the behaviour of these compounds *in vivo*, so 9,9-diethyl-3-phenyl-6,8-dioxo-2,3,4,5,6,7,8,9-octahydropyrimido[3,4-*a*]-*s*-triazine (TZ) (Fig. 1a, R = C_6H_5), which has the highest number of potential hydroxylation sites, was studied in dogs. Metabolic studies required the isolation of modified compounds from the urine of treated animals and their identification.

Before administering the drug to dogs, it was necessary to perform a study of the analytical behaviour of models of the studied compounds. Potential metabolites and degradation products were then studied in solution in urine, in order to identify any artefacts and to establish suitable conditions for the biological experiments.

TZ and its potential metabolites were dissolved in urine, extracted with ethyl acetate and/or chloroform, and then analysed by gas chromatography–mass spectrometry (GC–MS).

The potential metabolites were selected by a comparison of TZ with related molecules with

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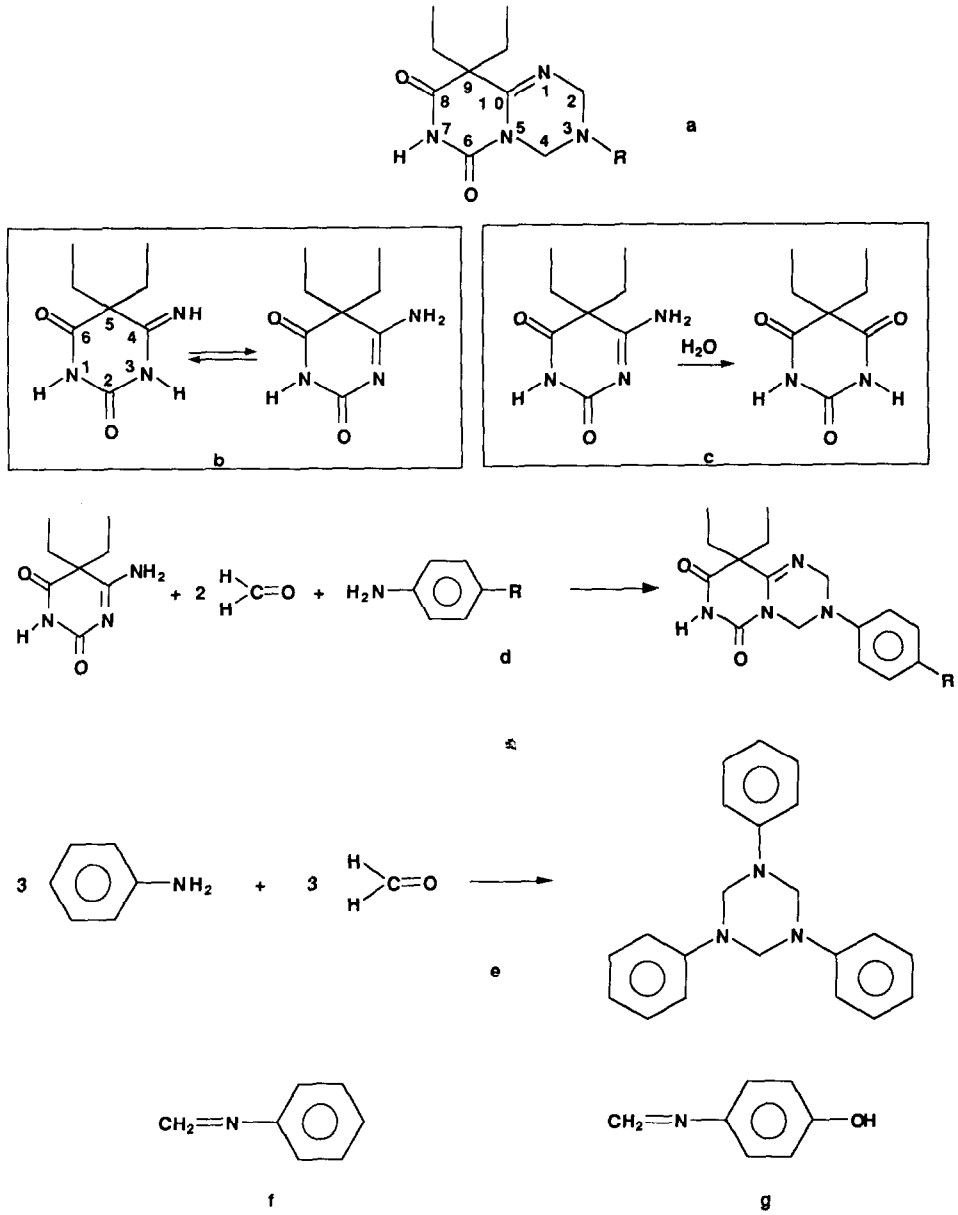

