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MASS FRAGMENTOGRAPHIC ASSAY OF NANOGRAM AMOUNTS OF THE ANTIDEPRESSANT DRUG MIANSERIN HYDROCHLORIDE (Org GB 94) IN HUMAN PLASMA

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SUMMARY

For the assay of the antidepressant compound mianserin hydrochloride (Org GB 94) in human plasma, a mass fragmentographic method, using the deuterated analogue as internal standard and a high-performance liquid chromatographie sample clean-up procedure has been developed. The assay specifications obtained are a lower limit for reliable measurements of 1 ng/ml, an accuracy of ca. 0.01 ng/ml, a precision of 6—7% and a capacity of about 60 samples per day. The applicability of the assay method is illustrated by measurements of single-dose and steady-state plasma levels in clinical experiments, demonstrating the possibility of monitoring plasma levels during at least 24 h after a single dose of 15 mg of Org GB 94. The mean steady-state plasma levels after a daily dose of 3 × 20 mg of Org GB 94 appeared to be remarkably constant with time: 38, 36 and 34 ng/ml after 2, 4 and 6 weeks of treatment of 18 depressed patients.

INTRODUCTION

To permit a reliable pharmacokinetic and pharmacological evaluation of the new antidepressant drug mianserin hydrochloride (Org GB 94; 1,2,3,4,10,-14b-hexahydro-2-methyldibenzo[*c*,*f*]pyrazino[1,2-*a*]azepine monohydrochloride), a method for its assay in human plasma has been developed. This compound, the chemical structure of which is depicted in Fig. 1, showed potent antidepressant activities in EEG profile analysis and in therapeutic studies [1].

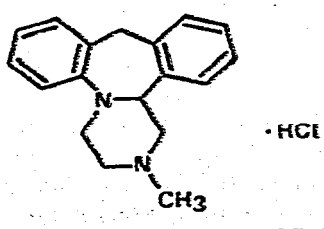

Fig. 1. Structure of Org GB 94.

In view of expected low levels after a therapeutic dose (nanogram per millilitre range) and the extensive biotransformation [2], a highly sensitive and specific assay method is required. Because of its sensitivity and inherent specificity, mass fragmentography (MF) [3] was selected as the detection method. The applicability of the assay has been illustrated by measuring plasma levels after a single oral dose and during chronic treatment.

MATERIALS AND METHODS

Internal standard

[10,10-$^2H_2$]Org GB 94, used as an internal standard, was prepared by hydrogen—deuterium exchange in the system hexamethylphosphoric triamide (HMPT) with NaO^2H and ^2H_2O at 80°. According to mass spectrometric analysis, the deuterium content was 4.2% $^2H_0$, 21.0% $^2H_2$, 73.3% $^2H_1$, 1.3% $^2H_3$ and 0.2% $^2H_4$.

Solvents

All solvents used were purchased from Merck (Darmstadt, G.F.R.). *n*-Hexane and isopropanal were of Uvasol quality, ethanol and methanol were of analytical-reagent grade and the ammonia was of Suprapur grade.

Glass-ware

Ten-millilitre glass-stoppered tubes were cleaned ultrasonically and subsequently rinsed with *n*-hexane and methanol. One-millilitre disposable glass tubes were cleaned by rinsing with methanol and subsequent heating in a gas burner until red hot.

Equipment

A Waters Assoc. (Milford, Mass., U.S.A.) high-performance liquid chromatograph equipped with a 30 cm × 4 mm I.D. μPorasil column (10 μm, Waters Assoc.) and a 280-nm UV detector was used for sample purification. The instrument was operated at a pressure of about 800 p.s.i. and a flow-rate of 2 ml/min of *n*-hexane—isopropanol (80:20, v/v) to which 4% of ethanol and 0.1% of concentrated ammonia were added.

