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QUANTITATIVE DETERMINATION OF TIFLOREX IN HUMAN FLUIDS USING ELECTRON-CAPTURE DETECTION

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SUMMARY

A procedure is described for the determination of tiflorex and its metabolite nortiflorex in biological specimens. The compounds are converted into their trichoroacetyl derivatives, which are separated on a glass column packed with 3% OV-17 on Gas-Chrom Q, and measured with an electron-capture detector. The mechanism was investigated by gas chromatography–mass spectrometry. The method is rapid, sensitive for concentrations of 1 ng/ml and has been used to measure tiflorex and its metabolite in rat plasma after intravenous administration and in human volunteers after administration by the oral route.

INTRODUCTION

Tiflorex* [± 1-(3'-trifluoromethylthiophenyl)-2-ethylaminopropane] (Fig. 1) is a new synthetic molecule that has been shown to produce a definite effect on hunger in volunteers without inducing anxiety or altering the mood[1,2]. In rats, the compound appears to be twice as potent as fenfluramine in reducing food intake[3] and also significantly reduces intestinal absorption of triglycerides[4]. In human isolated striatal muscle, tiflorex increases glucose uptake[5]. Observations in volunteers have also shown that the compound does not modify the sleep pattern and that when given as a slow-release formulation it has no effect on the cardiovascular system[2].

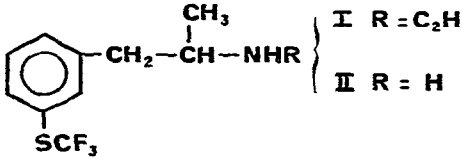

Fig. 1. Structural formulae of tiflorex (I) and nortiflorex (II).

* Previously known as Flutiorex.

Preliminary pharmacokinetic studies by Giudicelli and co-workers[6,7] using gas chromatography (GC) with flame-ionization detection, showed that the compound and its de-ethylated metabolite nortiflorex [± 1-(3′-trifluoromethylthiophenyl)-2-aminopropane] are present in blood at very low concentrations; in several instances the compound was present at concentrations below 5 ng/ml.

Giudicelli and co-workers' method had limited sensitivity and required large plasma samples (10 ml), and was therefore unsuitable for accurate kinetic studies. We therefore developed a more sensitive GC procedure based on trichloroacetylation of the molecule[8], with the use of an electron-capture detector (ECD), which permits a sensitivity of 1 ng/ml with a 1-ml plasma sample.

EXPERIMENTAL

Chemicals and reagents

Tiflorex hydrochloride and nortiflorex hydrochloride were synthesized in our laboratory and fenfluramine hydrochloride was a gift from Dr. Frigerio of the Mario Negri Institute (Milan, Italy). Toluene (chromatographic grade) and sodium hydroxide were obtained from Merck (Darmstadt, G.F.R.), trichloroacetyl chloride from Fluka (Buchs, Switzerland) and trimethylchlorosilane (TCAC) from Applied Science Labs. (State College, Pa., U.S.A.).

Stock solution. Standard solutions of tiflorex (base) (0.5 μg/ml), nortiflorex (base) (0.5 μg/ml) and the internal standard, fenfluramine (1 μg/ml), were prepared in double glass-distilled water and kept at 4°. Under these conditions the solutions were stable for up to 3 weeks.

Calibration graphs and quantitation

Standard graphs for tiflorex and nortiflorex were prepared by adding 2.5, 5, 10, 15 and 25 ng of tiflorex, 2.5, 5, 10, 15 and 25 ng of nortiflorex and 20 ng of fenfluramine to 0.5 and 2 ml of blank plasma. The samples for both standard graphs were extracted according to the method described below, and the extracts were derivatized by heating with TCAC at 80° for 1 h and then chromatographed.

The ratio of the peak areas of tiflorex to fenfluramine and of nortiflorex to fenfluramine were used to calculate response factors, from which the amounts of the drugs in the unknown samples were then calculated using a Perkin-Elmer PEP-2 data system.

