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Methods for study of fluphenazine kinetics in man

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Fluphenazine and its principal metabolites, fluphenazine sulphoxide, and 7-hydroxyfluphenazine were identified and quantified in human plasma, urine and faeces following intramuscular and oral administration of ^{14}C-fluphenazine dihydrochloride. The presence of a conjugate fraction was also noted. Unmetabolized fluphenazine was selectively extracted into n-heptane. The metabolites were separated by solvent extraction into toluene. Conjugates were hydrolysed back to fluphenazine, fluphenazine sulphoxide and 7-hydroxyfluphenazine. Fluphenazine and fluphenazine conjugates were also measured in the urine of patients receiving long term non-radioactive fluphenazine decanoate therapy. The urinary excretion rate of the conjugate fraction was systematically related to the plasma concentration, regardless of urine flow rate or pH, providing a convenient method for the assessment of fluphenazine kinetics by urinary excretion studies not involving administration of labelled drug.

Attempts to study fluphenazine kinetics in man include the work of Forrest, Forrest & Mason (1961) who devised a semi-quantitative spot test for fluphenazine in urine and McIsaac (1971) who administered ^{14}C-labelled fluphenazine to human subjects but reported only total radioactivity measurements. Fluphenazine is converted to a number of metabolites (Ebert & Hess, 1965; Dreyfuss, Ross & Schreiber, 1971a, b; Gaertner & Breyer, 1972; Gaertner, Breyer & Liomin, 1974; Breyer, Gaertner & Prox, 1974a; Breyer, Prox & others, 1974b). Smulevitch, Minsker & others (1973) reported fluorescence measurements on patients using a method which we have been unable to reproduce. Larsen & Naestoft (1973) and Kelsey, Keskiner & Moscatelli (1973) described g.l.c. methods neither of which has been applied to routine clinical determinations. Curry (1970) reported isotope dilution techniques applied to solutions prepared at high concentrations in the laboratory. We now report a new study in which data from investigations with [^{14}C]fluphenazine were used as the basis for the quantitative assessment of fluphenazine kinetics from urinary excretion studies in patients receiving non-radioactive fluphenazine. A new g.l.c. method was also used.

METHODS

Materials

Reagents (analytical grade, if available) were purchased from BDH, Limited, Poole, Dorset. β-Glucuronidase/aryl sulphatase (*Helix pomatia*) was purchased from Boehringer (Mannheim), Limited, Lewes, Sussex. *N,O*-Bis-(trimethylsilyl)-acetamide (BSA) and *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Pierce and Warriner (U.K.) Ltd, Chester. Non-radioactive reference materials (fluphenazine dihydrochloride, fluphenazine enanthate, fluphenazine decanoate and 7-hydroxyfluphenazine) and fluphenazine-(ethanol 1,2-[^{14}C] dihydrochloride (2 mCi mmol^{-1}), synthesized at the Radiochemical Centre (Amersham) according to Yale, Cohen & Sowinski (1963) were donated (Squibb).

Fluphenazine sulphoxide was prepared in solution in the laboratory by shaking a solution of fluphenazine in heptane with a solution of H_2O_2 (approx. 10%). The heptane was discarded and the aqueous solution treated with potassium metabisulphite solution (M) to reduce any fluphenazine *N*-oxides. The solution was made alkaline with sodium hydroxide and washed twice with heptane before the product was extracted into toluene. Thin-layer chromatography (t.l.c.) of the toluene extract showed only one compound which was confirmed as fluphenazine sulphoxide by t.l.c. (Dreyfuss & others, 1971a) fluorescence (Ragland & Kinross-Wright, 1964), g.l.c. (Kelsey & others, 1973) and by its reactions with potassium metabisulphite (no effect) and $TiCl_3$ (reduction to fluphenazine).

Fluphenazinyl acetate was prepared by heating fluphenazine dihydrochloride with acetic anhydride for 30 min at 100°. On cooling, the product precipitated and it was filtered and washed with petroleum spirit (40°–60°). The identity of the product was confirmed with its t.l.c., ultraviolet spectroscopy and extraction characteristics. Hydrolysis with NaOH(N) at 100° regenerated fluphenazine.