The gas chromatograph—mass spectrometer—computer system consisted of the following:

(i) A Varian Aerograph 2740 gas chromatograph equipped with a 4 m × 2 mm I.D. glass column filled with 1% JXR on Gas-Chrom Q (80—100 mesh). The injector, column and detector oven temperatures were ca. 270, 260 and 270°, respectively. Helium was used as the carrier gas at a flow-rate of 30 ml/min. The gas chromatograph was coupled via a dual-stage Watson—Biemann separator to a mass spectrometer (see below).

(ii) A Varian-MAT CH7 mass spectrometer equipped with a peak-matching device. The spectrometer was focused at *m/e* 264 and the peak matcher was set to display alternatively the *m/e* 264 and *m/e* 266 signals, representing the molecular ion peaks of Org GB 94 and the internal standard, respectively. A home-made filter system allowed continuous, (pseudo)-simultaneous registration of the two signals on a two-pen potentiometer recorder. The mass

spectrometer was operated at electron energy 70 eV, ionizing current 300 μA, ion accelerating voltage 3 kV, electron multiplier voltage 2 kV and ion-source temperature 135°.

(iii) The computer system was a Varian-MAT SpectroSystem 100 MS with a 2.4-M-word disc backing store. Calculations were also performed with this computer system using a BASIC program.

Clinical experiments

Single-dose experiments were performed by administration of 15 mg of Org GB 94 to three healthy male volunteers aged between 21 and 35 years under the supervision of Prof. M. Fink, New York, U.S.A. Each volunteer was treated twice with a minimum time interval of 10 days between the two sessions.

Chronic therapy was performed by administration of a daily dose of 60 mg of Org GB 94 (20 mg three times a day) for 6 weeks to 18 patients suffering from depressive illness, under supervision of Dr. A. Coppen, Epsom, Great Britain. Blood samples were taken with heparinized syringes at the time intervals indicated in Tables II and III. After the addition of saturated sodium citrate solution, the blood was centrifuged and the plasma was transferred to separate sample tubes and stored at −20° until required for further analyses. Plasma was prepared within 40—100 min after withdrawal of each blood sample.

Assay methods

From the administered dose and the sampling time, the Org GB 94 concentration was estimated. For expected levels of less than 1 ng/ml in plasma, 2-ml samples were processed, while for higher levels 1-ml samples were used. An amount of [10,10-$^2H_2$]Org GB 94 more or less equal to the expected Org GB 94 concentration, dissolved in 0.1—2 ml of water and 100 μl of concentrated ammonia (to obtain pH 11) was added to the sample. After equilibration for at least 3 h, the plasma was extracted with tho 5-ml portions of *n*-hexane by thoroughly mixing and centrifuging for 5 min at 1200 *g*. The combined extracts were evaporated to dryness in a 10-ml conical tube at 45° under a gentle stream of nitrogen.

The residue was re-dissolved in 0.5 ml of the solvent system used for sample clean-up by high-performance liquid chromatography (HPLC). The collected fraction containing the Org GB 94 and internal standard was evaporated to dryness at 45° under nitrogen.

The residue was transferred into 1-ml glass tubes using one 200-μl portion and one 100-μl portion of methanol. The contents of each glass tube were again evaporated to dryness at 45° under nitrogen and the residue was subsequently re-dissolved in 8 μl of methanol. From this methanolic solution, the maximum possible amount (6—7 μl) was injected into the gas chromatograph—mass spectrometer for quantification. Intermittently, 2-μl portions of standard solutions containing Org GB 94 and [10,10-$^2H_2$]Org GB 94 in concentration ratios of 4:1, 2:1, 1:1, 1:2 and 1:4 (ng/μl) were analyzed in triplicate for establishment of the calibration graph. Peak heights (occurring at the retention time for both compounds: ca. 2.5 min) were measured manually. From these results, the amount of Org GB 94 per millilitre of plasma was calculated using a BASIC computer program. Because the amount of internal standard was adapted to each

individual sample in order to achieve optimal accuracy and precision, a relative calibration graph had to be used.