Extraction procedure

All glassware was previously silanized with a 1% solution of trimethylchlorsilane in toluene, then washed twice with methanol and finally with acetone. A 20-ng amount of fenfluramine (20 ng ≡ 20 μl of the aqueous solution) as internal standard, 2–3.5 ml of distilled water, 100 μl of 5 *N* sodium hydroxide solution and 5 ml of toluene were added to either 0.5 or 2 ml of plasma in a 20-ml test-tube. The test-tubes were gently shaken on a rotating shaker for 30 min. After centrifugation at 4° for 5 min at 1000 *g*, 4.5 ml of the organic phase were transferred into a second series of test-tubes and 50 μl of a 0.1% solution of trichloroacetyl chloride in toluene (prepared immediately before use) were then added. The test-tubes were capped and heated at 80° for 1 h.

The toluene solution was then concentrated to 0.5 ml under a gentle stream of nitrogen. After cooling at room temperature, 1–2 μl of the solution was injected into the gas chromatograph.

For blood analysis, red cells were haemolysed by freezing and the amount of water added to these samples was always twice the sample volume. Otherwise the procedure was the same as for plasma.

Gas–liquid chromatographic conditions

The analysis was performed under isothermal conditions on a Perkin-Elmer Model 3920 B gas chromatograph equipped with a nickel-63 linear electron-capture detector (ECD) operating at −55 V (pulse current) with a width of 250 nsec. The glass column (2 m × 4 mm I.D.) was packed with 3% OV-17 on Gas–Chrom Q (80–100 mesh) and was conditioned for 1 h at 270° (40 ml/min nitrogen flow-rate), 4 h at 320° (no nitrogen) and 24 h at 280° (40 ml/min nitrogen flow-rate). The column temperature was 210°, injector port temperature 250° and interface and detector temperatures 275°. The carrier gas (nitrogen) flow-rate was 50 ml/min.

Mass spectrometric (MS) conditions

An LKB 9000 GC–MS mass spectrometer was used. Spectra were obtained under the following conditions in the electron-impact mode: electron energy, 70 or 12 eV; trap current, 60 μA; accelerating voltage, 3.5 kV, ion source temperature, 290°; molecular separator temperature, 290°.

For GC introduction, the same column was used as described above under *Gas–liquid chromatographic conditions*, but the helium flow-rate was reduced to 20 ml/min.

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms of extracts from plasma and red cells. No interfering peaks from endogenous substances were found in samples obtained from rats or human volunteers and peaks corresponding to the three substances are well resolved. Retention times were 4.6 min for fenfluramine, 5.8 min for nortiflorex and 8.6 min for tiflorex, and the heights equivalent to a theoretical plate (HETP) were 0.59, 0.61 and 0.59 mm for fenfluramine, nortiflorex and tiflorex, respectively. The resolution factors were 3.1 for fenfluramine–nortiflorex and 8.2 for fenfluramine–tiflorex. The minimum detectable amounts in plasma were 0.5 ng/ml for nortiflorex and 1 ng/ml for tiflorex.

Calibration graphs are shown in Fig. 3 for (a) tiflorex and (b) nortiflorex. These graphs were prepared by plotting the ratios of the peak areas of the drugs to the internal standard against known amounts of the drug added to the biological specimens. The calibration graphs in Fig. 3 cover the concentration range 0–25 ng/ml, which is the range found in man after therapeutic administration. Another calibration graph, prepared for determining tiflorex in plasma and red cells of the rat, was linear from 25 to 200 ng/ml.

