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LC determination of moclobemide and three metabolites in plasma

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Abstract

Moclobemide and three metabolites were quantified using 1 ml of plasma and solid-phase extraction with Bakerbond CN column after the addition of nadolol as the internal standard (I.S.). Separation and detection the analysed substances were achieved isocratically with acetonitrile–methanol-0.067 M phosphate buffer pH 2.65–0.4% triethylamine (12.7:1.9:85:0.4, v/v/v/v), a Nova-pak C_8 column and UV detection at 230 nm. The lower limits of quantitation for moclobemide was 10 ng ml^{-1}, for M1 (Ro 16-3177)–8 ng ml^{-1}, for M2 (Ro 12-5637)-10 ng ml^{-1} and for M3 (Ro 12-8095)-15 ng ml^{-1} (as the metabolites). Accuracies calculated of three concentrations in each of three separate runs were between 84.55 and 93.68 for moclobemide, 83.28 and 92.30 for M1, 86.31 and 92.66 for M2 and 88.42 and 93.40 for M3. Precision data within day were between 5.71 and 7.50 for moclobemide, 2.91 and 6.58 for M1, 4.98 and 6.40 for M2 and 0.94 and 4.73 for M3.

Keywords: Moclobemide; HPLC; Plasma; Assay; Solid phase extraction

1. Introduction

Moclobemide [p-chloro-*N*-(2-morpholinoethyl)benzamide (Scheme 1) is a new type of reversible and selective inhibitor of the enzyme monoamine oxidase subtype A [1]. This drug is videly prescribed for the treatment of depression and it is rapidly absorbed from the gastrointestinal tract [2,3]. The principal pathways of moclobemide metabolism involve C- and *N*-oxidation of its morpholine ring to yield its two major metabolites in plasma (which were also found to be active MAO [monoamine oxidase] (Scheme 1) Ro 12-8095 and Ro 12-5637. Metabolite III Ro-16-3177 is pharmacologically inactive, but it is the main metabolite in human plasma [4].

Only a few methods of the literature can be found on moclobemide determination in human plasma.

Geschke et al. [5] described a HPLC method with Spherisorb hexyl stationary phase and acetonitrile–phosphate buffer at pH 3.9 (30:320, v/v) as mobile phase, with liquid-liquid extraction

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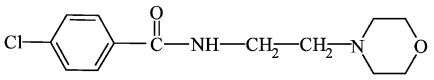
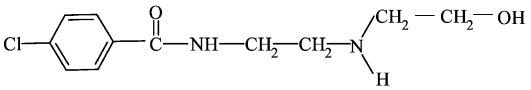
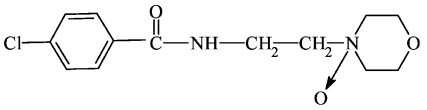


Moclobemide



Ro 16-3177 (M1)



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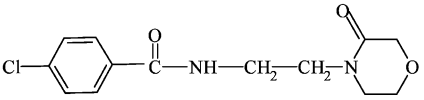

Ro 12-8095 (M3)

Scheme 1. The structures of moclobemide and its three metabolites in plasma.

(dichloromethane) and detection UV 240 nm for determination moclobemide and three its metabolites in plasma.

Maguire et al. have been published for the determination of moclobemide in human plasma by gas chromatography with nitrogen-selective detection [6].

Two papers have been elaborated HPLC methods for determination only moclobemide in human serum. In one case, it has been used diethyl ether to extraction of moclobemide and metaclopramide (as internal standard (I.S.)).

The authors in these papers have been used the reversed phases with detection UV at 254 nm. As the mobile phases they have been applicated: acetonitrile-0.023M tetramethylethylenediamine (20:80, v/v) and methanol–water–glacial acetic acid (70:30:0.44, v/v/v) [7,8].

A sensitive liquid chromatography–electrospray ionisation mass spectrometry (HPLC–ESI–MS) assay has been developed for the measurement of moclobemide and metabolitites, Ro 12-5637 and Ro 12-8055 in human plasma by Hoskinsa et al. [9]. They have been used Nova-Pak phenyl column, a mixture 0.2 M ammonium formate buffer pH 3.57-actonitrile (55:45, v/v) as the mobile phase. Sample preparation involved solid-phase extraction, using C_{18} cartriges.

