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SIMULTANEOUS DETERMINATION OF CLOMIPRAMINE AND ITS N-DESMETHYL METABOLITE IN HUMAN WHOLE BLOOD BY CAPILLARY GAS CHROMATOGRAPHY WITH MASS-SELECTIVE DETECTION

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SUMMARY

A method for the simultaneous determination of clomipramine and its N-desmethyl metabolite at concentrations down to ca. 2 nmol/l in human whole blood is desribed. After addition of a known amount of deuterium-labelled internal standards, compounds are extracted into *n*-heptane–isoamyl alcohol (99:1, v/v) at basic pH, back-extracted into an acidic aqueous solution and re-extracted at basic pH into *n*-heptane. N-Desmethylclomipramine and the internal standard are derivatized with pentafluoropropionic anhydride. The compounds are determined by capillary gas chromatography with mass-selective detection. The technique was applied to determine the human blood concentrations of clomipramine and its N-desmethyl metabolite after oral administration of Anafranil; mean blood concentrations are reported.

INTRODUCTION

Clomipramine, 3-chloro-5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine hydrochloride, the active ingredient of Anafranil® (Ciba-Geigy) is a tricyclic antidepressant agent.

Several methods have already been proposed for the quantitative assay of clomipramine and its N-desmethyl metabolite in biological fluids in a review [1]. Individual methods use the double-radioisotope derivative technique [2], liquid chromatographic procedures [3–6] and gas chromatography with nitrogen-selective detection [7,8]. The more sensitive methods were based on gas chromatography–mass spectrometry [9–13] with packed columns.

This paper describes a method using the extraction procedure previously published by Dubois et al. [9] but adapted to a Hewlett-Packard gas chromatograph and mass-selective detector with a fused-silica capillary column. The limits of

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quantitation in whole blood could be improved and are 2 nmol/l (1 ng/ml) for clomipramine and N-desmethylclomipramine.

EXPERIMENTAL

Chemicals and reagents

Clomipramine hydrochloride, N-desmethylclomipramine and the corresponding deuterium-labelled internal standard (Fig. 1) were supplied by Ciba-Geigy (Basle, Switzerland). The solvents and reagents used were all of analytical grade: *n*-heptane (Uvasol, Merck, Darmstadt, F.R.G.), isoamyl alcohol (Merck), pentafluoropropionic anhydride (Pierce, Rockford, IL, U.S.A.), pyridine (Pierce) and N,N-dimethylformamide (Merck). A solution of 2 *M* sodium carbonate, pH 11, (Merck) was used as buffer.

Equipment

A Hewlett-Packard 5890A gas chromatograph equipped with a capillary inlet system was used. The column was a 12.5 m×0.2 mm I.D. fused-silica capillary column coated with cross-linked methyl silicone (Hewlett-Packard 19091A, Op-

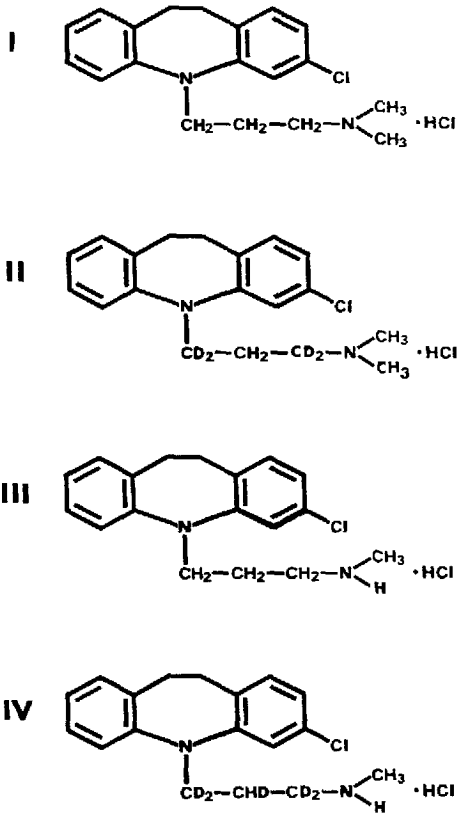


Fig. 1. Chemical structures of clomipramine (I), [$^2H_4$]clomipramine (II), N-desmethylclomipramine (III) and [$^2H_5$]N-desmethylclomipramine (IV).

tion 101). The carrier gas was helium at an inlet pressure of 48 kPa. Splitless injection was used, with a 0.30-min splitless period. The injection temperature was 250°C. The column was initially at 200°C for 0.5 min, and the temperature was then raised at a rate of 50°C/min up to 280°C.