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Apparatus

Ultraviolet spectra. Fluphenazine exhibited major peaks at 262 nm and 315 nm, uncorrected.

Gas liquid chromatography was with a Pye, Series 104 chromatograph equipped with a standard flame ionization detector and with a Perkin-Elmer Model F-17 chromatograph equipped with an alkali bead detector (nitrogen sensitive). High purity nitrogen was the carrier gas in both instances; we found helium not to be necessary for the latter detector. Temperature and flow rates were adjusted to give suitable retention times. Silanized glass columns were packed with either 1% or 3% OV-17 on Chromosorb W, HP (80–100 mesh) prepared by standard methods in the laboratory. The compounds were reacted with excess BSA or BSTFA at room temperature before chromatography and the reaction mixture injected directly on the column. The reference materials gave Gaussian curves well separated from the solvent signal. Minimum detectable samples were 5 ng (alkali bead).

Thin layer chromatography was carried out using plastic backed silica gel plates, 0·1 mm thick (Eastman Kodak) or 0·25 mm thick (Merck) containing fluorescent indicators. The solvent systems were those of Dreyfuss & others (1971a) (chloroform–methanol, 90:10; and chloroform–methanol–0·88 ammonia 80:10:1; were the most used). The spots were located as shadows under short wave ultraviolet light (250 nm) and, additionally, the sulphoxides were characterized by their blue fluorescence under long wave ultraviolet light (350 nm). When sprayed with Folin and Ciocalteu's reagent, fluphenazine and fluphenazine sulphoxide produced pink spots and 7-hydroxyfluphenazine a purple spot. Radioactive material was located by cutting the plates into vertical strips and cutting these horizontally into 0·5 cm segments which were placed in vials for liquid scintillation counting. When chromatographing radioactive samples from biological fluids, non-radioactive material (approximately 10 μg) was added as a carrier and internal standard.

Radioactivity measurements were made with a Tracerlab Corumatic Series 2000 liquid scintillation spectrometer. Dioxane based scintillation fluid (Nuclear Enterprises, N.E.250) was used. Quenching was determined by the channels ratio method.

Solvent extraction. The compound under test was dissolved in n-heptane or toluene and aliquots of the solution were shaken with equal volumes of aqueous solutions with different pH values. Suitable pH values were obtained from dilutions of HCl and NaOH for the extreme values and from 0·1M buffers (glycine–HCl, phosphate and glycine–NaOH) for the intermediate values. After centrifugation the concentrations in both phases were determined. The partitioning, expressed as the percentage remaining in the organic phase, was plotted against the pH of the aqueous phase. This produced curves characterstic of each compound (Whelpton & Curry, 1976).

Biological material was from a group of patients, under the care of Dr S. Vrankcx at the University of Leuven, Belgium, who were receiving oral and intramuscular doses of labelled fluphenazine from the same batch as our reference standards (the supply of samples was arranged by Dr A. Schiff of E. R. Squibb & Sons, Ltd), and patients, under the care of Dr D. M. Lewis at St. Bernard's Hospital, Southall, Middlesex, U.K., who were receiving routine doses of non-radioactive fluphenazine decanoate as part of their treatment. Blood was collected by venepuncture into tubes containing heparin and centrifuged to separate the plasma. Urine and faeces were 24 h collections. Blood bank plasma and the urine from one of us (R.W.) was used for reference purposes.

Quantitative analysis of radioactive fluphenazine

After intramuscular or oral dosing of [^{14}C]fluphenazine dihydrochloride (25 mg), plasma collected at selected times up to 96 h after dosing, was pipetted into test tubes and non-radioactive fluphenazine dihydrochloride (100 μg in 0·5 ml of water) was added as internal standard. Sodium hydroxide (1 ml, 10 N) and n-heptane (5 ml) were added and the stoppered tubes were shaken mechanically for 30 min. After centrifugation, the heptane (4 ml) was removed and the ultraviolet absorption of the solution was measured before it was transferred, along with cuvette washings (2 × 0·5 ml) into a counting vial.

