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DETERMINATION OF (+)-(3*R*),(4*S*)-3-[(4-METHOXYPHENOXY)METHYL]-1-METHYL-4-PHENYLPIPERIDINE HYDROCHLORIDE (FG 4963) IN BIOLOGICAL FLUIDS USING COMPETITION FOR ADSORPTION TO GLASS

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SUMMARY

A procedure has been developed for the gas chromatographic determination of a structurally new 5HT-uptake inhibitor with antidepressant properties (FG 4963) in plasma and urine. The method involves an extraction from alkaline solution with *n*-pentane, and a quantitative determination by gas chromatography using an internal standard and a nitrogen-sensitive detector. The binding of FG 4963 to glass during storage and evaporation necessitates the addition of a structurally similar compound to the vials used for collection of the blood and urine, in order to compete for the binding sites of the glass. The method is suitable for pharmacokinetic and clinical studies, the sensitivity being *ca.* 5 ng/ml in plasma and 1 ng/ml in urine and the precision being 10–15% for concentrations greater than 10 ng/ml.

INTRODUCTION

The need for more sensitive analytical methods, caused by the increasing potency of new drugs, has created problems in handling small volumes or very dilute solutions from which substances have a tendency to be adsorbed to the walls of the containers. For some substances this problem has been solved by treatment of the glass tubes with dichlorodimethylsilane and the addition of trimethylamine or ethyl acetate[1–4]. In other cases glassware coated with poly(tetrafluoroethylene) has prevented or reduced adsorption[5].

The present paper describes an analytical procedure in which the adsorption of a structurally new serotonin (5HT) uptake inhibitor, (+)-(3*R*),(4*S*)-3-[(4-methoxyphenoxy)methyl]-1-methyl-4-phenylpiperidine hydrochloride (FG 4963), has been prevented by the addition of an analogous compound to compete for the binding sites of the glass.

EXPERIMENTAL

FG 4963 (Fig. 1) is a tertiary amine with MW 347.87 and pK_a 8.7. Its synthesis has been described in *Belg. Pat.*, No. 810310, 1974. FG 4963, FG 4915 and FG 4948

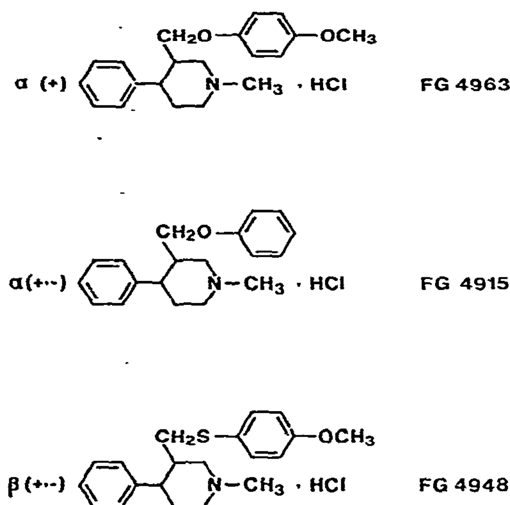

Fig. 1. Structural formulae of FG 4963, FG 4915 (adsorption inhibitor) and FG 4948 (internal standard).

(Fig. 1) were all synthesized by Dr. J. A. Christensen in the research laboratories of A/S Ferrosan, Copenhagen, (Denmark). The pharmacological and antidepressant properties of FG 4963 have been studied by Buus Lassen *et al.*[6–8] and by Heltberg *et al.*[9].

Gas chromatography

A Packard 419 gas chromatograph equipped with a nitrogen detector (Type No. 713) and Texas FLO 1-W recorder was used. The column of silanized glass (1.5 m × 4 mm I.D.) was packed with 1.5% SP 2250 (or OV-17) on Chromosorb G-AW-DMCS (80–100 mesh). Operating conditions: column temperature, 275°; injection port temperature, 280°; detector temperature, 285°; flow-rates of nitrogen, hydrogen and air, *ca.* 25, 25 and 280 ml/min, respectively.

Reagents and glassware

n-Pentane (pract.; Fluka, Buchs, Switzerland) was purified on a column of aluminium oxide, when necessary. All of the other reagents were of analytical quality. Disposable test-tubes (100 × 16 mm), surmounted with aluminium foil under a plastic cap, were used for the blood sampling and extraction procedures. All of the other glassware was rinsed in ethanol and *n*-pentane. The test-tubes for blood sampling were pre-treated by heparinization, addition of 125 μg of FG 4915 (100 μl of a solution containing 1.25 mg/ml of water) and subsequently dried at 110°.