A Hewlett-Packard 5970B mass-selective detector was interfaced with the gas chromatograph, with the capillary column inserted directly into the ion source. The interface was maintained at 280°C. The detector was calibrated with the Autotune® program at the beginning of each day using perfluorotributylamine.

The detector was turned on from 2.5 to 3.5 min after injection. The electron multiplier voltage applied was 400 V above the Autotune value.

Calibration curves

Aliquots of solutions of clomipramine hydrochloride and N-desmethylclomipramine hydrochloride in 0.01 *M* hydrochloric acid were added to 1 ml of human whole blood to produce reference samples in the concentrations range 1.99–159.40 nmol/l for clomipramine and 1.78–142.30 nmol/l for N-desmethylclomipramine. A constant amount of internal standards in 0.01 *M* hydrochloric acid was added to each reference sample: 42.15 pmol of [$^2H_4$] clomipramine and 32.12 pmol of [$^2H_5$] N-desmethylclomipramine.

Extraction

A 100-μl volume of the internal standards was measured into a glass tube, to which 1 ml of whole blood, 1 ml of alkaline buffer and 4.5 ml of *n*-heptane–isoamyl alcohol (99:1, v/v) were added. The tube was shaken mechanically (Infors shaker) for 15 min at 300 rpm and centrifuged at 2500 *g* for 5 min. An aliquot of the organic phase was transferred to a 10-ml conical glass tube, shaken with 500 μl of 0.05 *M* sulphuric acid for 10 min at 300 rpm and briefly centrifuged (2 min at 2500 *g*).

The organic phase was discarded, and 300 μl of 1 *M* sodium hydroxide and 1.6 ml of *n*-heptane were added. The mixture was shaken for 10 min at 300 rpm. After centrifugation (2 min at 2500 *g*), the organic phase was transferred into a 10-ml conical glass tube.

Derivatization of N-desmethylclomipramine and chromatography

To the organic phase were added 20 μl of dimethylformamide, 20 μl of pyridine and 100 μl of pentafluoropropionic anhydride. The tube was stoppered and shaken on a Vortex mixer for 15 s. After 1 h at 60°C, 2 ml of alkaline buffer were added, and the mixture was shaken for 5 min at 300 rpm, then centrifuged. The organic phase was transferred into a conical vial and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 10 μl of pyridine, and 2 μl of the solution were injected into the gas chromatograph.

Study in humans

Six healthy subjects, who had been advised to take no drugs for two weeks prior to the study and none besides clomipramine throughout its duration, received 75 mg of clomipramine hydrochloride as one 75-mg Anafranil commercial tablet.

Blood samples were collected before and 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h after administration and stored at −20 °C until analysis.

RESULTS AND DISCUSSION

Mass spectra

Electron-impact spectra of clomipramine, [$^2H_4$]clomipramine and the pentafluoropropionyl derivatives of N-desmethylclomipramine and [$^2H_5$]N-desmethylclomipramine are shown in Figs. 2 and 3.

The selected ions monitored were the molecular ions at *m/z* 314 and *m/z* 318 for clomipramine and [$^2H_4$]clomipramine, respectively, and the molecular ions of the pentafluoropropionyl derivatives at *m/z* 446 and *m/z* 451 of N-desmethylclomipramine and [$^2H_5$]N-desmethylclomipramine, respectively.