A wide-range calibration graph (concentration ratio may vary by a factor of 16) was obtained by plotting the fractional peak height $h(m/e\ 264)/[h(m/e\ 264) + h\ (m/e\ 266)]$ versus the fractional concentration [Org GB 94]/[Org GB 94] + [[10,10^{2}H$_2$]Org GB 94]) in the range 1:4 to 4:1. A typical example of such a calibration graph with calculated slope of 0.89 is shown in Fig. 2.

Using this calibration graph, the measured peak heights and the data on sample volume and amount of internal standard added, the program calculates the level of Org GB 94 (free base) in nanograms per millilitre of plasma. Samples in which the actual concentration ratio of Org GB 94 to internal standard exceeds the boundaries of the calibration graph have to be re-processed with different amounts of internal standard.

RESULTS AND DISCUSSION

The merits of mass fragmentographic analyses have been discussed elsewhere

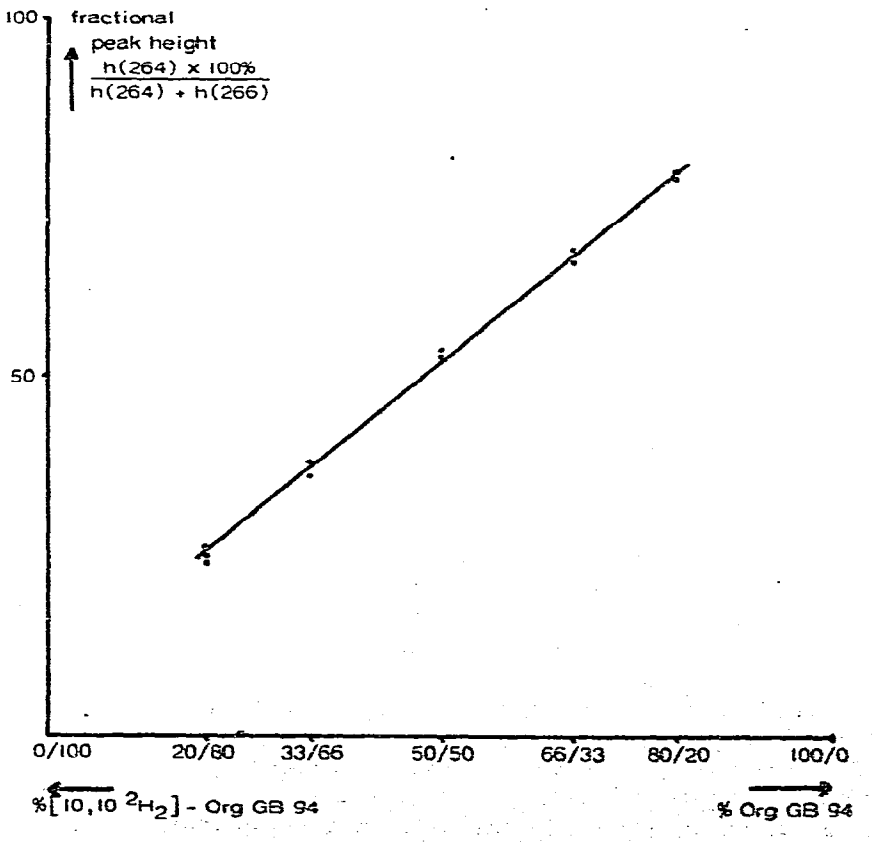

Fig. 2. Mass fragmentographic calibration graph for 1:4 to 4:1 ratios of Org GB 94 and [10,10-^{2}H$_2$]Org GB 94 calibration mixtures.

[3], and the discussion of the present method is limited to the description of the assay specifications.

