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Determination of *N*-(2-benzoyloxyethyl)-norfenfluramine (JP 992) and its metabolites in urine

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A gas-liquid chromatographic method of determination of norfenfluramine and *N*-2-hydroxyethylnorfenfluramine is described. Also a method is reported to determine *N*-(2-benzoyloxyethyl)norfenfluramine and its metabolites containing the *m*-trifluoromethylbenzyl and -benzoyl moieties in urine, by oxidation of these to m-trifluoromethyl benzoic acid followed by methylation and gas-liquid chromatography.

Preliminary investigations indicated that *N*-(2-benzoyloxyethyl)norfenfluramine (JP 992; compound 1d) is metabolized in man to *N*-2-hydroxyethylnorfenfluramine (compound 1c), norfenfluramine (compound 1a) and other metabolites, while no unchanged drug was detected in urine.

A method was therefore sought to determine quantitatively in urine, the metabolites of compound 1d containing the *m*-trifluoromethyl-benzyl and -benzoyl moieties, as well as the above bases (compound 1c and a).

Bruce & Maynard Jr. (1968) described a method of oxidizing these compounds to *m*-trifluoromethyl benzoic acid, but in our hands the method only gave a 55% conversion of compound 1d to the acid in solution in urine.

The possibility of rearrangement of compound 1d upon changing the pH of an aqueous solution of the compound was also investigated, since it is known that esters of β-aminoalcohols in acid solution rearrange to the amides upon making the solution alkaline (Immediata & Day, 1940; Kanao, 1928; Phillips & Baltzly, 1947; Fodor, Bruckner & others, 1949) as under conditions of extraction of the above bases into organic solvents.

MATERIALS AND METHODS

Apparatus

Perkin-Elmer Model F.11 Gas Chromatograph (F.I.D.), Hitachi Perkin-Elmer Model 159 recorder.

Materials and reagents

Compounds Ia, b, c, d, e hydrochlorides and *m*-trifluoromethyl benzoic acid were supplied by Selpharm Laboratories Ltd., England; Aletamine HCl by National Drug Co., Philadelphia; m-toluic acid by Hopkin and Williams Ltd., England. Freshly distilled Analar diethylether. 5N HCl, 20% NaOH, 10% $NaHCO_3$. Freshly prepared diazomethane in ether (Vogel, 1957) saturated $KMnO_4$ solution ($\simeq$0·4M)

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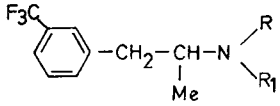

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Ia	R=H; R_1=H	Norfenfluramine
Ib	R=H; R_1=Et	Fenfluramine
Ic	R=H; R_1=C_2H_4OH	*N*-2-Hydroxyethyl norfenfluramine
Id	R=H; R_1=C_2H_4OCO–C$_6$H$_5$	*N*-(2-Benzoyloxyethyl)-norfenfluramine (JP 992)
Ie	R=CO–C$_6$H$_5$; R_1=C_2H_4OH	*N*-(2-Hydroxyethyl)-*N*-benzoyl norfenfluramine (Rearranged from of JP 992).

Gas-liquid chromatography. The chromatographic columns and conditions of chromatography are described in Table 1.

Recovery of m-*trifluoromethyl benzoic acid by oxidation of compound 1d*

(a) Urine or water (5 ml) containing known quantities (4–50 μg/ml) of *m*-trifluoromethyl benzoic acid was placed in Quickfit tubes, the solution acidified, internal standard added and the organic acid analysed as in the general procedure below.

(b) Urine (5 ml) containing known quantities (10 μg/ml and 50 μg/ml) of compound 1d was placed in Quickfit tubes, NaOH (0·5 ml), and saturated $KMnO_4$ solution (2·5 ml to 12 ml) were added, the solution oxidized, and the organic acid analysed as below.

(c) Urine (5 ml) containing known quantities (4–50 μg/ml) of *m*-trifluoromethyl benzoic acid was oxidized with $KMnO_4$ (2·5 ml) and the solution analysed as below.

Reserve capacity of $KMnO_4$ *solution for oxidation in the general procedure*

To urine samples (5 ml) collected from five subjects at different times of the day was added 100 μg/ml of compound 1d and the solution then oxidized with $KMnO_4$

Table 1. *Gas-liquid chromatographic conditions and retention times of* N-(2-*benzoyloxyethyl*)*norfenfluramine, metabolites and related oxidation products.*

Column	Support material	Stationary phase	Operating temp. °C	Column length	Hydrogen pressure lb/in²	Air pressure lb/in²	Nitrogen flow rate at room temp.	Retention times (min) of
A	Chromosorb G A/W, DMCS treated 80–100 mesh	10% Apiezon L 10% KOH	140	1 metre S.S. ⅛″ o.d.	15	26	27 ml/min	NF = 2·3 JPA = 15·2 ALT* = 8·4
B	Chromosorb G A/W, DMCS treated 80–100 mesh	2% SE 30	195	2 metre glass ¼″ o.d.	20	30	60 ml/min	JP992 = 6·3 JPR = 9·2
C	Gas-chrom Q A/W, DMCS treated 100–120 mesh	10% Carbowax 20M	110	2 metre glass ¼″ o.d.	15	25	80 ml/min	TMB = 4·0 MB′ = 7·0 MMT* = 11·9