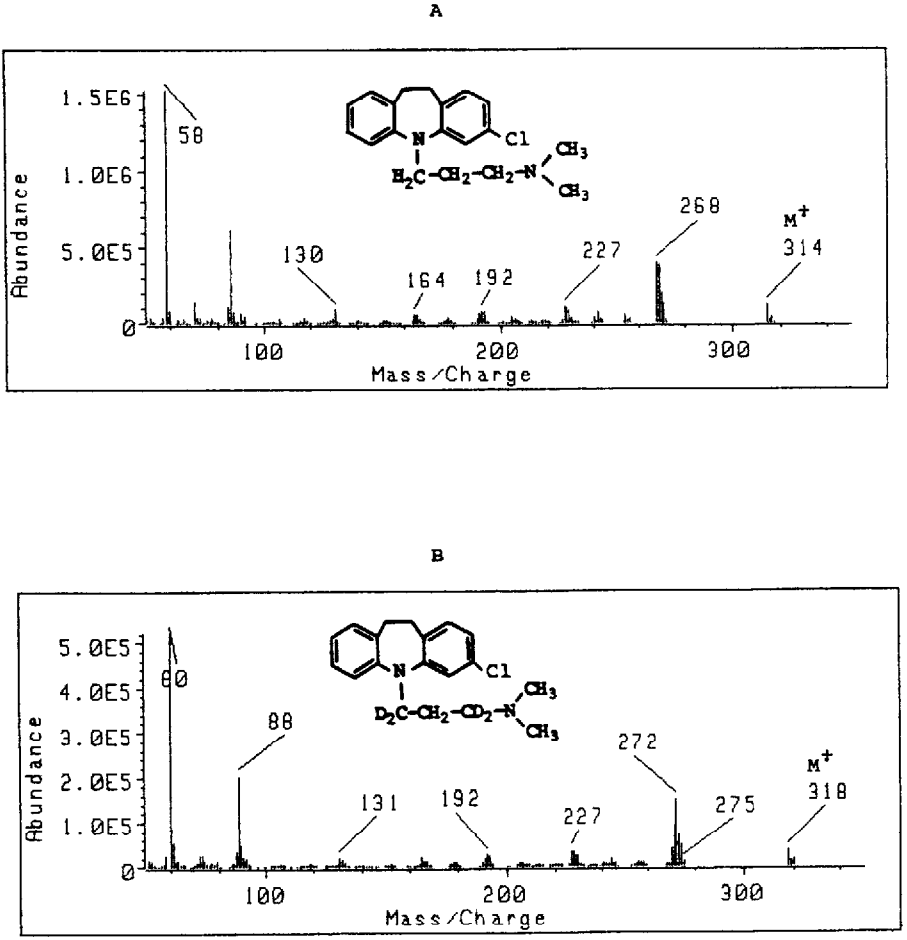


Fig. 2. Electron-impact mass spectra of clomipramine (A) and [$^2H_4$]clomipramine (B).