Assay specifications

To test the method, 1-ml samples of human plasma pools spiked with 1 or 15 ng/ml of Org GB 94 were processed. A possible disintegration of Org GB 94 during storage was investigated by analyzing a pool of plasma spiked with 15 ng/ml, after 7 months of storage at −20°. The results of these analyses are presented in Table I and are discussed in terms of accuracy, precision and sensitivity.

Accuracy and precision

The accuracy of the measurements, defined as the deviation from the true value, was found to be independent of the concentration in the range investigated. During 7 months of storage at −20° the plasma concentration of 15 ng/ml decreased by not more than 5%. The high degree of accuracy, inherent in MF assays with internal standardization, is even enhanced by the use of a sliding relative calibration graph, which allows adaptation of the amount of internal standard to each individual sample.

The precision, expressed as standard deviation, was 6—7% and was independent of the concentration and storage time. From these precision data, it can be derived that for a level of 1 ng/ml the measurements have a probability of only 0.05 of being outside the range 0.86—1.13 ng/ml. It should be borne in mind, however, that these analyses were performed with a 1:1 ratio of compound to internal standard. In separate experiments, it was demonstrated that a 1:4 or 4:1 ratio does not affect the precision significantly.

The relatively high accuracy and precision are due mainly to the method of internal standardization employed, with the stable isotope-labelled analogue added directly to the plasma sample. Because of equal recovery of drug and internal standard from plasma, any loss of drug is automatically corrected for by proportional losses of the internal standard, which makes the accuracy independent of the Org GB 94 recovery. Further, it should be realized that by using the same stock solution of [10,10-$^2H_2$]Org GB 94 for addition to the samples as for preparing the calibration graph, any inaccuracy in concentration of the stock solution is cancelled out in the calculations.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF Org GB 94 IN HUMAN PLASMA

Org GB 94 plasma concentration (ng/ml)	Internal standard added (ng/ml)	Mean of measured concentration (ng/ml)	Number of determinations	Accuracy (%)	Precision (S.D.) (%)
1	1	1.01	10	1	6.5
15	15	15.01	20	0.1	6.0
15*	15	14.26	10	−4.9	6.8

*This pool had been prepared 7 months before analysis and had been stored at −20° in order to investigate possible disintegration of Org GB 94 during storage.