The reproducibility of the method was checked by quadruplicate analyses of plasma samples to which known amounts of tiflorex had been added. As can be seen from the results in Table I, the greatest deviation (*ca.* 14%) occurs at the lowest con-

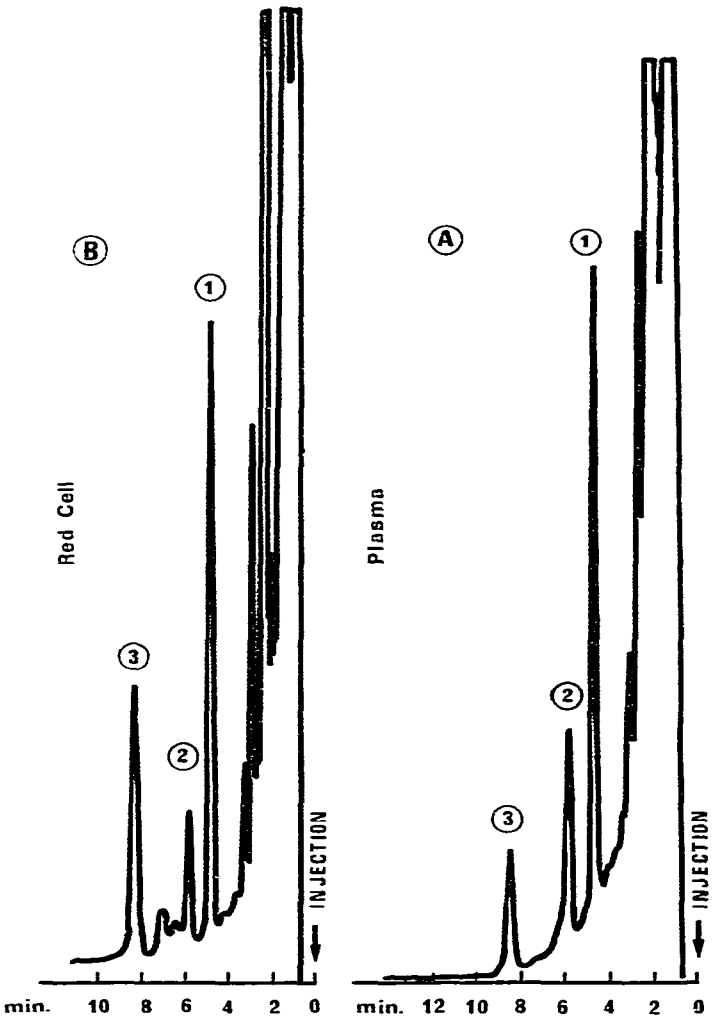

Fig. 2. Gas chromatograms of the trichloroacetyl derivatives of tiflorex (3), nortiflorex (2) and fenfluramine (1) extracted from plasma (A) and red cells (B).

centrations; the error was less at the higher concentrations. The same range of variability (3–5%) was found for the calibration graph for concentrations ranging from 25 to 200 ng/ml.

GC–MS analysis of tiflorex using the electron-impact mode at 70 eV gave a mass spectrum with an apparent molecular ion with *m/e* 262. This corresponds to the M—1, ion which is probably formed by loss of H$^{\cdot}$. The base peak is represented by the ion at *m/e* 72, produced by cleavage α,β to the nitrogen atom; the complementary fragment containing the aromatic ring is at *m/e* 191. It was not possible to obtain a spectrum containing the molecular ion even when the energy in the source was reduced to 12 eV.

