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Note

Fluorescence assay of citalopram and its metabolites in plasma by scanning densitometry of thin-layer chromatograms

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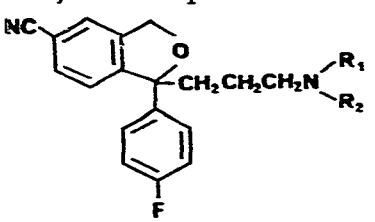
The drug citalopram (Fig. 1, I), Lu 10-171, is a specific, potent serotonin re-uptake inhibitor [1—4], now under clinical trials as an antidepressant [5].

Pharmacokinetic studies have been performed in man [6, 7] and in various safety-related animal trials, one of which has been published [8]. The analytical technique used in these studies was based on the isolation and purification of citalopram (I) and its demethylated metabolite (II) by extraction and thin-layer chromatography (TLC) followed by fluorimetric quantitation by ion-pair formation with a fluorescent anion [6]. The method was fairly reproducible (standard deviation ca. 10%) and sensitive (limit of detection 20 ng of citalopram in 2-ml plasma samples), but lengthy. In addition, quantitation of the demethylated metabolite (II) was less reliable and less sensitive. Assay of the didemethylated metabolite (III), present in both animals and man, was not possible.



		R_1	R_2	
Citalopram	(I)	CH_3	CH_3	
Monodesmethyl metabolite	(II)	CH_3	H	
Didesmethyl metabolite	(III)	H	H	
Citalopram N-oxide	(IV)	CH_3	CH_3	$-N^{+}-O^{-}$

Fig. 1. Formulae of citalopram (I), a potent and specific serotonin re-uptake inhibitor, and its metabolites.

For these reasons, efforts have been made to improve the assay, especially with regard to quantitation of the metabolites present and sample throughput. A method involving direct scanning of the native fluorescence of drug and metabolite spots on the thin-layer plate is described here and compared with the previous technique.

EXPERIMENTAL

Extraction procedure

Plasma samples (2.0 ml) were adjusted to pH 10 with 100 μl of 1 *N* sodium hydroxide solution. As standards were added in ethanol solution, all samples were diluted with 75 μl of ethanol. The samples were extracted twice with 6-ml portions of *n*-hexane containing 1% of triethylamine by mechanical shaking for 10 min in stoppered glass tubes, after which the samples were centrifuged and placed in a dry-ice—ethanol mixture. The aqueous phase was frozen and the *n*-hexane phase transferred into 10-ml conical glass tubes and evaporated to dryness under a stream of air in a 50°C water-bath.

For each set of sixteen samples, four standards (0, 25, 100 and 250 ng of I, II and III added to drug-free plasma) were included.

Thin-layer chromatography

The residues were dissolved in 50-μl portions of chloroform by vigorous shaking and transferred together with a 50-μl rinse by autopipette into Sanz micro-tubes, ready for application onto 0.25 mm thick, pre-coated HPTLC silica gel plates without a fluorescence indicator (Merck, Darmstadt, G.F.R.).

Twenty samples were applied on a 10 × 20 cm plate with a Desaga Autospotter as spots of diameter about 2.5 mm. In addition, a reference mixture containing I, II and III was applied on each side of the plate. Ascending chromatography was then performed with dichloroethane—ethyl acetate—ethanol—acetic acid—water (15:26:12:8:7.5); the development time was about 45 min for a height of 70 mm. This system ensures the separation of citalopram (I) (R_F = 0.43) and its monodemethylated (II) (R_F = 0.51) and didemethylated (III) (R_F = 0.58) metabolites from each other and from endogenous impurities. The plates were dried in an oven at 80°C for 10 min. The references on the margins were made visible by spraying with Dragendorff reagent (No. 87 [9] after covering the sample part of the plate.

Florescence scanning

The intrinsic fluorescence of citalopram (I) and its metabolites (II and III) was then quantitated using a Perkin-Elmer MPF-3L fluorescence spectrophotometer, equipped with a TLC scanning device. The excitation and emission wavelengths were 240 and 295 nm, respectively. Slits of 12 nm were used with a sensitivity setting of 1; the background was corrected for by zero suppression. Sensitivity and zero suppression settings were chosen so as to provide a stable baseline (Fig. 2). The back face of the plate was wiped off with water and ethanol prior to scanning, which was performed along the path with a 3 × 6 mm slit at a scanning speed of 50 mm/min after optimization of the fluorescence of the spot.