Fig. 1. Molecular formulae and reaction schemes.

known metabolism, *i.e.* barbiturates [3] and iminobarbiturates [4]. The ability of the tetrahydro-*s*-triazine cycle to undergo ring-opening under relatively mild conditions [2] was also taken into account.

As well as TZ, the following compounds were also chosen: 9,9-diethyl-3-(4-hydroxyphenyl)-6,8-dioxo-2,3,4,5,6,7,8,9-octahydropyrimido[3,4-*a*]-*s*-triazine (OH-TZ) (Fig. 1a, R = *p*-C_6H_4OH) resulting from *p*-hydroxylation of the phenyl nucleus; 4-imino-5,5-diethylbarbituric acid (IB) (Fig. 1b) resulting from retro-Mannich opening of the tetrahydro-*s*-triazine ring [2] (in solution, this 3-unalkylated 4-iminobarbituric acid has an amidinoketone structure, and the imido tautomeric form cannot be observed [4]; 5,5-diethyl-

barbituric acid (VE) (Fig. 1c), which is produced by hydrolysis of IB.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a split capillary inlet system was used. The split ratio was 1:77. A fused-silica capillary column (25 m × 0.22 mm I.D., 0.11 μm film thickness) was coated with cross-linked 5% phenylmethylsilicone. The carrier gas was helium at an inlet pressure of 62 kPa.

A Hewlett-Packard 7673 A liquid autosampler, operated in the fast mode for split injection, was used in conjunction with the gas chromatograph. Before each injection, the 10-μl injection syringe was automatically rinsed out with six 10-μl volumes of methanol, and then with three 10-μl volumes of a sample solution. As a rule, 3.0 μl of the sample solution were injected.

A Hewlett-Packard 5970 MSD mass spectrometer, operated in electron-impact (EI) mode, was directly interfaced with the 5890 gas chromatograph by the capillary column and was used either in the full-scan mode or in the selected-ion monitoring (SIM) mode.

Reagents and chemicals

Chemicals were either synthesized, or purchased from companies. In both cases, their purity was checked by CHN microanalysis and ^{1}H NMR spectroscopy. MS was performed on a Nermag 12-12.

IB was prepared by condensing urea and diethylcyanoacetate in alkaline medium (sodium ethanolate–ethanol) [5]. TZ was synthesized from IB, treated by formaldehyde and aniline [2] (Fig. 1d, R = H). For IB and TZ, spectral data were identical with those previously published [1,4].

The synthesis of OH-TZ was an adaptation of the synthesis of TZ, using *p*-hydroxyaniline instead of aniline (Fig. 1d, R = OH). Barbital (VE) was a characterized product of Serva (Heidelberg Germany), and phenobarbital (PB) [used as internal standard, (I.S.), because of its structural similarity to VE and IB] was purchased from Specia (Paris, France). HPLC-grade methanol was purchased from Carlo Erba (Milan, Italy).

Sample preparation

The working I.S. solution of PB in methanol (1 mg/ml) was prepared weekly and stored at 4°C. Working solutions of each compound (2 mg/ml) in methanol was prepared every day before use. Dilutions of previous solutions with methanol were prepared to obtain concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/ml for each substance assayed, and of 0.25 mg/ml for I.S., for the determination of standard curves.

