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The Measurement of Diphenoxylic Acid in Plasma following Administration of Diphenoxylate

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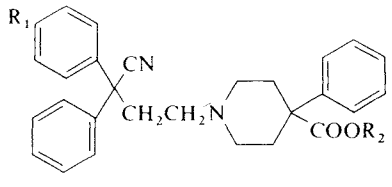
Abstract—The measurement of diphenoxylic acid, the major metabolite of diphenoxylate, has been performed in man using a deuterium labelled internal standard and multiple ion monitoring using a gas chromatograph mass spectrometer. The determination of plasma concentrations following administration of diphenoxylate to man is described.

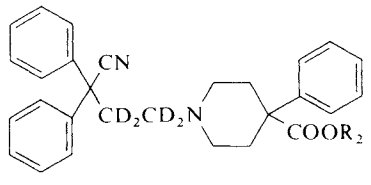
Introduction

DIPHENOXYLATE hydrochloride [1-(3-cyano-3,3-diphenyl propyl)-4-phenylpiperidin-4-carboxylic acid ethyl ester hydrochloride, **1a**] occupies an important place in the treatment of diarrhoea in man.[1–3] From earlier studies in both animals[4,5] and man[6] it was shown that the major metabolite in plasma was diphenoxylic acid (**1c**) and that considerable quantities of the acid were excreted in urine. It thus appeared of importance to measure diphenoxylic acid in plasma following administration of a standard solution of diphenoxylate and a similar dose of diphenoxylate as tablets, in order to assess the comparative availability from the two dosage forms.

This paper describes a method which was developed and evaluated for the measurement of diphenoxylic acid in plasma using its tetradeutero analogue (**2c**) as an internal standard.



1; **3**



2

1: R_1 = H
3: R_1 = Cl

a: R_2 = Et
b: R_2 = Me
c: R_2 = H

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Experimental

All the reagents used were of Analar grade from Hopkin and Williams. Etheral solutions of diazomethane were prepared from Diazald (Ralph N. Emmanuel, Ltd). A Finnigan 3200E gas chromatograph mass spectrometer with a chemical ionization (c.i.) source on-line to a Finnigan 6000 data system was used for g.c.m.s. analyses. Gas chromatography was performed on a 50 cm × 3.0 mm i.d. glass column packed with 1% Dexsil 300 GC on gas chrom Q 100/120 mesh (Phase Separations Ltd). Methane was used as carrier gas at a flow of 20 ml/min. The injector was kept at 300 °C and the column at 270 °C. The c.i. source was kept at 110 °C and source pressure of methane was in the range 800–1000 microns.

PREPARATION OF DEUTERODIPHENOXYLIC ACID

Deuterium labelled diphenoxylic acid was prepared by reacting diphenylacetonitrile with 1,2-dibromo-1,1,2,2-tetradeuteroethane (NMR Ltd, Bledlow Ridge, High Wycombe, Bucks., England) to yield 4-bromo-2,2-diphenyl-3,3,4,4-tetradeuterobutyronitrile. The bromonitrile was reacted with 4-phenylpiperidin-4-carboxylic acid ethyl ester to give tetradeuterodiphenoxylate (**2a**) n.m.r. (60 MHz) τ2.7 m (15H) 5.9 q (2H, J = 7 Hz), 6.2–7.7 m (8H), 8.85 t (3H, J = 7 Hz). Diphenoxylate (**1a**) contains an additional signal at τ6.10 integrating for four protons. Mass spectrum (**2a**) (c.i. using methane as reactant gas): *m/e* 458 (42), 457 (100), 248 (61). Tetradeuterodiphenoxylic acid (**2c**) was obtained from the ester by hydrolysis with methanolic potassium hydroxide.

EXTRACTION PROCEDURE

An aliquot of plasma (1 ml) was diluted with water (1 ml) and deuterodiphenoxylic acid (10 μg) in methanol (0.1 ml) added. The pH was adjusted to 8 with aqueous sodium hydroxide solution (1 M) and the mixture extracted with hexane (3 × 3 ml). The hexane extracts were discarded, the aqueous phase was acidified with hydrochloric acid solution (1 M) to pH 3 and then extracted with chloroform (2 × 5 ml). The combined

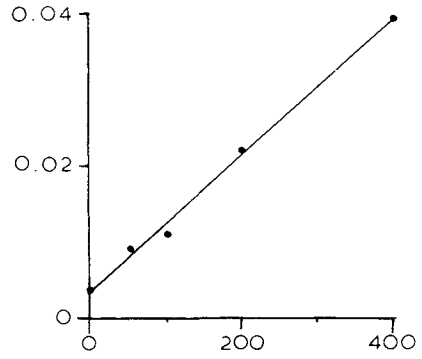

FIG. 1. Standard calibration curve for diphenoxylic acid in plasma, adding tetradeuterodiphenoxylic acid (10 μg) as internal standard.