NF = norfenfluramine, JPA = *N*-2-hydroxyethylnorfenfluramine, JP922 = N-(2-benzoyloxethyl)norfenfluramine, JPR = JP992 rearranged form, TMB = methyl *m*-trifluoromethyl benzoate, MB′ = methyl benzoate, MMT = methyl *m*-toluate, ALT = Aletamine, A/W = acid washed, DMCS = dimethylchlorosilane. S.S. = stainless steel. Injection block temperature *ca* 250°C. * Used as markers.

as in the general procedure. After cooling, dilute H_2SO_4 (10 ml) and potassium iodide (3 g) were added to the solution and the liberated iodine titrated with 0·1N sodium thiosulphate.

General procedure

Analysis of compound 1*d in urine by oxidation to* m-*trifluoromethyl benzoic acid.* Urine (5 ml) to which was added compound 1d was placed in a Quickfit tube, NaOH solution (0·5 ml) and saturated $KMnO_4$ solution (2·5 ml) were added and the solution refluxed in a water bath (90°) for 2 h. The solution was cooled, HCl solution (1·5 ml) added and the solution then refluxed for a further half hour.

The above solution was allowed to cool and the internal standard (1 ml, 20 μg/ml *m*-toluic acid in water) was added. The organic acids were extracted with ether (3 × 4 ml) and then repartitioned into $NaHCO_3$ solution (5 ml). The solution was made acidic with HCl and re-extracted with ether (3 × 2·5 ml). The bulked ether extracts were concentrated (*ca* 5 ml) on a water bath (42°), cooled and excess of ice-cold ethereal solution of diazomethane was added. After mixing for 2 min, the solution was washed with distilled water (3 × 5 ml). The ethereal layer was separated and concentrated in an evaporating tube (*ca* 50 μl) and 2 μl was injected on column C.

Calibration factor. Urine (5 ml) containing known quantities (4–50 μg/ml) of compound 1d was placed in Quickfit tubes, oxidized and assayed as above. The calibration factor (μg base/ml urine ÷ the drug to marker ratio) was calculated.

Reproducibility of general procedure. Urine (5 ml; 10 samples) from a bulked 24 h collection from a subject, who was given a dose of compound 1d, was oxidized and assayed as above.

Specificity of general procedure. "Blank" urine (5 ml; 10 subjects) was oxidized and assayed as above without incorporating the internal standard, to check for the absence of peaks chromatographing in the vicinity of the *m*-trifluoromethyl benzoic and *m*-toluic acid peaks.

Analysis in urine of compound 1*d*

Urine or water (5 ml) containing known quantities (0·2–10 μg/ml) of compound 1d was placed in centrifuge tube, NaOH (0·5 ml) or dilute ammonia (0·5 ml) internal standard (1 ml; 2 μg base/ml tripelennamine hydrochloride in water) added and extracted with ether (3 × 2·5 ml). The ether extracts were concentrated (*ca* 50 μl) on a water bath (42°) and 2 μl injected on column B.

Analysis in urine of compounds 1*a and c*

Urine or water (5 ml) containing known quantities (0·2–10 μg/ml) of compounds 1a and c was placed in a centrifuge tube, NaOH (0·5 ml) and internal standard (1 ml, 10 μg/ml aletamine hydrochloride in water) added and extracted with ether (8 × 2·5 ml). The ether extracts were concentrated (*ca* 50 μl) on a water bath (42°) and 5 μl was injected on column A.

Stability of compound 1*a and c in urine*

Compound 1a and c, 1 μg/ml, in acidic (pH 0·5), alkaline (pH 8·1) and neutral

urine (pH 7·2) were stored at 4° and the drug content determined every third day for two weeks.

Analysis of compounds 1*a and* 1*c in the presence of* 1*d and the total measured as* m-*trifluoromethyl benzoic acid by the general procedure*

Urine (5 ml) containing known quantities (2–12 μg/ml) of compounds 1a, 1c and 1d was placed in centrifuge tube, NaOH (0·5 ml) and the internal standard added and analysed for 1a and 1c as above.

A second sample (5 ml) was oxidized and analysed by the general procedure to determine the total as *m*-trifluoromethyl benzoic acid.

RESULTS AND DISCUSSION

Oxidation of compound 1*d*

The analysis by gas-liquid chromatography (g.l.c.) of the ethereal extracts of blank urine samples oxidized by the general procedure, showed no major interfering peaks in the vicinity of *m*-trifluoromethyl benzoic and *m*-toluic acids when chromatographed as their methyl esters, the retention times are recorded in Table 1. Benzoic acid formed in relatively large quantities on oxidizing urine gave a peak well separated from the peaks of the above acids (Fig. 1). Freshly prepared diazomethane solution ensured non interference with the g.l.c. analysis.