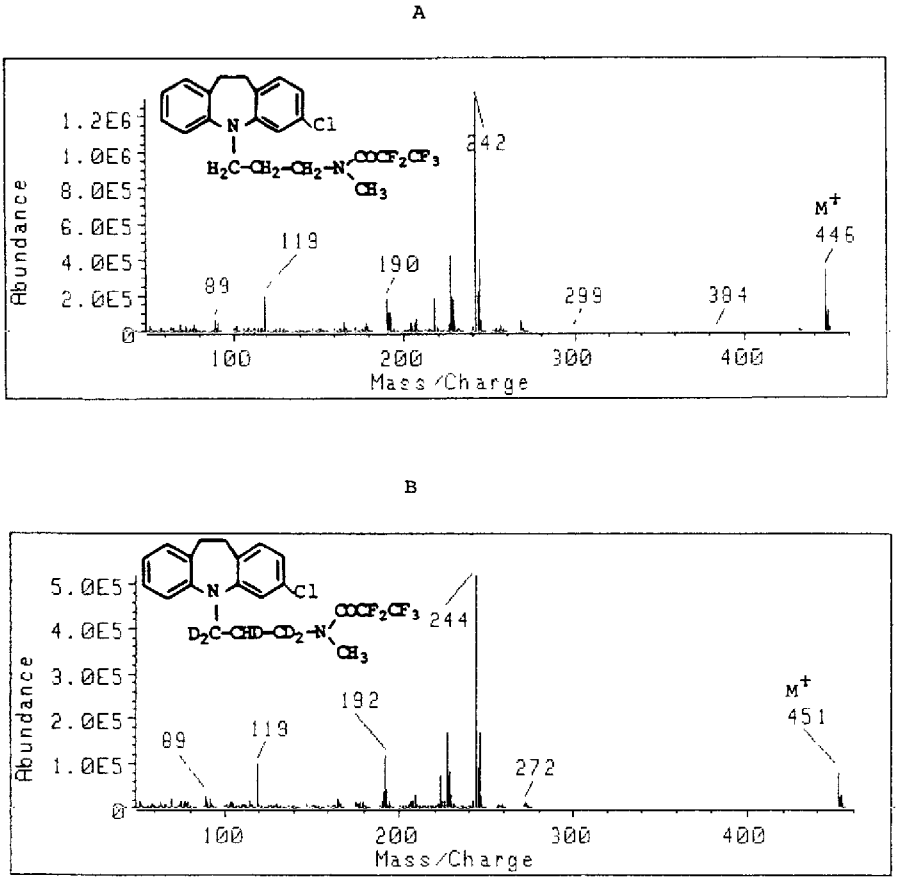


Fig 3 Electron-impact mass spectra of the pentafluoropropionyl derivatives of N-desmethylclomipramine (A) and of [$^2H_5$]N-desmethylclomipramine (B).

Blood interferences

The extract of blank human whole blood showed a clean baseline at *m/z* 314, 318, 446 and 451 (Fig. 4). Typical selected-ion current profiles obtained from human whole blood samples are shown in Fig. 5.

Calibration curves

Curves were obtained by plotting the peak-area ratio of clomipramine (*m/z* 314) and [$^2H_4$]clomipramine (*m/z* 318) versus the concentration of clomipramine in the range 1.99–159.4 nmol/l and by plotting the peak-area ratio of the derivative of N-desmethylclomipramine (*m/z* 446) and the derivative of [$^2H_5$]-N-desmethylclomipramine (*m/z* 451) versus the concentration of N-desmethylclomipramine in the range 1.78–142.30 nmol/l. Their equations were calculated by using weighted linear least-squares regression with a weighting factor of $1/(\text{concentration})^2$. They correspond to the regression equations $y=0.02344x-0.00438$ for clomipramine and $y=0.03314x-0.00357$ for N-desmethylclomipramine.

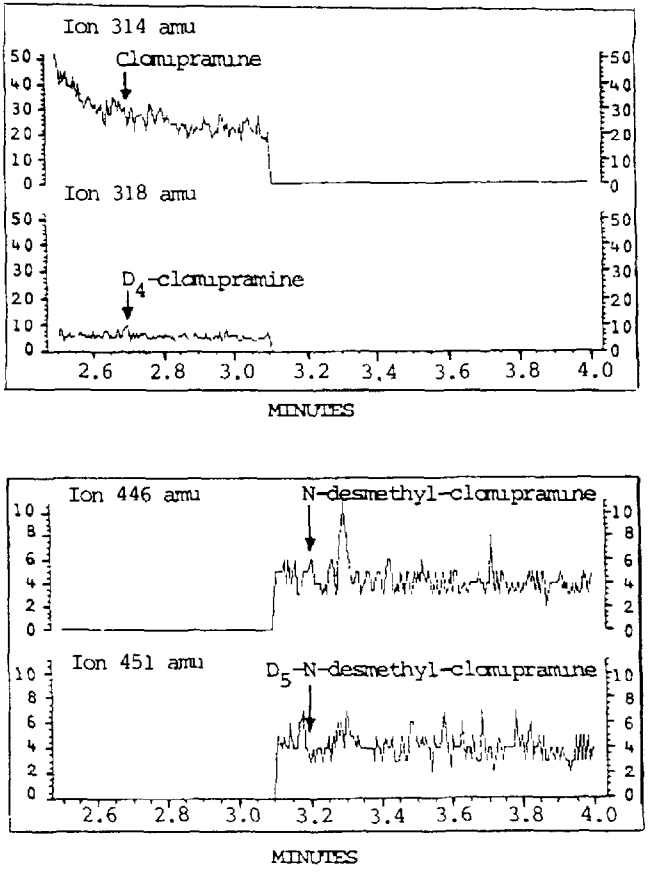


Fig. 4. Selected-ion current profiles of an extract of 1 ml of blank human whole blood.