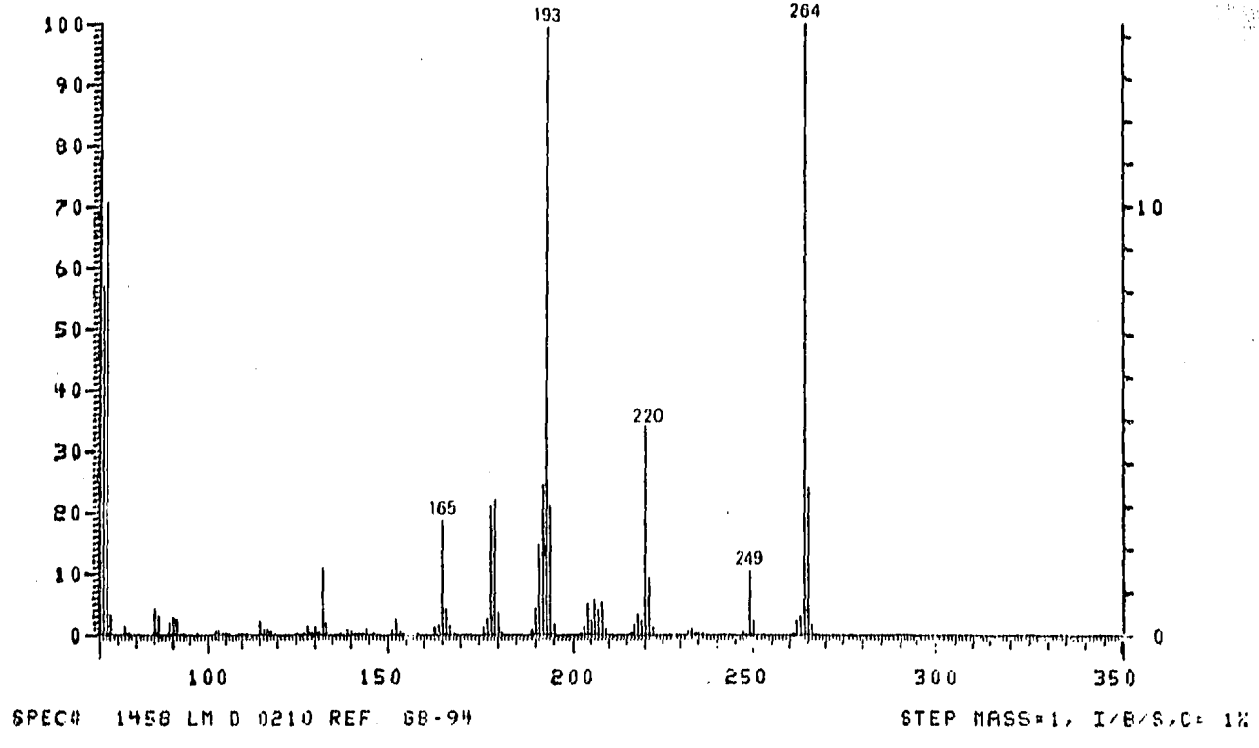

Fig. 3. Mass spectrum of Org GB 94 (Varian-MAT CH7, 70 eV).

Sensitivity

The sensitivity of the assay method is determined by (i) the detection limit of the gas chromatographic—mass spectrometric (GC—MS) system and (ii) the percentage recovery of Org GB 94 from the plasma in the extraction, purification and concentration procedure. The extreme sensitivity of the mass spectrometer in principle allows the detection of picogram amounts provided that the instrument is running under optimal conditions. In combination with a gas chromatograph, however, a decrease in sensitivity occurs owing to adsorption to the GC column packing material and disappearance of substance through the helium separator. Nevertheless, standard solutions containing 50 pg of Org GB 94 per injection yielded mass fragmentograms with a signal-to-noise ratio of better than 10:1.

The sensitivity is afforded by the relatively high abundance of the molecular ion of Org GB 94, as shown in Fig. 3. The use of the base peak at *m/e* 193 for quantitation should improve the sensitivity to only a small extent. In that event, however, the specificity is lost because ions with *m/e* 193 are also common fragments for Org GB 94 metabolites.

The percentage recovery in the sample working-up procedure was measured by processing plasma samples to which 1 ng of [^{14}C]Org GB 94 had been added. It was found that the extraction yield was almost 100% and that the HPLC purification was quantitative. Losses of 10—20% were observed during the various sample transfer, concentration and evaporation steps. Although in many instances amounts of less than 1 ng can be quantified (depending on the conditions of the equipment), for routine analyses of 1-ml samples with adequate accuracy and precision a level of 1 ng/ml in plasma is considered to be the lower limit for reliable measurements. When even lower levels are to be analyzed, decreased accuracy and precision must be accepted or larger plasma samples should be processed (permitting quantifications down to ca. 0.25 ng/ml with a 4-ml sample).

Specificity

The specificity of the method is obtained by the combination of all four successive steps in the analytical procedure: the *n*-hexane extraction, the HPLC purification, the GC separation and the MS detection. Analyses of blank plasma samples revealed vanishingly small peaks in the MF traces at *m/e* 264 and *m/e* 266 with Org GB 94 GC retention times. Moreover, of all known Org GB 94 metabolites, only the N-oxide shows a peak at *m/e* 264 in the mass spectrum and, owing to the decomposition into Org GB 94 at elevated temperatures, similar GC retention time. The N-oxide, however, cannot be extracted from plasma with *n*-hexane and consequently does not interfere in the determination. The specificity of the assay is automatically controlled during the detection by coincident onset and duration of both peaks in the mass fragmentogram*.