Urine (1 or 2 ml) was made alkaline with sodium hydroxide (1 ml) and extracted with n-heptane (5 ml). The heptane (4 ml) was removed for counting and toluene (4 ml) added to the residue. After extraction, the toluene (4 ml) was counted and the pH of the aqueous layer was adjusted to pH 9·0 by titrating to neutrality with HCl and then adding pH 9·0 glycine buffer (2M, 1 ml). A toluene extraction at this pH removed any 7-hydroxyfluphenazine, facilitating the separate estimation of fluphenazine sulphoxide and the 7-hydroxylated metabolite. Conjugates were determined by hydrolysing urine samples with β-glucuronidase/aryl sulphatase (0·1 ml) overnight at 37° before the extraction procedure. Faeces were homogenized with 3 volumes of methanol, filtered, the residues washed and the combined filtrate and washings measured, counted (0·1 ml), and aliquots

(1 ml) evaporated to dryness and fractionated as for urine.

Analysis of non-radioactive fluphenazine. The quantities of free and conjugated fluphenazine in 24 h urine samples were determined by g.l.c. using nitrogen sensitive detection. Non-conjugated fluphenazine was extracted from untreated urine and fluphenazine conjugates were determined by extracting the urine after enzymatic hydrolysis. Samples of urine (100 ml) were adjusted to pH 5 with glacial acetic acid (*ca* 0·5 ml) and hydrolysed with β-glucuronidase/aryl sulphatase (0·2 ml) for 18 h at 37°. Hydrolysed and unhydrolysed urines were transferred to screw topped Pyrex tubes containing 10N NaOH (2 ml) and hexane (10 ml) and shaken mechanically for 30 min. After standing, the aqueous layer was aspirated and the organic layer transferred to 15 ml polypropylene tubes which were stoppered and centrifuged at 3500 rev min^{-1} for 15 min. The hexane (9 ml) was pipetted into pointed glass tubes, placed in a waterbath at 60° and evaporated under a gentle stream of nitrogen. The dry residues were dissolved in 100 μl of hexane containing thioridazine (100 μg ml^{-1}) as an internal standard. BSTFA (20 μl) was added and the tubes stoppered and allowed to stand for 60 min before aliquots (1 to 5 μl) were injected into the chromatograph. The 6 ft glass column containing 3% OV-17 was maintained at 275° and the detector at 290°. With the carrier gas inlet pressure at 25 psig (1 psi = 6·9 Kpa) the retention times of the fluphenazine derivative and thioridazine were 5·9 and 9·5 min respectively and non-derivatized fluphenazine had a retention of 7·0 min. The peak height ratio was plotted against fluphenazine concentration to construct a calibration curve.

RESULTS AND DISCUSSION

Conditions used

A study of the partitioning of fluphenazine, its sulphoxide and 7-hydroxyfluphenazine in n-heptane and toluene allowed the selective separation techniques to be devised (see Whelpton & Curry, 1976). If an aqueous solution containing the three compounds was adjusted to pH 14 and extracted with n-heptane the fluphenazine was removed. Extraction of the residue with toluene removed fluphenazine sulphoxide and adjustment of the pH to 9 permitted 7-hydroxyfluphenazine to be extracted into a second toluene extract. When the compounds were added to urine and taken through the extraction procedure, the recoveries (100·5 ± 1·2 s.e.; n = 6) were not significantly different from those obtained using solutions in distilled water. However, when radioactive fluphenazine was added to blank plasma and extracted into n-heptane, the recovery was only approximately 50%. The percentage recovery was determined by adding a known amount of non-radioactive fluphenazine such that its ultraviolet absorption in the n-heptane extract could be measured without significant contribution being made by the radioactive fluphenazine. The added fluphenazine also acted as a carrier, preventing loss of radioactive material by adsorption onto glass.

Nitrogen sensitive detection for the g.l.c. of fluphenazine was an improvement over standard flame ionization detection. Under the conditions described it was possible to measure fluphenazine concentrations to 2 ng ml^{-1} of urine. Fluphenazine could be chromatographed without pretreatment, but derivatization increased the sensitivity of the g.l.c. approximately ten-fold. Whereas acetyl and trimethylsilyl (TMS) derivatives were both suitable for chromatographing fluphenazine, the acetyl derivatives of 7-hydroxyfluphenazine and fluphenazine sulphoxide were not completely resolved, and TMS ethers were used. Thioridazine was included as an internal standard to compensate for changes in the alkali bead detector response, which was linear for both fluphenazine and the internal standard. Although there was some decrease in sensitivity on repeated injections the peak height ratio for a particular sample remained constant. The sensitivity was restored by increasing the bead temperature setting for 2 min and then returning it to the operating position. The peak height ratio was the same at different settings of bead temperature.