The our assay described here provided a specific, sensitive and reproducible method for the determination of moclobemide and its three metabolites in human plasma with nadolol (I.S.) by high performance liquid chromatography, using UV detection. This method utilises standard Bakerbond CN SPE cartriges and has a higher analyte recovery ($>95\%$) as well as a lower limit of quantification (0.01 μg ml^{-1}).

2. Experimental

2.1. Materials

All solvents were of HPLC grade and all reagents were of analytical grade. Moclobemide and metabolites M1 (Ro 16-3177, M2 (Ro 12-5637), M3 (Ro 12-8095), were kindly donated by Hoffmann–La Roche Ltd. (Basel, Switzerland). Nadolol (I.S.) was purchased from Apotex, INC (Ontario, Canada).

Heparinised human whole blood was obtained from the District Blood Centre in Lublin (Poland). Water was deionised and glass distilled prior to the use.

Bakerbond CN sorbent columns for the extraction were purchased from Baker Inc. (USA). Acetonitrile LiChrosolv® for chromatography, methanol and triethylamine (E.Merck, Darmstadt, Germany) were applied.

Stock standard methanolic solutions of moclobemide, the methabolites (M1–M3) and I.S. at concentrations 10 μg ml^{-1} were prepared. There were stored at 4 °C and were stable up to 6 month. Stock solution for each analyte were serially diluted in methanol for each assay run and finally made up to the required concentration in drug free plasma.

2.2. Instrumentation

The following instruments and apparatus were used for the HPLC assay: a Waters HPLC system (Milford, MA, USA) consisting of a Model 515 high-pressure pump, and a Model 2487 variable wavelength detector (UV-Vis) dual λ absorbance; the analytical column (150 × 3.9 mm I.D.) packed with Nova-Pack C_8–dp 4 μm, were used. K 24D high-speed refrigereted centrifuge–(MLW, Engelsdorf, Germany) with 11–30 ml propylene centrifuge tubes were applied. The minicolumns for solid-phase extraction were Bakerbond CN packed with 500 mg cyanopropylsilane.

2.3. Chromatographic conditions

Each sample was analysed with detection at 230 nm.

The mobile phase was a mixture of acetonitrile–methanol-0.067M phosphoric buffer (adjusted to pH 2.65 with 65% ortophosphoric acid)-0.4% triethylamine (12.7:1.9:85:0.4, v/v/v/v). The flow rate was 1.3 ml min^{-1}. The substance nadolol was applied as the I.S. for the determination of moclobemide and its methabolites.

3. Sample preparation

To the centrifuge test tubes, each containing 1.0 ml of blood plasma, 0.1–4 μg of the methanolic solutions of moclobemide, 0.1–0.6 μg; M2, 0.1–3.0 μg; M3 and 2 μg I.S., 0.04–0.6 μg solution the metabolite M1 at concentration 1 μg ml^{-1}, were given. The methanol was added to a final volume of 3 ml and the mixture was centrifuged for 10 min at 2000 × g.

Then 1.5 ml of the supernatant and 1 ml of phosphate buffer pH 8.4 were injected into the Bakerbond CN extraction column which had previously been rinsed with 2 ml of methanol and 2 ml of phosphate buffer at pH 8.4. Sample solution was passed through the column at a flow rate of 50 μl ml^{-1}. Then the substances were eluted from the column with four portions of 0.5 ml of methanol. The eluates were evaporated to dryness in a nitrogen stream and after dissolving in 1000 μl of mobile phase, 20 μl of the solution was injected into the analytical column. All measurements were repeated five times at each concentratin. Drug free plasma was analysed in the same way a control.

3.1. Determination of moclobemide and three metabolites levels in patients plasma

Efficacy and plasma concentration date were available from four patients who received moclobemide 150 mg daily for 1 week, in next week—300 mg daily and 450 mg daily by third week.

The blood samples were taken from patients and then at once centrifuged for 10 min at 1100 × g in purpose to obtain of plasma, which stored deep frozen (−35 °C) until needed for analysis.

To 1 ml plasma of patients 2 μg of nadolol (I.S.) was added, and the volume was completed up to 3 ml using methanol. The mixtures were centrifuged for 10 min at 2000 × g; the supernatant was passed through the Bakerbond CN extraction column. Next steps of this procedure were as above mentioned.

4. Results and discussion

Resolution and sensitivity were determined by injection of an extracted plasma (Fig. 1).