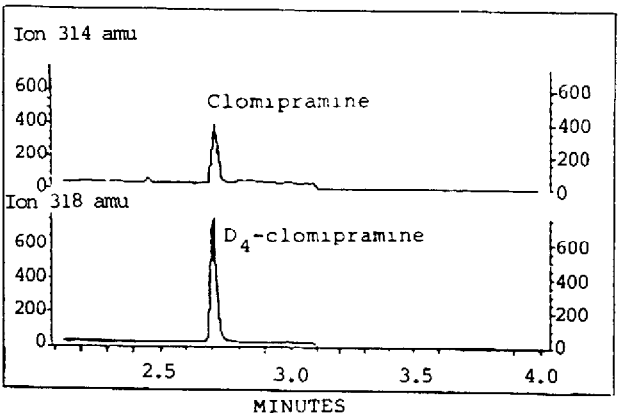
For routine analysis, the calibration curves are valid for one week. Every day the validity of the calibration curves was checked by analysis in duplicate of samples spiked with a low and a high amount of the compound. If these spiked samples gave results deviating too much, a new calibration was performed.

Within-day precision

The within-day precision was checked by determining six blood samples spiked with different concentrations of clomipramine and N-desmethylclomipramine. The relative standard deviation (R.S.D.) was used as a measure for the precision, and the relative difference between found and added amounts as a measure for the accuracy. The results obtained with the procedure described are given in Tables I and II.

Day-to-day precision

Two concentrations were determined in duplicate on days 2–5 with the same calibration curve obtained on day 1. The results obtained with the procedure described are given in Tables III and IV.



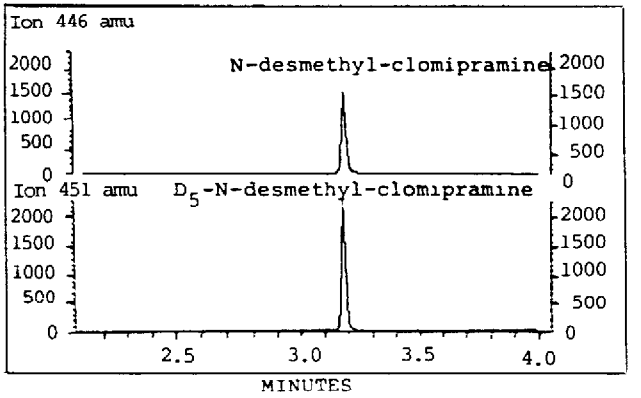


Fig. 5. Selected-ion current profiles of an extract of 1 ml of human whole blood spiked with 20 pmol (7 ng) of clomipramine hydrochloride, 42 pmol (15 ng) of [$^2H_4$]clomipramine hydrochloride, 18 pmol (6 ng) of N-desmethylclomipramine hydrochloride and 32 pmol (11 ng) of [$^2H_5$]N-desmethylclomipramine hydrochloride.

TABLE I

WITHIN-DAY PRECISION AND ACCURACY FOR CLOMIPRAMINE IN SPIKED BLOOD SAMPLES

Amount added (nmol/l)	Mean amount found (nmol/l)	R.S.D.* ($n=6$) (%)	Relative error (%)
1.99	2.24	3.9	+12.7
7.97	7.89	8.3	−1.0
13.95	14.86	4 0	+6 5
27.90	28.54	3.0	+2.3
55.79	54.39	5.2	−2 5

*R.S.D. = (S.D./mean) × 100%.

TABLE II

WITHIN-DAY PRECISION AND ACCURACY FOR N-DESMETHYLCLOMIPRAMINE IN SPIKED BLOOD SAMPLES

Amount added (nmol/l)	Mean amount found (nmol/l)	R.S.D. ($n=6$) (%)	Relative error (%)
1.78	1 61	12.8	− 9.5
7.12	8.05	5.7	+13.1
12.45	12.49	5.4	+ 0.4
24.90	25.74	3.4	+ 3.4
49.81	50.86	2.3	+ 2.1

TABLE III

DAY-TO-DAY PRECISION AND ACCURACY FOR CLOMIPRAMINE IN SPIKED BLOOD SAMPLES WITH A CALIBRATION CURVE OBTAINED ON DAY 1

Day of analysis	Amount found (nmol/l)	
	3.99 nmol/l added	79.70 nmol/l added
2	4.27	75.63
3	3.67	81.44
4	4.01	78.94
5	4.10	81.92
Mean	4.01	79.48
R.S.D. (%)	6.3	3.6
Relative error (%)	+0.6	−0.3