Selectivity

The specificity of the various extraction techniques was confirmed by t.l.c., g.l.c. and solvent partition characteristics (Whelpton & Curry, 1976). The extracts into heptane from pH 14 solution, into toluene from pH 14 solution, and into toluene from pH 9 solution, showed peaks corresponding to fluphenazine, fluphenazine sulphoxide, and 7-hydroxyfluphenazine respectively. The same extracts when combined and concentrated by evaporation under vacuum and chromatographed gave peaks corresponding to the 3 compounds (Fig. 1).

For pharmacokinetic analysis the most important extract is that which allows the amount of unchanged fluphenazine in plasma to be quantified. Solvent extraction (Table 1) and t.l.c. confirmed that the radioactivity extracted from plasma into n-heptane was unmetabolized fluphenazine. To date the only radioactive compound (among fluphenazine, flu-

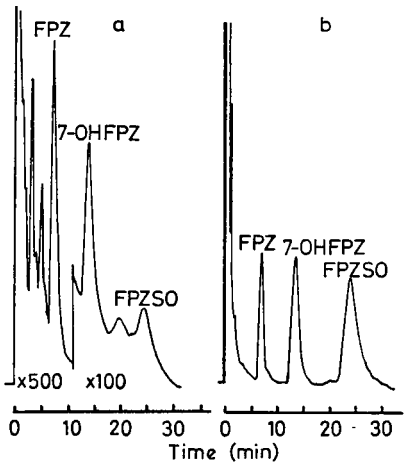

FIG. 1. G.l.c. trace of material extracted from a—faeces and b—reference compounds. Fluphenazine (FPZ) fluphenazine sulphoxide (FPZSO) and 7-hydroxyfluphenazine (7-OH FPZ) are clearly visible. Chromatograph equipped with FID using 1 ft column packed with 1% OV-17. Oven temperature 215°. Nitrogen flow rate 40 ml min^{-1}.

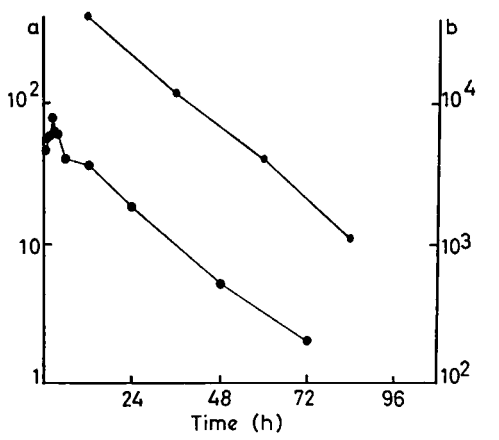

FIG. 2. a—Plasma concentrations (d min^{-1} ml^{-1}) of fluphenazine in a patient following 25 mg of [^{14}C]fluphenazine dihydrochloride (i.m.) (lower line) and b—urine excretion rate (d min^{-1} h^{-1}) of total fluphenazine (conjugated and non-conjugated) in the same patient (1 ng of fluphenazine = 8·8 d min^{-1}) (upper line).

phenazine sulphoxide and 7-hydroxyfluphenazine) identified in plasma is unmetabolized fluphenazine. Intramuscular injection of 25 mg of fluphenazine dihydrochloride resulted in a peak plasma concentration of 8·9 ng ml^{-1} at 2 h, and a half life of 15 h (Fig. 2). Fluphenazine, 7-hydroxyfluphenazine and fluphenazine sulphoxide in urine were mainly conjugated (Fig. 3). Fluphenazine and 7-hydroxyfluphenazine, but no conjugates, were found in faeces; it is probable that the conjugates were excreted in bile. Dreyfuss & others (1971b) have shown that 7-hydroxyfluphenazine glucuronide is excreted in the bile of dogs and is subsequently hydrolysed in the gastrointestinal tract so that only 7-hydroxyfluphenazine is found in the faeces.