A mixture acetonitrile–methanol-0.067M phosphate buffer pH 2.65–0.4% triethylamine (12.7:1.9:85:0.4, v/v/v/v) at a flow rate of 1.3 ml ml^{-1} was found to be appropriate mobile phase, allowing adequate and separating of analytes and the I.S. The retention times of moclobemide, M1, M2, M3 and nadolol at $\lambda = 230$ nm were 6.10, 4.60, 7.90, 14.70 and 3.25 min, respectively.

The linearity of both the extracton procedure and the detector response (determined from the peak heiht) was verified over the assay range (50–2000 ng ml^{-1}) for moclobemide, 20–300 ng ml^{-1} for M1, 50–400 ng ml^{-1} for M2, and 50–1500 ng ml^{-1} for M3. The linearity was determined by assaing pooled drug-free plasma (which had been previously screened for extraneous peaks) spike with known amounts of each at the analytes. Calibration curves were calculated for each ana-

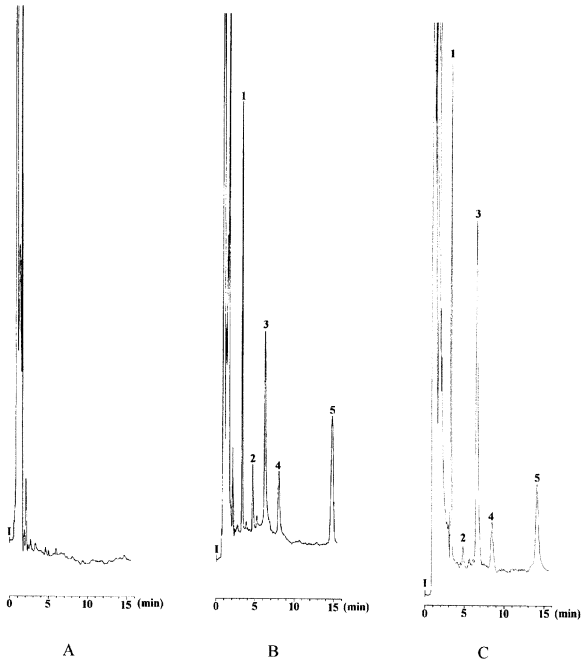

Fig. 1. Chromatograms obtained from a blank human plasma sample (A); from a spiked human plasma (B) with 500 ng ml^{-1} of moclobemide (3), 100 ng ml^{-1} of Ro 16-3177 (2), 200 ng ml^{-1} of Ro 12-5637 (4), 500 ng ml^{-1} of Ro 12-8095 (5), and 1000 ng ml^{-1} of nadolol (I.S.) (1); from patient after oral administration of an 450 mg of moclobemide in 'Aurorix' tablet (C); 3, (1526.8 ng ml^{-1}); 2, (54.5 ng ml^{-1}); 4, (236.4 ng ml^{-1}); 5, (584.8 ng ml^{-1}); 1, (1000 ng ml^{-1}).

lyte using its concentration and the peak height ratio of analyte to I.S. over the studied range.

The equation for each analyte calibration curve were:

$$y = 0.000897946(\pm 1.9 \times 10^{-5})x + 0.00609906(\pm 2.0 \times 10^{-2}),\ r = 0.9993 \text{ for moclobemide}$$

$$y = 0.00150208(\pm 9.6 \times 10^{-6})x - 0.00170151(\pm 1.6 \times 10^{-2}),\ r = 0.9999 \text{ for M1}$$

$$y = 0.000738415(\pm 2.8 \times 10^{-5})x - 0.00943293(\pm 6.9 \times 10^{-3}),\ r = 0.9978 \text{ for M2}$$

$$y = 0.000586606(\pm 1.1 \times 10^{-5})x + 0.00417821(\pm 9.5 \times 10^{-3}),\ r = 0.9994 \text{ for M3}$$

The mean inter-assay R.S.D. for moclobemide, M1, M2 and M3 were for a spiked plasma quality control pool containing of each analyte ($n = 5$). The absolute extraction recovery analyte of moclobemide ranged from 95.16–99.06, 98.87–101.13 for M1, 99.63–100.98 for M2, and 95.05–100.54. The 3-day validation of moclobemide and three metabolites in human plasma are presented in Table 1.

The intra- and inter-day precision are presented in Table 1 too. For intra-day precision five sets of samples (low, medium and high concentration) were analysed on 1 day. Precision was from 571 to 6.34% for moclobemide from 2.91 to 6.58% for M1, from 4.98 to 6.40% for M2 and 0.94 to 4.73% for M3.