TABLE IV

DAY-TO-DAY PRECISION AND ACCURACY FOR N-DESMETHYLCLOMIPRAMINE IN SPIKED BLOOD SAMPLES WITH A CALIBRATION CURVE OBTAINED ON DAY 1

Day of analysis	Amount found (nmol/l)	
	3.56 nmol/l added	71.15 nmol/l added
2	3.26	66.59
3	3.56	71.92
4	2.91	69.11
5	3.79	68.60
Mean	3.38	69.06
R.S.D. (%)	11.3	3.2
Relative error (%)	−5.1	−2.9

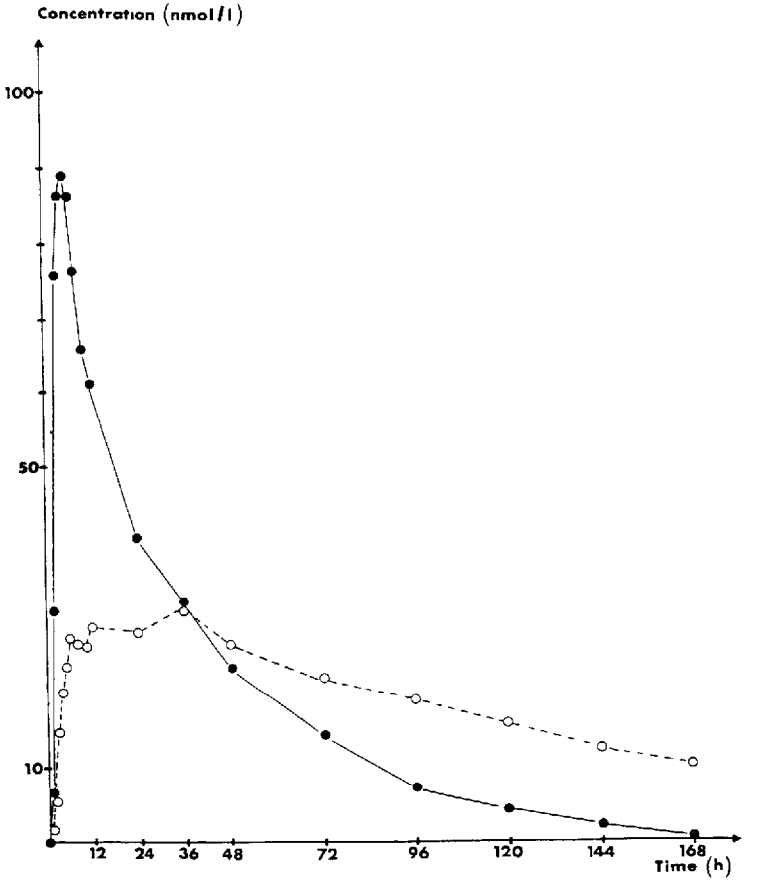


Fig. 6. Mean blood concentrations of clomipramine (●) and N-desmethylclomipramine (○) obtained in six healthy subjects after oral administration of one 75-mg Anafranil commercial tablet.

Limit of quantitation

The limit of quantitation in whole blood was estimated at 2 nmol/l (1 ng/ml) for clomipramine and N-desmethylclomipramine. Lower concentrations could still be detected with a coefficient of variation of greater than 13%.

Application

The present method was used to determine the blood concentrations of clomipramine and its N-desmethyl metabolite after oral administration of 75 mg of clomipramine hydrochloride as a single Anafranil commercial tablet. Fig. 6 shows the curves of mean blood concentrations over 168 h of clomipramine and N-desmethylclomipramine obtained in six healthy subjects.

CONCLUSIONS

The proposed technique permits the quantitative assay of clomipramine and its N-desmethyl metabolite in human whole blood at concentrations down to 2

nmol/l for clomipramine and N-desmethylclomipramine. It is specific, reproducible and sensitive for determination of clomipramine and its N-desmethyl metabolite in bioavailability, pharmacokinetic and clinical pharmacology studies.

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