The mass spectrum of the tiflorex derivative prepared by reaction with TCAC

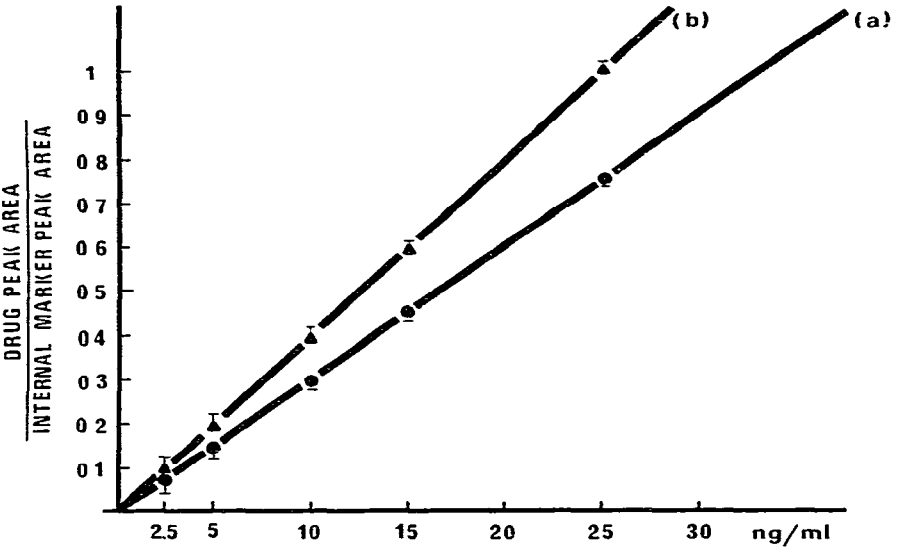

Fig. 3. Calibration graphs for trichloroacetyl derivatives of tiflorex (a) and nortiflorex (b), with standard deviations, obtained after extraction from plasma.

is similar to that for tiflorex. There appears to be only one extra peak, of low relative intensity, at *m/e* 264 (M + 1). However, because the retention time of the derivative when chromatographed is lower than that of tiflorex and because the response of the ECD is greater, it is reasonable to assume that tiflorex chromatographs as the trichloroacetyl derivative after reaction with TCAC.

GC–MS analysis of nortiflorex under similar conditions gave mass spectra analogous in all respects. An apparent molecular ion was observed at *m/e* 234 (M−1) and, after derivatization with TCAC, an M + 1 ion of low intensity was present at *m/e* 236. It is therefore also assumed that the trichloroacetyl derivative of nortiflorex was chromatographed in these studies.

TABLE I

CONCENTRATION OF TIFLOREX FOUND IN PLASMA FOR KNOWN AMOUNTS OF THE DRUG ADDED

In each case four determinations were made. The largest variation is found at the limit of sensitivity of the method.

Sample No.	*Amount added to plasma (ng/ml)*	*Amount detected (ng/ml)*	*Mean ± S.D. (ng/ml)*	*Coefficient of variation,* $\frac{S.D.}{Mean} \cdot 100$ *(%)*
1	2.5	2.2, 2.0, 2.3, 2.8	2.2 ± 0.3	14
2	5	4.5, 4.9, 5.4, 6.0	5.2 ± 0.6	12
3	10	9.5, 9.8, 10.2, 10.6	10 ± 0.5	5
4	15	14 , 14.6, 14.8, 15.7	14.7 ± 0.7	4.7
5	25	23 , 24.2, 25.5, 25.7	24.6 ± 1.25	5
6	50	48 , 49.5, 50.5, 52.5	50.1 ± 1.9	3.8

The efficiency of the derivatization reaction was established by checking the consistency of the GC response after different periods of reaction with trichloroacetyl chloride. Although the reaction was almost complete after an incubation of 30 min, we preferred to prolong the reaction time to 1 h.

The derivatization of a molecule that already contains three atoms of fluorine allows a greater sensitivity and therefore better detection by the ECD. Although the retention times of tiflorex and nortiflorex derivatives were increased to 8.60 and 5.80 min, respectively, at 210° instead of 6.80 and 5.25 min at 195° for the underivatized compounds, the sensitivity was doubled and the resolution between tiflorex and nortiflorex was greatly improved (see Fig. 4). The derivatization procedure was therefore preferred.

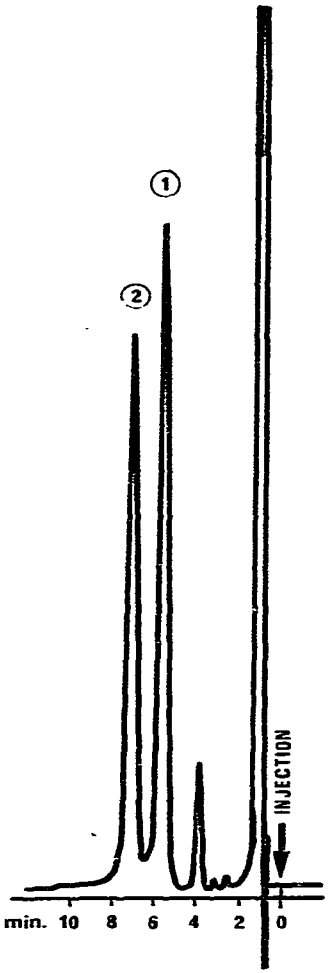

Fig. 4. Gas chromatogram of tiflorex (2) and nortiflorex (1) (bases) injected at 195° without derivatization.