Analytical procedure

Plasma. 10-ml samples of blood were taken by vein puncture, collected in the pre-treated test-tubes and centrifuged within 10 min. 250 ng of the internal standard (FG 4948), 250 μl of phosphate buffer (saturated, pH 12) and 5 ml of *n*-pentane were

added to 3.0 g of plasma and the mixture was shaken for 30 min at 50 rpm (Desaga 3 D, Model 13300, modified), the opening of the tube being covered by aluminium foil and a plastic cap. After centrifugation (10 min, 1000 *g*), the water phase was frozen in solid CO_2 and the organic phase was decanted into a new disposable test-tube, in which it was evaporated to dryness in a vacuum. The wall of the test-tube was washed twice using 500 μl and 250 μl of *n*-pentane, respectively, each time followed by evaporation to dryness. Finally, the bottom of the tube was washed with 8 μl of *n*-heptane, added by means of a Hamilton (701) syringe, and as much as possible (*ca.* 5 μl) was withdrawn and injected into the chromatograph. The retention times were: FG 4915, 2.3 min; FG 4963, 3.8 min; and FG 4948, 6.3 min.

Standard solutions containing 0–100 ng/ml of FG 4963 in water were treated and analysed simultaneously with the plasma samples. The plasma concentration was calculated on the basis of the peak-height ratio of FG 4963 and FG 4948 by reference to the standard graph obtained.

Urine. Samples were stored in glass containers pre-treated with FG 4915 (*ca.* 50 μg per 7 ml of urine). 250 μg of the internal standard (FG 4948), 1 ml of phosphate buffer and 3 ml of *n*-pentane were added to 7.0 g of urine. The remaining procedure was as described for plasma.

Testing of the analytical procedure. The accuracy and precision of the method were determined using spiked samples of human urine and plasma analysed at random. The precision under "routine conditions" was calculated from 250 determinations carried out in duplicate (on different days) on plasma and urine from patients and volunteers to whom FG 4963 had been administered.

RESULTS AND DISCUSSION

General procedure

Determinations of tricyclic antidepressants in the nanogram range, using gas chromatography and a nitrogen-sensitive detector, have recently been described[3,4,10,11]. The methods involved extraction with heptane or hexane from an alkalized sample of plasma or serum, followed by evaporation of the organic phase and, if convenient, derivatization before the gas chromatography. Adsorption of the extracted substances during the evaporation was prevented by silanization of the glass tubes and/or addition of trimethylamine, triethylamine, alcohols or ethyl acetate. This principle has also been used in the analysis of other amines and of alcohols in the picogram–nanogram range[1,2]. Besides adsorption, loss of amphetamine due to volatilization has been reported by O'Brien *et al.*[5], who eliminated this by forming the non-volatile hydrochloride prior to evaporation of the organic solvent.

FG 4963 was found to be adsorbed to glass, not only during evaporation but even during storage of plasma samples. For instance, 40 ng of FG 4963 base was completely adsorbed when added to 5 ml of pentane and then evaporated. Pre-treatment of the tubes, with triethylamine or benzylaniline (1% in pentane) or silanization (5% dichlordimethylsilane in toluene), did not prevent the binding of FG 4963 base to glass. Moreover, 25 μg of benzylaniline or 1.5% butanol did not prevent the complete adsorption of FG 4963 base when added to the pentane phase previous to the evaporation procedure. FG 4963 (the hydrochloride), dissolved in 5 ml of dichlormethane, was adsorbed during evaporation. However, addition of

25–50 μg of FG 4915 to each test-tube completely inhibited the adsorption of FG 4963 to the glass.

More than 50% of the FG 4963 contained in plasma samples (25 ng/ml) was adsorbed during storage for 16 h at 5° followed by 2 h at 25° (Fig. 2). This considerable binding could be prevented by the addition of FG 4915 to the test-tubes in amounts corresponding to 25–50 μg per analysis of blood. However,the addition of FG 4915 caused haemolysis, and the blood samples had to be centrifuged immediately, preferably within 10 min. Similarly, FG 4915 was added to urine before storage of samples. For instance, the whole urine fraction could be collected and a sample immediately transferred to test-tubes or vials containing FG 4915 (corresponding to 25–50 μg per analysis) without loss of FG 4963.

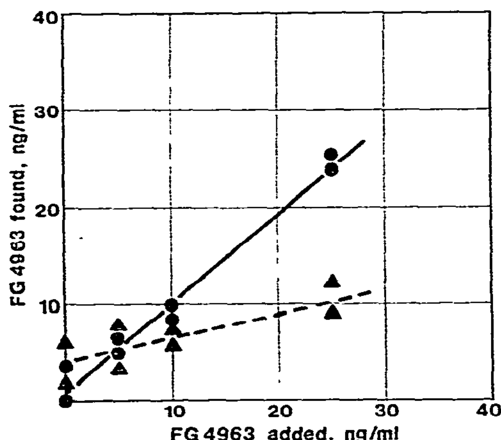

Fig. 2. The influence of FG 4915 on the binding to glass of FG 4963 in plasma samples during storage (16 h at 5° and 2 h at 25°): ●, 2 ml of plasma to which 50 μg of FG 4915 were added; ▲, 2 ml of plasma without FG 4915.