Dogs' urine was spiked with the various compounds and extracted: 100 ml of urine, spiked with 25 mg of one of the compounds, were adjusted to the optimum pH for extraction by addition of hydrochloric acid (pH 9 after congelation). The urine was then extracted with chloroform or ethyl acetate. Each extraction was repeated three times. The organic phase was dried with anhydrous sodium sulphate, and then evaporated under vacuum at a temperature lower than 40°C, in order to avoid degradation of the compounds. Residues were dissolved in methanol, and compounds were identified by GC–MS and assayed in order to evaluate the recovery.

Before administration to dogs, TZ powder was placed in gelatine capsules. The mass of powder was adjusted so that each dog (female) received every day 20 mg/kg. Dog 1 (30 kg), dog 2 (12 kg), dog 3 (38 kg) and dog 4 (25 kg) were treated for five, eleven, four and six days, respectively, so that each dog received a total of *ca.* 3 g of drug (Table I).

The four dogs were placed in separate Pajon metabolism cages with free access to food and water. Every morning, each animal received orally one capsule containing the daily dose. Urine was collected every morning and every evening and frozen (−18°C) during the administration period and for the following two days. When the experiment was over, the various samples were allowed to warm up to room temperature and then pooled. The extraction was then performed, first with chloroform at pH 7, and then with ethyl

TABLE I

RESULTS OF THE DETERMINATION OF METABOLITES AFTER ADMINISTRATION OF TZ TO DOGS

Dog	TZ dose (mg)	Metabolite	Mass (mg)	R.S.D. (%)
pH 7				
1	3000	VE	19.5	3.21
2	2640	VE	16.6	4.48
3	3040	VE	15.8	1.70
4	3000	VE	29.5	3.18
pH 5				
1	3000	VE	27.6	1.50
		IB	1030	2.36
2	2640	VE	33.2	2.95
		IB	823	1.20
3	3040	VE	56.3	9.72
		IB	983	4.51
4	3000	VE	32.8	4.47
		IB	876	8.91
pH 1				
1	3000	VE	237	2.25
2	2640	VE	163	1.70
3	3040	VE	185	1.46
4	3000	VE	217	0.70

acetate at pH 5 and 1, in order to break conjugation. The solvent was evaporated under vacuum at room temperature, and then the two kinds of extract were studied separately by GC–MS.

Chromatographic conditions

For IB, VE and OH-TZ, the oven temperature was 110°C for 1 min after injection, then increased at 15°C/min to 210°C, and then held at 210°C for 7 min. The injector and transfer line temperatures were maintained at 240°C.

For TZ, the oven temperature was 160°C for 1 min after injection, then increased at 5°C/min to 200°C, and then held at 200°C for 15 min. The injector and transfer line temperatures were maintained at 200°C.

Validation

The validation results, established for six injections per concentration and three concentrations for each product are listed in Table II.

RESULTS AND DISCUSSION

In the full-scan mode, the scan range was selected from *m/z* 50 to 400. The mass spectra (scan mode) of IB, VE, PB, OH-TZ and TZ (Fig. 2) showed that the following ions should be monitored in the SIM mode: *m/z* 140, 155 for IB, corresponding to $[M-C_2H_4]^{\ddagger}$ = 155, $[M-NHCO]^{\ddagger}$ = 140; *m/z* 141, 156 for VE, corresponding to $[M-C_2H_4]^{\ddagger}$ = 156, $[M-HNCO]^{\ddagger}$ = 141; *m/z* 204, 232 for PB, corresponding to $[M]^{\ddagger}$ = 232, $[M-C_2H_4]^{\ddagger}$ = 204;

TABLE II

VALIDATION

Compound	Theorical concentration (mg/ml)	Found concentration (mg/ml)	Intra-assay variability, R.S.D. (%)	Accuracy (%)	Inter-assay variability, R.S.D. (%)
VE	1.0	0.984	1.92	98.4	3.07
	0.5	0.490	2.14	98.0	3.16
	0.125	0.122	3.29	97.6	3.96
IB	1.0	1.016	3.47	101.6	4.07
	0.5	0.503	2.75	100.6	3.13
	0.125	0.121	4.12	96.8	4.78
TZ	1.0	1.024	3.28	102.4	4.23
	0.5	0.511	4.12	102.2	4.52
	0.125	0.124	4.21	99.2	5.24
OH-TZ	1.0	1.015	3.94	101.5	4.87
	0.5	0.520	4.22	104.0	5.21
	0.125	0.129	4.37	103.2	4.98

