Journal of Chromatography, 420 (1987) 111–120
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3737

DETERMINATION OF THE NEW MONOAMINE OXIDASE INHIBITOR MOCLOBEMIDE AND THREE OF ITS METABOLITES IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. GESCHKE, J. KÖRNER and H. EGGERS*

Biological and Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd., Basle (Switzerland)

(Received March 13th, 1987)

SUMMARY

A sensitive high-performance liquid chromatographic method has been developed for the determination of moclobemide and three of its metabolites in plasma and urine. The four substances and the internal standard were extracted from basified plasma (pH 11) with dichloromethane, with recoveries of generally more than 70%. A column packed with 5-μm Spherisorb hexyl phase and an eluent consisting of acetonitrile and aqueous phosphate buffer 30:320 (v/v) with a pH of 3.9 were found most suitable for the chromatographic separation of the four compounds of interest. Non-ideal interactions of the basic compounds and the N-oxide metabolite occurred with many other reversed-phase materials, as indicated by broad and tailing peaks. Limits of quantitation for moclobemide and its three metabolites in the range 20–30 ng/ml and a good intra-assay reproducibility ranging between 2 and 5% for concentrations over 100 ng/ml could be achieved for plasma, which increased to ca. 8–10% at a concentration of 50 ng/ml.

INTRODUCTION

Moclobemide [*p*-chloro-N-(2-morpholinoethyl) benzamide, Fig. 1] is presently under clinical investigation as a new antidepressant. Its pharmacological effect is based on a reversible inhibition of the monoamine oxidase (MAO), with preference for type A of the enzyme [1]. The drug has been shown to be completely and rapidly absorbed from the gastrointestinal tract [2], but it undergoes rapid and extensive metabolism in the liver with the formation of numerous metabolites [3]. Two of these metabolites (II, III) were also found to be active MAO inhibitors. Metabolite IV is pharmacologically inactive, but was found to be the main metabolite in human plasma (for structures, see Fig. 1).

Until now only one method has been published for the determination of moclobemide in human plasma, by gas chromatography with nitrogen-selective detec-

0378-4347/87/$03.50

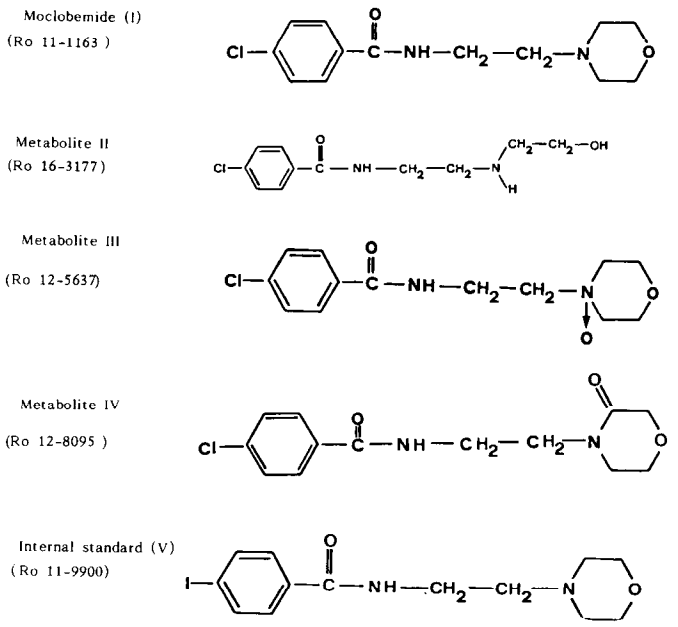

Fig. 1. Chemical structures of the substances determined and the internal standard.

tion [4]. Despite the limitation of this method to the determination of only the unchanged drug the reproducibility, reported as 15.7% for 50 ng/ml, seemed unsatisfactory for pharmacokinetic trials where such low plasma concentrations had to be expected after the administration of the envisaged therapeutic doses of moclobemide. Another disadvantage of this method is the lack of information about possible analytical interferences of metabolites with the parent drug.

Therefore, a sensitive and selective new high-performance liquid chromatographic (HPLC) method was developed, suitable for the simultaneous determination of moclobemide and three of its important metabolites in human plasma with a high precision (8–10%) even at low concentrations (50 ng/ml). By a modification of the method it was also possible to analyse urine samples.