chloroform extracts were taken to dryness and methylated with an etheral solution of diazomethane overnight. The solution was evaporated and the residue taken up in methanol (20 μl). Aliquots (3–5 μl) were injected onto the gas chromatograph. Calibration curves were drawn up using plasma containing known amounts of diphenoxylic acid. A straight calibration from 50–400 ng ml^{-1} was obtained (Fig. 1). Regression analysis gave the equation of the line as $y = 0.342 + 0.009x (p = 0.99)$. Controls containing known amounts of diphenoxylic acid were run with each batch of samples in a random manner as a check on the assay.

The coefficient of variation and standard error of the mean were calculated for a number of replicate injections (Table 1). Both the methyl ester analogue of

TABLE 1. Coefficient of variation and standard error for standard solutions of diphenoxylic acid in plasma

Sample	Concn ng ml^{-1}	Mean Ratio[a]	n	Coeff. var. %	S.E.
1	0	0.35	6	25.4	0.036
2	50	0.89	5	13.0	0.032
3	100	1.08	5	9.7	0.030
4	200	2.18	6	3.4	0.026
5	400	3.93	4	2.8	0.050

[a] Mean ratio = area m/e 439/area m/e 443.

diphenoxylate and its tetradeutero derivative gave $[M + 1]^+$ as the base peak in their c.i. spectra (Fig. 2). These ions (m/e 439 and 443, respectively) were monitored and the ratio of peak areas used to calculate the quantity of the methyl ester analogue of diphenoxylate. Although the pseudomolecular ions have nominal masses m/e 439, 443, four ions at m/e 439.3, 439.4, 443.3 and 443.4 were monitored using the Finnigan 6000 data system. Careful inspection of the peak height ratios for the ion pairs 439.3, 439.4; and 443.3, 443.4, enabled correction for instrument drift to be made thus optimizing sensitivity.

Blank plasma samples containing no internal standard showed no interfering peaks at m/e 439 and 443. Interfering peaks were visible at m/e 232, the base peak in the e.i. spectrum.

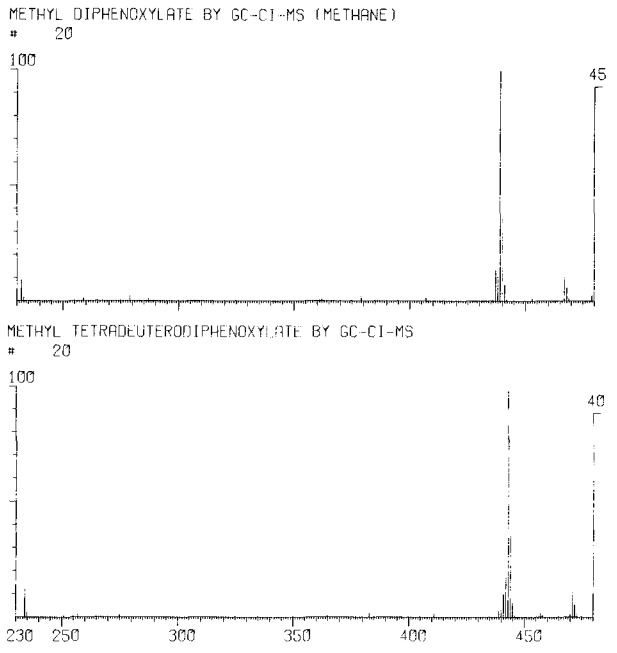

FIG. 2. The partial c.i. spectra (m/e 230–480) of the methyl ester analogue of diphenoxylate and the corresponding tetradeutero compound.

Results

The method was used to measure diphenoxylic acid in the plasma of six male volunteers following treatment with diphenoxylate hydrochloride tablets (four tablets each containing 2.5 mg) and a standard solution (containing 10 mg) on a random cross-over basis. Although this work will be reported in detail elsewhere, results from one subject are shown (Fig. 3). The areas under the two curves were 810 ng h ml^{-1} for the tablet and 1490 ng h ml^{-1} for the solution, giving a comparative bioavailability of 54%. The availability of diphenoxylate from tablet or solution showed considerable inter-subject variation, but in all cases the tablet was less available.