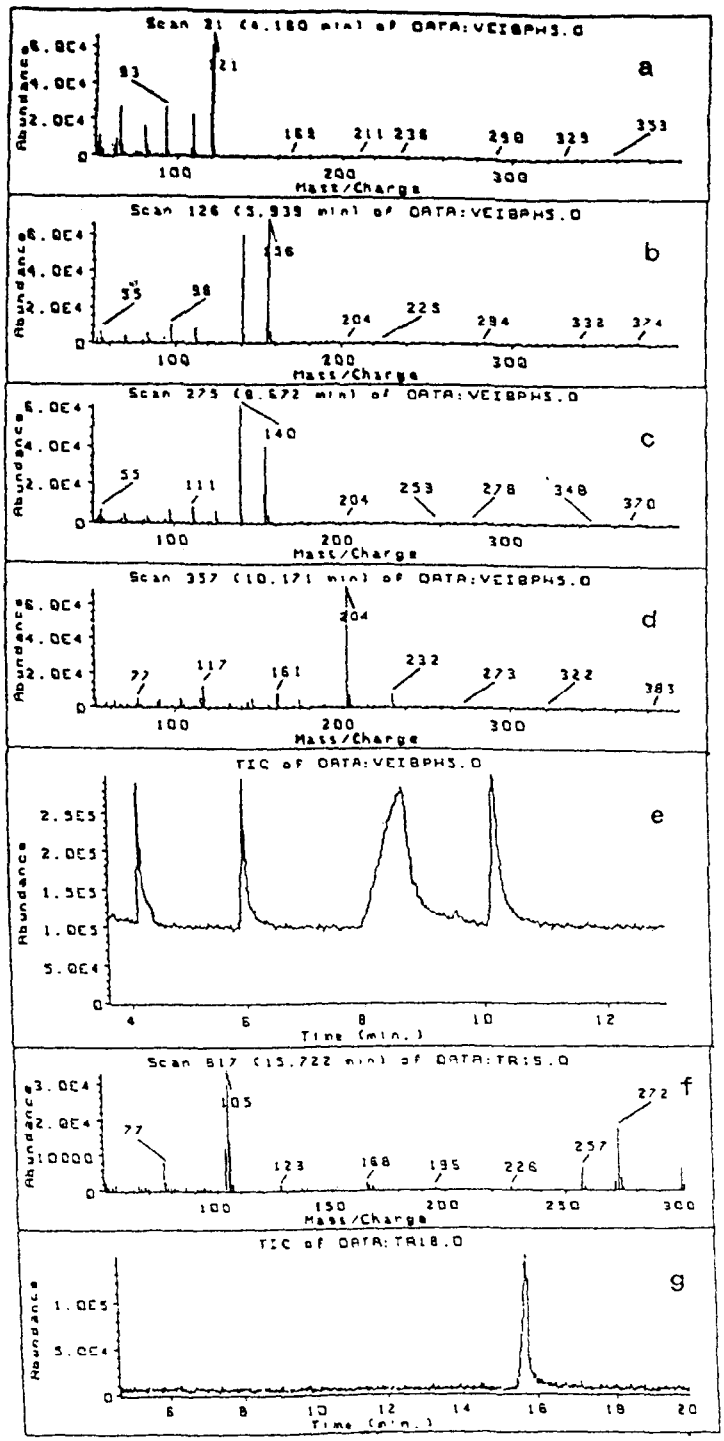

Fig. 2. Mass spectra of OH-TZ (a), VE (b), IB (c), PB (d) and TZ (f). Chromatograms of extracts of urine containing (e) OH-TZ, VE, IB and PB and (g) TZ.

m/z 105, 272 for TZ, corresponding to $[M-C_2H_4]^{\ddagger}$ = 272, $[PhNCH_2]^{\ddagger}$ = 105; m/z 121, 93 for OH-TZ, corresponding to $[HOC_6H_4NCH_2]^{\ddagger}$ = 121, $[HOC_6H_4]^{+}$ = 93.