Only glassware could be used be used for the storage of the samples and for the analytical procedure. Plastic and glass tubes having plastic caps caused interference during the subsequent gas chromatography. Therefore the openings of the test-tubes, used for blood sampling and the extraction procedure, were covered with aluminium foil and then with plastic caps. Disposable glass test-tubes were always used, and pipetting of samples was avoided since a normal washing-up procedure gave varying blank values. An unusually large fraction of the extract was transferred to the gas chromatograph (5 μl out of 8 μl). 50–60% of the initial content of FG 4963 in a plasma or urine sample could be carried through to the chromatograph, provided that the inside of the tube was properly washed.

The nitrogen-sensitive detector (TID) had almost the same sensitivity for FG 4963 as the usual flame-ionization detector (FID) (*ca.* 1 ng injected under the conditions described). Even if the TID was a little more difficult to operate it was preferred to the FID because of a much smaller response from the solvent and from non-nitrogen-containing impurities. A typical gas chromatogram of an extracted plasma sample spiked with FG 4963 is given in Fig. 3.

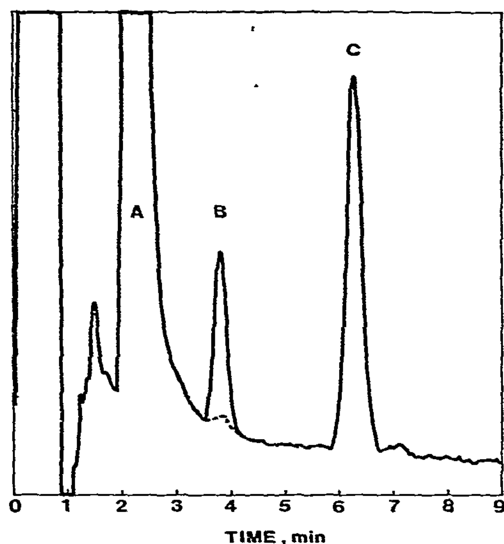

Fig. 3. A typical gas chromatogram of an extract from a plasma sample spiked with 25 ng/ml of FG 4963. The broken line shows the response from an extracted plasma blank. Peaks: A = FG 4915, B = FG 4963 and C = FG 4948 (internal standard). For experimental conditions see text.

Accuracy and precision

The analytical procedure for FG 4963 in urine is accurate and precise, the lower limit being *ca.* 1 ng/ml (Table I). The recovery was between 103 and 106% in the range 2.5–50 ng/ml, and the relative standard deviation was less than 6% for concentrations greater than 2.5 ng/ml. Plasma blanks higher than indicated in Table I were occasionally found in a few individuals, so that the limit is considered to be *ca.* 5 ng/ml. The recovery from plasma was 91–104% in the range 5–100 ng/ml, and the relative standard deviation was less than 10% for concentrations greater than 10 ng/ml. Determinations carried out in duplicate "under routine conditions" on plasma and urine from humans administered FG 4963 gave the relative standard deviations of 25, 15 and 10% in the concentration ranges < 10, 10–20 and > 20 ng/ml, respectively.

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF FG 4963

Spiked human urine and plasma were used for six determinations at each level.

Urine			*Plasma*		
FG 4963 added (ng/ml)	*FG 4963 found ($\bar{x}$) (ng/ml)*	*S.D. (ng/ml)*	*FG 4963 added (ng/ml)*	*FG 4963 found ($\bar{x}$) (ng/ml)*	*S.D. (ng/ml)*
0	0.1	0.1	0	2.8	1.4
2.5	2.6	0.2	5	5.2	0.6
5	5.3	0.3	10	9.1	0.7
12.5	13.3	0.3	25	24.6	1.7
50	51.5	1.4	100	97.3	9.1

Frozen urine and plasma samples containing FG 4915 have been stored for more than 6 months without loss of FG 4963.

Specificity

The metabolism of FG 4963 has not yet been investigated, but possible metabolites such as the N-desmethyl, the N-oxide and the *p*-hydroxy compounds do not interfere with the determination of FG 4963. Interference from other drugs in the assay has been studied in a number of patients during their treatment with these and before medication with FG 4963. Interference has not been seen from bisacodyl, clopenthixol, chloralodolum (chloral hydrate), chlormezanone, chlorpromazine, diazepam, diphenoxylate, disulphiram, glutethimide, haloperidol, hydroflumethiazide, lactulose, melperone, nitrazepam, orphenadrine, oxprenolol, periciazine, perphenazine or sodium levothyroxine. Only one metabolite in plasma from patients treated with dextropropoxyphene gave an interfering peak in the gas chromatogram.

Capacity

The described method involves rather simple procedures but is relatively time-consuming and requires skillful and careful execution. The present capacity is about 15 samples at a time (exclusive standards), carried out in two sequences: (1) the pre-chromatographic procedures are performed in one day; (2) the gas chromatography is carried out on the following day. In order to increase the capacity, work is in progress on automation of the chromatographic procedure using a solid-injection technique and an on-line calculator.

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