That fluphenazine is converted to a conjugate or conjugates (from which it can be regenerated by hydrolysis) is important since it is this which allows the indirect assessment of its kinetics. This conjugated material must arise by direct combination of the alcoholic group with the conjugating molecule(s). It will not be readily reabsorbed in the renal tubule, and, as a consequence it will be excreted at a rate independent of urine pH and volume. Thus its urinary excretion rate will be directly proportional to its plasma concentration. Furthermore, the formation of this material is dependent on the presence of

Table 1. *pH partition of heptane extractable radioactivity from plasma.*

	Percent in heptane	
pH	u.v.	Radioactive
5·20	20	13
5·52	36	37
6·08	53	56

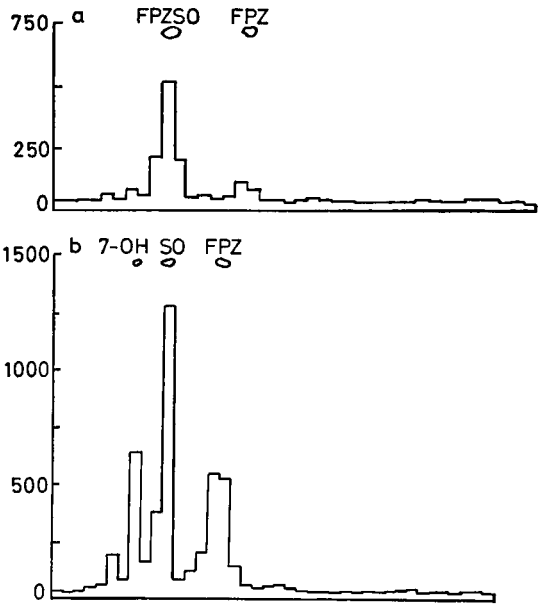

FIG. 3. T.l.c. of radioactivity from urine, a—before and b—after hydrolysis, extracted to remove the three reference compounds. y axis—Counts min^{-1}.

fluphenazine in plasma. Thus there must exist a relation between the excretion rate of fluphenazine conjugates and the concentration of unconjugated fluphenazine in plasma. In practice, we showed a linear relation between plasma fluphenazine and the urinary excretion rate of total material (conjugated plus non-conjugated) (Fig. 2). The two measures declined in parallel with the same half-time. The conjugates comprised more than 80% of the total material, and their clearance was high indicated by their virtual absence from plasma.

Until a satisfactory plasma assay for non-radioactive fluphenazine is developed, studying the urinary excretion pattern will permit useful investigation of the pharmacokinetics of fluphenazine. As an example, Fig. 4 shows the excretion rate (24 h urine samples) of combined fluphenazine (fluphenazine and fluphenazine conjugates) in urine from a patient receiving fluphenazine decanoate (50 mg) at weekly intervals. Doses were given at day 0 and day 7 (arrows). The excretion rate rose and fell with each dose. At present, measurement of urinary excretion in this way is the best approach to the obtaining of an estimate of the time course of changes in fluphenazine plasma concentrations. At worst, urinary excretion will indicate the time course of fluphenazine in the body. At best it will allow one to compute the exact plasma concentration.

Finally, this approach may have general applicability to compounds that are transformed directly into water soluble conjugates, free of urine pH and volume influences. Perphenazine, flupenthixol, morphine and other drugs may meet this criterion.

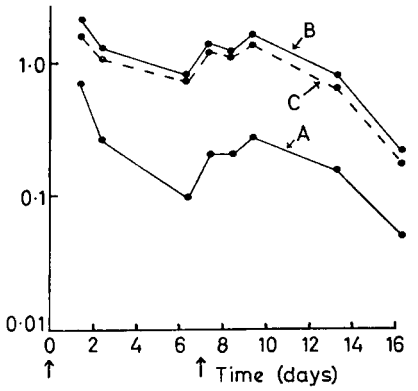

FIG. 4. The urinary excretion rate (μg h^{-1}) of fluphenazine (A), fluphenazine conjugates (C) and fluphenazine plus conjugates (B), in a patient receiving non-radioactive fluphenazine decanoate (50 mg per week).

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