Calculation

Amounts of drug and metabolites were calculated from calibration graphs generated by linear regression analysis of the peak height versus amount of standard (normally 0, 25, 100 and 250 ng of each compound, added to drug-free plasma prior to extraction and analysed on each plate). Two plates were normally run simultaneously and a common calibration graph was constructed.

RESULTS AND DISCUSSION

Calibration graphs

The calibration graphs obtained (Fig. 2) showed that the fluorescence emitted was linear with concentration of drug or metabolite in the range 10—300 ng and was identical for the three compounds. Fig. 2 shows regres-

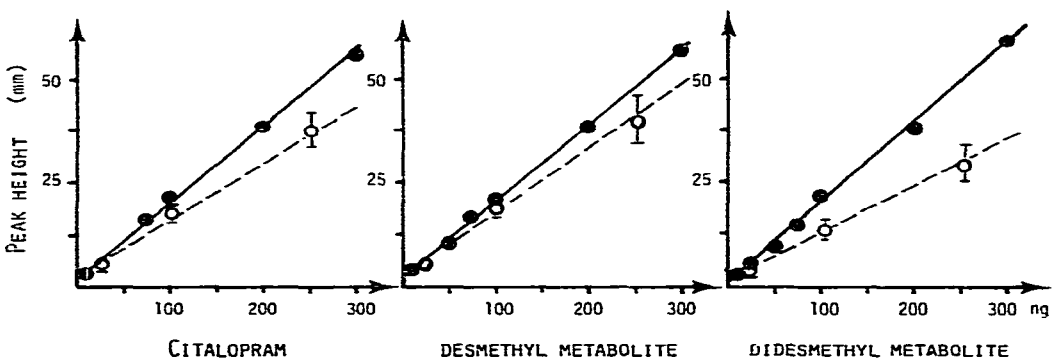


Fig. 2. Fluorescence of citalopram and its metabolites after application directly on HPTLC plates (•, single values) and after addition to drug-free plasma, extraction and TLC (○, mean ± S.D. from a series of 11 replicate experiments).

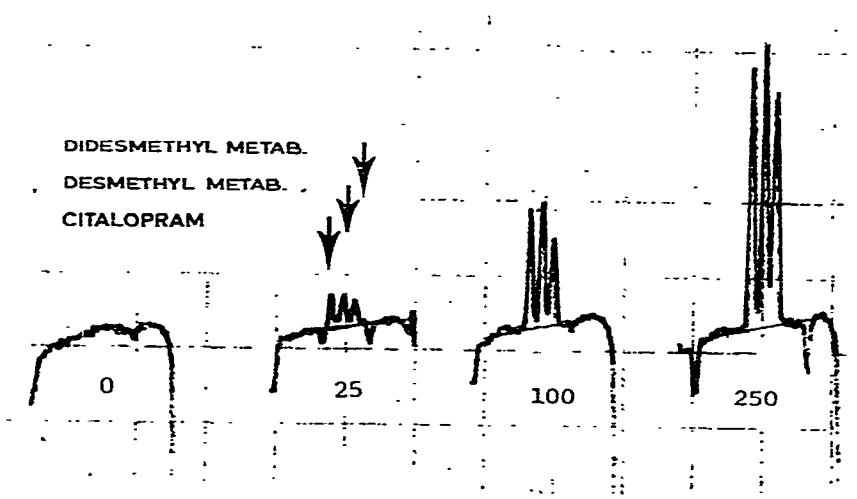


Fig. 3. Recorder trace from HPTLC scanning of fluorescence of citalopram and its mono- and didemethylated metabolites added to drug-free plasma in amounts of 0, 25, 100 and 250 ng.

sion lines for directly chromatographed standards and also for standards added to control (drug-free) plasma and assayed after extraction and chromatography. Typical chromatograms of authentic standards added to blank plasma are shown in Fig. 3. The correlation coefficients for the calibration lines were generally 0.99. With few exceptions they were reproducible from day to day or plate to plate as indicated (Fig. 2) by the variation of the average values from eleven replicate assays. The relative standard deviations were 10—15% for citalopram and 10—20% for the metabolites.