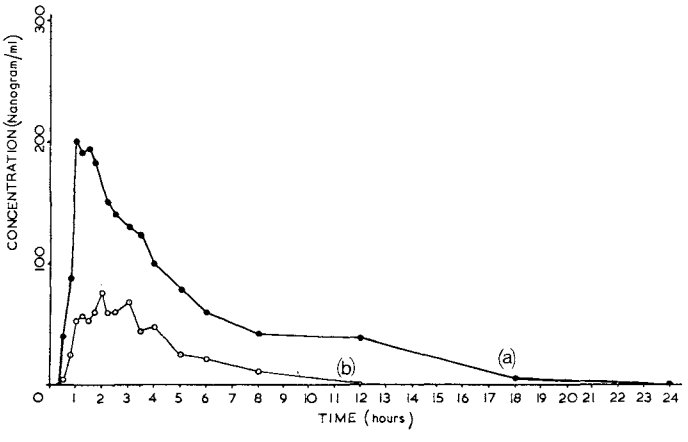

FIG. 3. Diphenoxylic acid plasma concentrations after oral administration of diphenoxylate (10 mg) as (a) a standard solution or (b) four tablets.

Discussion

The measurement of diphenoxylic acid in plasma has not been accomplished previously, due to the relatively insensitive methods available. The most sensitive gas chromatographic method[7] using chlorodiphenoxylic acid (**3**) [1-(3-4-chlorophenyl-3-cyano-3-phenyl propyl)-4-phenylpiperidin-4-carboxylic acid] as an internal standard has been used successfully to measure the urinary excretion of diphenoxylic acid. However, its limit of detection (about 200 ng ml^{-1}) is not sufficiently low to measure the levels of diphenoxylic acid in plasma.

The method described here utilizes tetradeuterodiphenoxylic acid both as an internal standard and as a carrier. By using a column sufficiently long to separate the solvent front, which is diverted to a backing pump, but which is short enough to minimize the thermal degradation of the methyl ester analogue of diphenoxylate, sufficiently high recoveries were obtained to allow detection down to about 10 ng ml^{-1}. Volatile fats and lipids which could contaminate the source were largely removed by the hexane washes. Involatile substances were trapped on the column. An experiment was performed to check the recovery of diphenoxylic acid. Diphenoxylic acid (10 μg) containing ^{14}C diphenoxylic acid (13 000 disintegrations min^{-1}) in plasma (1 ml) was extracted and recoveries at each stage of the extraction procedure were checked. The final chloroform extract contained 90% of the original radioactivity.

Low levels of diphenoxylate (<10 ng ml^{-1}) were detected in early plasma samples by monitoring the ion *m/e* 453 $[M + 1]^+$. No attempt was made to measure these as the levels were too low.

No peak for *m/e* 439 was observed in blank samples containing deuterodiphenoxylic acid other than that due to the small quantity of diphenoxylic acid (0.4%) present in the internal standard. A significant peak could be observed in the plasma of a volunteer dosed with diphenoxylate (Fig. 4). Using this assay plasma levels down to 20 ng ml^{-1} can be measured and useful pharmacokinetic data obtained.

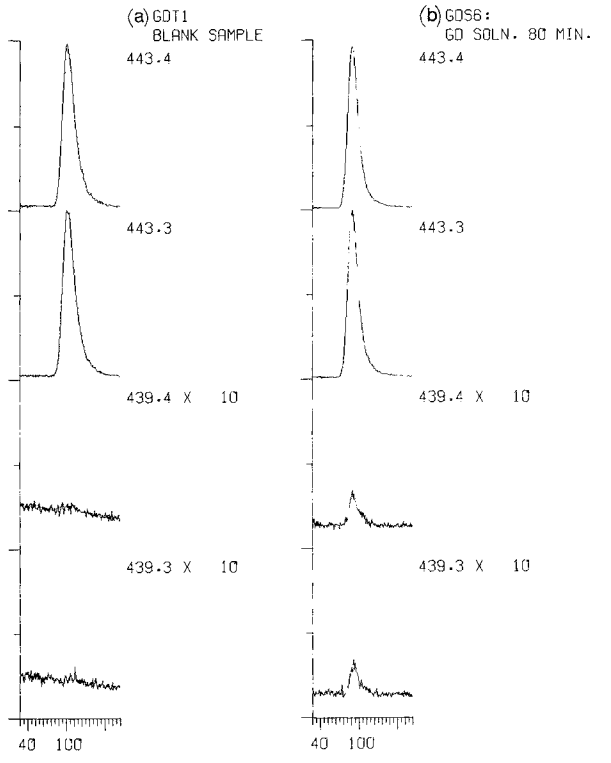

FIG. 4. Multiple ion recordings of plasma extracts taken (a) pre-dose and (b) 80 min after dosing with diphenoxylate solution. The traces for *m/e* 439.3, 439.4 are multiplied by 10.

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