For both compounds, as well as for the internal standard, the recovery was very close to 90% (90 ± 1.5%). The derivative is very stable and no degradation could be observed even when the samples were left for 2 weeks at 4°.

The method has been employed for the determination of tiflorex and nortiflorex in the blood of rats and humans. Plasma and red cell levels of tiflorex and nortiflorex found in rats after an intravenous injection of 10 mg/kg of tiflorex are shown in Table II; tiflorex had an apparent half-life of about 55 min in plasma and the cell to plasma ratio for tiflorex after initial phase of equilibration was between 3:1 and 5:1, confirming previous results by Giudicelli *et al.* obtained in man[3]. Nortiflorex appeared in significant amounts in rat plasma within 10 min after an injection at concentrations similar to that of the parent drug. Its penetration in the red cells, however, appeared to be slower than that of the parent compound.

TABLE II

PLASMA AND RED CELL CONCENTRATIONS (ng/ml) OF TIFLOREX (TF) AND NORTIFLOREX (NTF) IN SPRAGUE DAWLEY RATS (BODYWEIGHT 200 ± 10 g) AFTER I.V. ADMINISTRATION OF TIFLOREX HYDROCHLORIDE (10 mg/kg)

Rat No.	*Time after i.v. administration (min)*							
	10		*30*		*60*		*100*	
	TF	*NTF*	*TF*	*NTF*	*TF*	*NTF*	*TF*	*NTF*
Plasma 1	640	710	294	246	214	166	108	26
Plasma 2	790	574	328	150	132	138	78	11
Plasma 3	580	730	318	270	118	200	80	13
Mean ± S.E.	670 ± 62	671 ± 49	313 ± 10	222 ± 36	155 ± 30	135 ± 39	89 ± 10	17 ± 5
Red cell 1	1812	100	1550	137	762	130	445	172
Red cell 2	2250	150	1212	106	500	85	280	140
Red cell 3	1525	112	1125	118	585	115	260	70
Mean ± S.E.	1862 ± 210	120 ± 15	1295 ± 129	120 ± 9	614 ± 77	110 ± 13	328 ± 58	127 ± 30

TABLE III

PLASMA CONCENTRATIONS (ng/ml) OF TIFLOREX AND NORTIFLOREX IN TWO HEALTHY VOLUNTEERS AFTER ADMINISTRATION OF TIFLOREX HYDROCHLORIDE (20 mg)

Subject	*Body weight (kg)*	*Compound*	*Time after administration (h)*											
			1	*1.5*	*2*	*2.5*	*3*	*3.5*	*4.5*	*6*	*8*	*12*	*24*	*27*
G.B.	75	Nortiflorex	N.D.*	N.D.	N.D.	N.D.	N.D.	N.D.	1.2	2	2.5	3	5	3.5
		Tiflorex	N D	N.D.	N.D.	1	4	2.8	8	6	7	9	7.5	6.2
P.M.S.	82	Nortiflorex	N.D.	N.D.	1	2.5	3	3.5	4	6	5	6.5	11.5	11
		Tiflorex	0.5	0.8	4	6.5	8.5	9.5	13	13	13	12	8	7

* N.D. = not detected.