The recovery of citalopram and its demethylated metabolite (II) was 80—90%. The recovery of the more polar didesmethyl metabolite (III) was about 70%. Extraction of the more polar citalopram N-oxide (IV), identified in animals and man as a minor metabolite, was negligible.

Sensitivity and precision

The limit of detection, corresponding to peak heights of about 3 mm, was 10 ng. In 2-ml samples this corresponds to a concentration limit of about 5 ng/ml or 10—15 nmol/l.

The intra-assay variation as calculated from a series of duplicate determinations [10] of standards was 6—8% (standard deviation as a percentage of the mean) for 100- and 250-ng amounts of the three compounds, and higher (about 15%) at the 25-ng level. The duplicates were spotted on different plates, but otherwise processed simultaneously as is the routine procedure for standards and unknowns. From the calibration graph it can be calculated that a 6—8% variation in fluorescence corresponds to a 7—9% variation in concentration. This result agrees well with that from 25 identical samples (1.0 ml) containing 100 ng of citalopram (I), assayed frequently during several months, viz., 103 ± 12 ng/ml (mean ± S.D.).

Selectivity

The chromatographic separation of drug and extractable metabolites ensures complete selectivity in this respect. As regards potential interference from other drugs, only a few (hitherto encountered in clinical use) have been tested so far. Diazepam, nitrazepam, oxazepam, estazolam were all excluded on the plate. Dextropropoxyphene had an R_F value close to that of the didemethylated metabolite but showed no fluorescence at the given wavelength. In general one would expect little interference because of the TLC step and the specificity of fluorescence.

Advantages over previous technique

A comparison between the new and the previous technique was made by the assay of sixteen samples from a dog study at a sub-lethal dosage. The citalopram concentrations ranged from 1000 to 9000 nmol/l and the samples were diluted accordingly prior to assay. A single determination was performed with the previous technique and duplicates with the new scanning method. The correlation coefficient between the two methods was 0.93 and the regression line had a slope of 0.95 and an almost zero intercept (Fig. 4). The two methods were thus considered to produce identical citalopram data.

The advantages of the new assay are four-fold: greater simplicity, greater

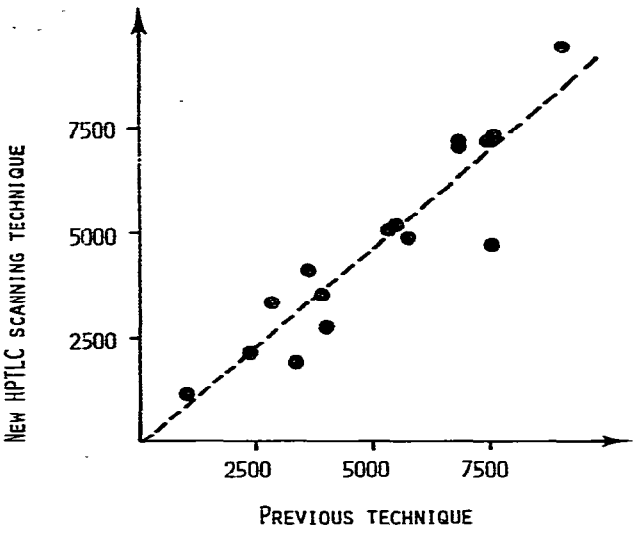


Fig. 4. Data from identical samples assayed by the coupling method (abscissa) and the new HPTLC method (ordinate). Concentrations in nmol/l.

accuracy, comprehensive metabolite assay and increased sensitivity. The method is considerably less time- and effort-consuming than the previous method, and 40 samples (including 8 standards) can be assayed by one assistant per day. Whereas with the coupling method the determination of the demethylated metabolite was less accurate and sensitive than that of citalopram, the scanning technique enables virtually the same accuracy and sensitivity for the drug and metabolites, including the didemethylated metabolite, which could not be determined by the previous technique. The limit of detection by the previous technique was stated [6] to be 10 and 25 ng/ml for citalopram and the demethylated metabolite, respectively. In comparison, the new scanning method is capable of detecting about 5 ng/ml of the drug and its metabolites. The improvement is thus limited for citalopram, but considerable for the metabolites. The sensitivity is satisfactory for clinical plasma levels and also for pharmacokinetic studies.

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