For inter-day precision five sets of samples (three levels) were analysed on 3 separate days. The accuracy of the method, calculated by determining five concentrations of moclobemide, M1, M2 and M3 is given in Table 1.

The detection limit (i.e. peak height equal to three times baseline noise) was 10 ng ml^{-1} for moclobemide, 8, 14, and 15 ng ml^{-1} for M1, M2 and M3, respectively.

The calibration curves were linear and reproducible over the analysed concentration range with correlation coefficients$>$0.990.

In fact, a content of 0.4% triethylamine in the mobile phase strongly improved the peak symmetry for moclobemide and its metabolites. Nova-Pak C_8 column was sufficiently deactivated to enable symmetrical peaks to be obtained for all compounds. There were also tested other columns: Nova-Pak C_{18}, LiChrosorb C_{18}, but by using these columns we were unable to separate all the analysed substances.

In addition, an influence of the mobile phase pH on the peak symmetry and the flow of the mobile phase of the studied compounds was evident. The peak tailing occured above an eluent pH 4 for M3 and M1, and above pH 6 for moclobemide and M2.

Table 1
Three-day validation of moclobemide and three metabolites in human plasma

Compounds	Normal concentration (ng ml^{-1})		Day 1	Day 2	Day 3	Average within day	Between day precision	Average accuracy
Moclobemide	100	Mean	99.01	100.03	98.14			
		S.D.	6.19803	6.2104	6.4143			
		R.S.D.	6.26	6.21	6.54	6.34	6.41	
		ACC	99.01	100.03	98.14			99.06
		n	5	5	5			
	500	Mean	483.55	470.43	491.16			
		S.D.	34.96067	35.97417	37.41341			
		R.S.D.	7.23	7.65	7.62	7.50	6.53	
		ACC	96.71	94.09	98.23			96.34
		n	5	5	5			
	1000	Mean	954.29	910.11	990.39			
		S.D.	53.63109	49.63413	60.14319			
		R.S.D.	5.62	5.45	6.07	5.71	4.23	
		ACC	95.43	91.01	99.04			95.16
		n	5	5	5			
M1	50	Mean	52.06	45.13	54.51			
		S.D.	3.90971	2.91098	3.14515			
		R.S.D.	7.51	6.45	5.77	6.58	9.62	
		ACC	104.12	90.26	109.02			
		n	5	5	5			101.13
	100	Mean	99.26	91.16	106.19			
		S.D.	2.45172	3.15171	2.96104			
		R.S.D.	2.47	3.46	2.79	2.91	7.60	
		ACC	99.26	91.16	106.19			98.87
		n	5	5	5			
	200	Mean	199.39	192.13	202.87			
		S.D.	6.67956	6.91435	7.14823			
		R.S.D.	3.35	3.60	3.52	3.49	2.76	
		ACC	99.69	96.07	101.44			99.07
		n	5	5	5			
M2	100	Mean	100.44	104.11	97.16			
		S.D.	3.57566	5.21041	6.21413			
		R.S.D.	3.56	5.00	6.39	4.98	6.91	
		ACC	100.44	104.11	97.16			100.57
		n	5	5	5			
	200	Mean	201.87	192.92	202.98			
		S.D.	10.71929	9.41321	11.98415			
		R.S.D.	5.31	4.88	5.90	5.36	2.77	
		ACC	100.94	96.46	101.49			99.63
		n	5	5	5			

Table 1 (*Continued*)

Compounds	Normal concentration (ng ml^{-1})		Day 1	Day 2	Day 3	Average within day	Between day precision	Average accuracy
	300	Mean	304.14	309.14	295.56			
		S.D.	23.41878	19.41361	15.41621	6.40	2.27	
		R.S.D.	7.70	6.28	5.22			100.98
		ACC	101.38	103.05	98.52			
		n	5	5	5			
M3	100	Mean	100.20	106.18	95.23			
		S.D.	5.18034	4.98111	4.11321			
		R.S.D.	5.17	4.69	4.32	4.73	5.54	
		ACC	100.20	106.18	95.23			100.54
		n	5	5	5			
	500	Mean	472.07	460.32	493.41			
		S.D.	18.12749	15.31231	14.32138			
		R.S.D.	3.84	3.33	2.90	3.35	3.53	
		ACC	94.41	92.06	98.68			95.05
		n	5	5	5			
	1000	Mean	984.68	960.42	998.43			
		S.D.	4.72646	10.41839	12.43081			
		R.S.D.	0.48	1.08	1.25	0.94	1.96	
		ACC	98.47	96.04	99.84			98.12
		n	5	5	5			

Another effect dependent on the pH of the mobile phase was masked increase of the capacity factors of moclobemide starting above 6 (Fig. 2), which has already been reported for other basic drugs [10]. The reasons for these observed effects appear to be complex, with contributions from protonation equilibria of residual silanol groups [11] as well as those of the basic substances [12] possibly playing important roles.