Oral administration of 20 mg of tiflorex hydrochloride, in a slow-release form, to two volunteers fasted overnight, gave the plasma concentrations shown in Fig. 5. Maximal plasma tiflorex concentrations of 8–13 ng/ml were achieved at 4 and 5 h and were then followed by a plateau of up to 24 h. The nortiflorex time course differed

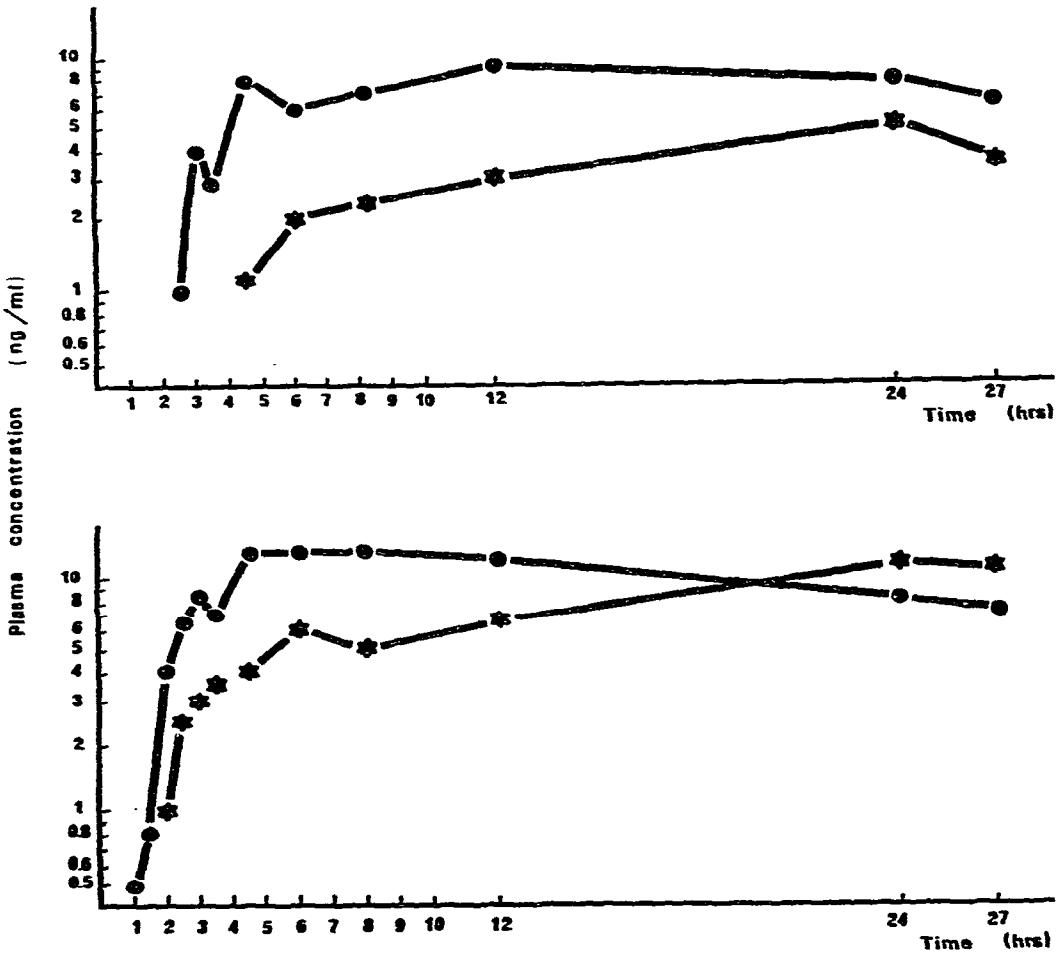

Fig. 5. Plasma concentrations of tiflorex (●) and nortiflorex (✳) in two subjects after administration of 20 mg of tiflorex in a slow-release formulation.

significantly between the two subjects and its formation was slower than that observed in rats. In man the red cell to plasma ratio for tiflorex was also of the order of 4–6.

The analytical procedure described here for tiflorex and its metabolite nortiflorex seems very suitable for both single-dose pharmacokinetic studies and the monitoring of drug plasma levels during repeated dose treatment. Further, its simplicity, sensitivity and specificity and the small amount of plasma required for analysis are considerable advantages over the previously existing method.

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