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*Minute differences in GC retention times of compounds and their deuterated analogues have been reported for compounds deuterated in the vicinity of a polarity centre; with Org GB 94, such differences were not observed.

Capacity

The method described permits routine measurements with a capacity of ca. 60 samples a day. The capacity can be improved by automation of HPLC and GC injection, automatic collection of the HPLC fractions and computerized digitization of the mass fragmentogram peaks.

The applicability of the assay method to the pharmacological and pharmacokinetic evaluation of the drug was illustrated by the measurement of plasma levels in patients after 2, 4 or 6 weeks of treatment with 60 mg of Org GB 94 per day and of plasma levels as a function of time after a single oral dose to male volunteers.

The steady-state plasma levels shown in Table II display a considerable inter-patient variation in the range 4—98 ng/ml, which is covered by the sensitivity of the assay. The individual levels, however, were remarkably constant during the 6 weeks of treatment, as reflected by the constant mean plasma levels over this period: 38, 36 and 34 ng/ml after 2, 4 and 6 weeks of treatment respectively.

The single-dose plasma levels in Table III demonstrate that the sensitivity of the assay is sufficient to monitor Org GB 94 in plasma from 1 h up to 24 h after a single dose of 15 mg. From these results, it can be concluded that the method permits measurements during at least 24 h following an oral dose of 15 mg, which is a prerequisite for acceptable pharmacokinetic analyses.

TABLE II

INDIVIDUAL AND MEAN EARLY MORNING Org GB 94 PLASMA LEVELS IN DEPRESSED PATIENTS AFTER 2, 4, AND 6 WEEKS OF DAILY TREATMENT WITH 3 × 20 mg OF Org GB 94

Patient	Org GB 94 plasma levels (ng/ml; free base) after treatment for		
	2 weeks	4 weeks	6 weeks
G.H.	23.6	24.3	23.9
M.Mr.	14.4	14.5	4.3
H.O.	22.1	29.9	31.4
E.T.	20.3	28.9	26.5
W.F.	98	57	52
N.L.	21.7	20.8	19.0
V.G.	35.2	—	—
R.L.	32.8	34.5	33.9
R.C.	41.3	60	45.6
M.Ms.	36.3	32.3	27.8
D.K.	15.4	16.5	22.9
M.S.	56	49.4	43.0
K.O.	28.5	23.6	20.4
J.M.	21.5	15.5	20.4
D.T.	69	64	60
S.G.	28.1	28.5	29.0
V.H.	35.8	44.2	48.7
G.S.	84	69	65
Mean ± S.D.	38 ± 24	36 ± 18	34 ± 16

TABLE III

Org GB 94 PLASMA LEVELS AFTER A SINGLE DOSE OF 15 mg OF Org GB 94 TO FOUR MALE VOLUNTEERS IN TWO SESSIONS

Subject	Session	Org GB 94 (free base) plasma levels as a function of time following administration (ng/ml)						
		25 min	1 h	2 h	3 h	5 h	7 h	24 h
J.G.	1	0.6	13.2	19.4	13.2	9.2	7.4	1.6
	2	0.4	7.6	19.5	18.4	11.7	8.8	2.3
M.P.	1	0.6	4.9	10.2	8.6	6.3	4.8	2.6
	2	1.5	6.5	11.1	10.9	8.6	5.1	1.1
M.G.	1	0.6	3.3	6.5	5.6	5.1	3.4	1.1
	2	0	0.5	8.1	8.9	7.4	6.4	1.4
S.K.	1	0.1	4.0	10.8	8.7	6.1	4.4	1.3
	2	0.3	1.0	11.8	11.4	7.5	5.2	1.8

CONCLUSIONS

The method described for the assay for Org GB 94 in human plasma, based on HPLC purification and MF quantitation, meets the requirements for routine analyses for evaluating clinical experiments and for monitoring patients' drug kinetics.

ACKNOWLEDGEMENTS

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