Liquid–solid extraction with Bakerbond CN minicolumns packed with 500 mg cyanopropylsilane was selective, efficient and involves minimal handling of the sample therefore, it saves time, glassware and reagents, and was found to be faster and more reliable than method liquid-liquid extraction.

In most cases, the extraction column has been used only one as suggested by the manufacturer. We analyzed plasma spiked with moclobemide, its three metabolites and nadolol (I.S.), each with five new Bakerbond CN columns. However, we repeated the extraction of plasma control using the same columns four times. The conditioning, washing and elution steps with the used columns were carried out in the same manner as with the new columns. The absolute recovery of drugs and within batch CV of the ratios of moclobemide and its metabolites/I.S. were similar in all five sets of extraction.

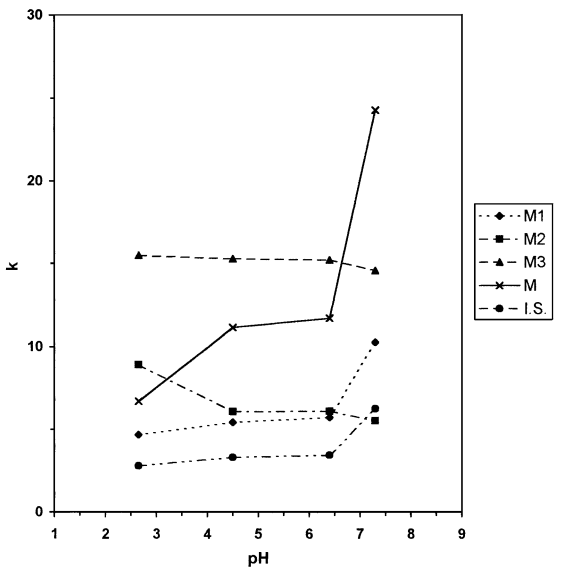

Fig. 2. Influence of the pH of the mobile phase on the k values of M (moclobemide), its metabolites, M1–M3 and the I.S.

It has been established that the ratio between the analytical recovery of the analytes and that of the I.S. submitted to the same operations were constant over a wide concentration range. Also, the detector response for the all compounds was linear over the range used. During the analytical procedure, the stability of the analytes has been proved.

The following solvents were tested for protein precipitation: 6% perchloric acid (plasma solvent ratio (1:1), acetonitrile (1:3) and methanol (1:3). Methanol was chosen as the best solvent for protein precipitation, because the recovery of the analytes were according to requirements. Methanol was also used to conditioning of the columns CN (for SPE) and to the elution of the analysed substances.

The influence on the separated moclobemide and its metabolites of the following drugs was tested in chromatographic conditions. It has been proved, that the retention time of fluoxetine, paroxetine, fluvoxamine, nefazodone, citalopram, diazepam, chlordiazepoxide, chlorprothixene, and amitriptiline was very long (approximately 30–120 min). Only the zopiclone had the retention time shorter (about 8 min) similar the retention time of M2.

Blood sample (3 ml) obtained from four patients, who received 'Aurorix' tablets ā 150 mg of moclobemide for 1 week, 300 mg for second week, and 450 mg for third week, were taken for the analysis 12 h after oral administration of drug. The following concentrations moclobemide, M1, M2 and M3 were found: from 61.5 to 157.7, 29.6–32.6, 17.3–61.6 and from 86.3 to 620.3 ng ml^{-1} respectively, after receiving 150 mg moclobemide; from 84.6 to 205, 14.4–29.7, 17.8–64.4 and from 52.2 to 669.8 ng ml^{-1}, respectively after receiving 300 mg moclobemide-from 107.9 to 1526.6, 27.4–54.5, 60.0–236.4 and from 388.8 to 795.5 ng ml^{-1} respectively, after receiving 450 mg moclobemide daily.

Representative chromatograms of drugs-free plasma spiked with moclobemide, its metabolites and I.S. and a plasma sample collected 12 h after

450 mg oral dose of moclobemide are shown in Fig. 1B,C.