Linearity studies were performed in the SIM mode. Linearity was observed for concentrations between 1.0 and 0.0625 mg/ml. Straight-line equations were: $y = 13.2x - 0.259$ for VE, $y = 2.78x - 0.86$ for IB and $y = 12.3x + 0.41$ for TZ (y = response ratio, compound/PB; x = concentration ratio, compound/PB). The respective regression coefficients, r^2, were 0.991 for IB, 1.000 for VE and 0.992 for TZ. These results showed that the assays could be performed under the conditions described, because these proved to be suitable for the evaluation of these compounds.

In the case of TZ, another titration was possible under the following chromatographic conditions. The oven temperature was kept at 110°C for 1 min after injection, then increased at 15°C/min, and then held at 210°C for 7 min. The injector and transfer line temperatures were maintained at 240°C. Under these conditions, TZ underwent, proportionately to its concentration, a thermal degradation to yield a compound of lower retention time (3.55 min) and corresponding to a mass of 105. The straight-line equation was $y = 3.133x + 0.0116$, and the regression coefficient, r^2, was 0.990. The same peak was observed, under the same conditions, from a compound obtained from a trimeric condensation [2] of aniline and formaldehyde (Fig. 1e).

All these data were in agreement with the formation of N-phenylformimine (Fig. 1f).

The injection of OH-TZ in the full-scan mode, gave a chromatogram with a peak (Fig. 2) that corresponded to a compound formed by analogous thermal degradation of OH-TZ. The mass of this degradation product was 121, and this peak could be attributed to N-*p*-hydroxyphenylformimine (Fig. 1g).

Consequently m/z 121, together with another fragment, m/z 93, were chosen for analysis in SIM mode. The relation between the concentration of injected OH-TZ and the integration of the peak corresponding to both m/z 121 and 93 proved to be linear. The straight-line equation was $y = 1.875x + 0.2129$ and $r^2 = 0.993$.

Extraction from urine samples

Extraction yields from urine spiked with the compounds were tested from pH 1 to 11. For IB, VE and OH-TZ the best yields, 86, 92 and 81%, respectively, were obtained at pH 5 with three 300-ml volumes of ethyl acetate. For TZ, the best results (65%) were obtained by extraction with three 100-ml volumes of chloroform at pH 7. Injection of extracts in full-scan mode gave the chromatograms shown in Fig. 2.

Extraction from urine of treated dogs

The amounts of metabolites found in urine are presented in Table I. Neither TZ nor OH-TZ was detected in dog urine, under conditions that usually permit both compounds to be extracted. The main metabolite was IB (52.05 ± 1.73% of administered TZ), which was extracted at pH 5. At pH 1 and at room temperature, a degradation product, VE, corresponding to 11.17 ± 0.76% of administered TZ, was extracted. VE could have been produced either by direct hydrolysis of TZ or by hydrolysis of IB. The same degradation could explain the very small amounts of VE extracted at pH 7 and pH 5.

Another extraction, from urine boiled for 1 h at pH 1, gave an amount of VE corresponding to 13.2 ± 1.22% of administered TZ. The formation of VE under these drastic conditions could be interpreted as caused by the hydrolysis of conjugated derivatives of IB, followed by the well known conversion [1] of IB into VE in an acidic medium.

The total amount of metabolites and degradation products extracted from urine corresponded to more than 75% of administered TZ.

CONCLUSION

All these data obtained by a GC–MS procedure show that *p*-hydroxylation of the phenyl ring, which is quite common metabolic pathway, does not occur for TZ. On the other hand, TZ biodegradation consists mainly of an opening of the tetrahydro-*s*-triazine ring, leading to IB. This information will be useful for the design of new compounds in the series.

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