Compound 1d was chosen as a model in the oxidation studies and it was assumed that the *m*-trifluoromethyl-benzyl and -benzoyl compounds present in the urine

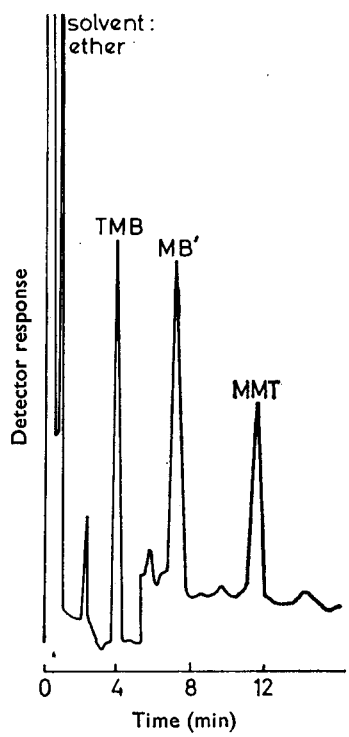

FIG. 1. Chromatogram of an ether extract of oxidized urine from a subject after a dose of JP992, showing *m*-trifluoromethyl benzoic acid (TMB), benzoic acid (MB′) and *m*-toluic acid (MMT; internal marker) chromatographed as methyl esters.

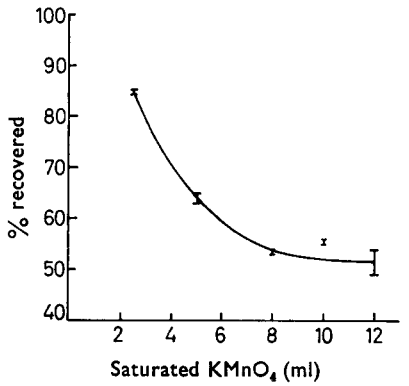

FIG. 2. The effect of concentration of $KMnO_4$ on the recovery of *m*-trifluoromethyl benzoic acid by oxidation of JP992 (compound Id) in urine.

Table 2. *Determination of compounds Ia and Ic in urine in the presence of Id and the total by oxidation to* m-*trifluoromethyl benzoic acid.*

Amount of compound added to urine (μg/ml)				Amount of compound determined in urine (μg/ml)			
Ia	Ic	Id	*Total in terms of Id	Ia	Ic	Id	Total by oxidation
2·0	2·0	6·0	12·26	1·80	2·14	ND	10·85
6·0	2·0	2·0	15·14	6·12	2·20	ND	15·30
2·0	6·0	2·0	13·90	2·12	5·83	ND	15·03
4·0	4·0	12·0	24·52	4·21	3·91	ND	23·72
12·0	4·0	4·0	30·28	11·70	4·20	ND	30·29
4·0	12·0	4·0	27·81	4·10	11·88	ND	27·14

ND—not determined.
*—calculated on molar basis.

following a dose of this compound would not be more difficult to oxidize to *m*-trifluoromethyl benzoic acid than was the parent drug. The product of oxidation, *m*-trifluoromethyl benzoic acid was not oxidized further under the conditions of the general procedure.

On oxidizing compound Id, increasing amounts of *m*-trifluoromethyl benzoic acid were produced as the amount of excess $KMnO_4$ was reduced (Fig. 2). Oxidizible substances are found in urine and inter subject variation in these occur; a compromise was therefore sought between the amount of excess $KMnO_4$ required for oxidizing compound Id quantitatively and the need for excess $KMnO_4$ to deal with the inter-subject variation in oxidizable substances; 2·5 ml/5 ml of urine for normal doses of drug (compound Id) represents this compromise in the general procedure. When varying amounts of compound Id (4–50 μg/ml) were present in the urine, analysis by the general procedure gave linear calibration graphs. Comparison of the results obtained by adding compound Id and *m*-trifluoromethyl benzoic acid to urine samples indicated that there was 85% conversion of the former to the latter (co-efficient of variance (c.v.) ±4%).

When known amounts of compounds Ia, Ic and Id were added to urine, Ia and Ic could be recovered quantitatively and the general procedure gave quantitative

conversion of the total of these compounds to *m*-trifluoromethyl benzoic acid (Table 2).

Analysis of a urine sample from a subject who had taken a dose of compound 1d was reproducible (c.v. $\pm$ 3·8%).

Analysis of compound 1a and c

Linear calibration graphs were obtained for compounds 1a and c over the range 0·2–10 μg/ml in urine and water (c.v. $\pm$ 2·2% and 1·8% respectively); retention times are shown in Table 1. No substance interfering with the determination of the amines was found in urine. Both the amines were stable in urine at 4° for at least two weeks.

Analysis of compound 1d

When compound 1d was placed in urine, the solution made alkaline and immediately extracted into ether, some conversion to compound 1e occurred. Storage of the solution at an alkaline pH increased this conversion but prolonged storage did not result in complete conversion.

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