The time curve of the concentration in plasma of the four patients (ABCD) of moclobemide (M), M1, M2, and M3 after 1, 2 and 3- week curation is shown in the Fig. 3.

In conclusion, the elaborated HPLC method for determining of moclobemide and its three metabolites in plasma is sensitive, linear over a wide range and reproducible. This method can be used both pharmacokinetic studies and for monitoring therapy of moclobemide.

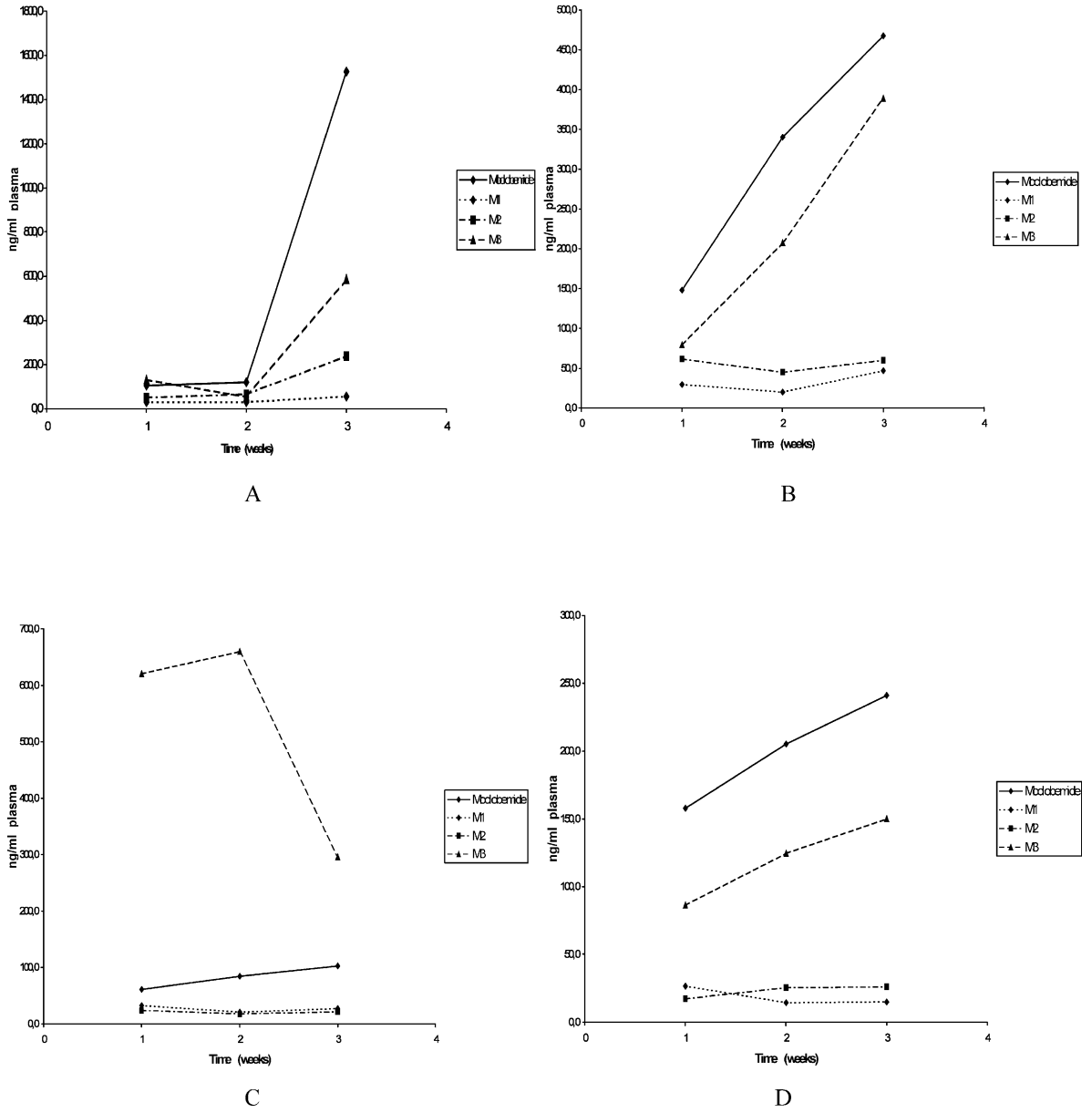

Fig. 3. The time curve of the concentration in plasma of the four patients (ABCD) of M, M1, M2, and M3 after 1–3-week giving of 'Aurorix' tablets.

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