EXPERIMENTAL

Chemicals and methods

Acetonitrile (p.a.), dichloromethane (p.a.), sodium phosphate ($Na_3PO_4 \cdot 12H_2O$ p.a.), potassium dihydrogenphosphate (KH_2PO_4 p.a.) and Extrelut 1 glass columns were all purchased from E. Merck (Darmstadt, F.R.G.). Doubly distilled water was used for the preparation of all aqueous solutions.

Moclobemide (I) and its metabolites II, III, and IV were supplied by F. Hoffmann-La Roche (Basle, Switzerland).

The 0.067 *M* potassium dihydrogenphosphate solution was prepared by dissolving 9.07 g of the salt in 1000 ml of water. A saturated solution of sodium phosphate (ca. 13%, w/v) was prepared by adding an excess of the salt to 100 ml of water.

Chromatography

The HPLC apparatus consisted of a Kontron 414T pump (Kontron, Eching, F.R.G.) combined with a pulse dampener (Orlita, Giessen, F.R.G.), a variable-wavelength UV detector (Type 87.00, Knauer, Berlin, F.R.G.) and an autosampler (Type ISS 100, Perkin-Elmer, Überlingen, F.R.G.). The 125×4 mm I.D. stainless-steel column was packed in our laboratory with Spherisorb S5 C6 (Phase Separations, Queensferry, U.K.) by forcing a 10% (w/v) methanolic slurry of the reversed-phase material into the column at 5.8 MPa with methanol. The detection wavelength was 240 nm.

The mobile phase was a mixture of acetonitrile and 0.067 *M* potassium phosphate buffer solution (30:320, v/v); the apparent pH was adjusted to 3.9 by dropwise addition of dilute hydrochloric acid. After degassing in an ultrasonic bath, the eluent was pumped at a flow-rate of 1.3 ml/min. The system was operated at room temperature. Under these conditions the retention times were ca. 5.3 min (II), 7.2 min (I), 8.6 min (III), 12.7 min (V) and 15.1 min (IV).

Extraction procedure

A 0.5-ml plasma sample was pipetted into a 20-ml conical glass tube, followed by 400 µl of a saturated sodium phosphate solution (pH 11) and 100 µl of an aqueous solution containing the internal standard. The components were mixed for a few seconds with a Vortex-Genie mixer and then transferred with an Eppendorf pipette onto the packing of an Extrelut 1 glass column. The mixture was allowed to soak into the column and distribute homogeneously for 10 min. The substances were then eluted from the column with two 6-ml portions of freshly distilled dichloromethane. The organic extract was evaporated to dryness in a thermostatted water-bath at 37°C under nitrogen. The residue was dissolved in 250 µl of the mobile phase by vortexing the conical glass tubes for 30 s. Finally a 75-µl aliquot was injected for analysis.

The same procedure could be applied to urine samples, the only modification being the use of 0.2 ml of urine and 0.7 ml of sodium phosphate solution. In this case, 50 µl from a final extract volume of 750 µl were injected, owing to the higher concentration of metabolite III present in urine samples.

Solutions and calibration standards

Stock solutions of moclobemide (I) and the metabolites II–IV were made by weighing accurately 10 mg of each substance into a 10-ml volumetric flask, which was made up to volume with methanol. A working solution suitable for the usually different calibration ranges of each substance was prepared by pipetting 2, 0.2, 0.2 and 1 ml of the stock solutions of I, II, III and IV, respectively, into a 10-ml volumetric flask and making it up to volume with doubly distilled water. A second working solution was obtained by a 1:10 dilution of the first one with water. Calibration standards as well as quality-control samples were prepared by spiking drug-free plasma from a blood bank (Blutspendezentrum, SRK, Basle, Switzerland) with 15–150 µl of the working solution, corresponding to a total volume of 10 ml of plasma standard. Thus, the following concentration ranges were covered: 30–3000 ng/ml for I, 30–250 ng/ml for II and III and 30–1250 ng/ml for IV. The

stock solution for urine standards contained 5 mg of moclobemide (I) and 50 mg of metabolite III dissolved in 10 ml of methanol–water (1:1). Working solutions and standards were prepared as described for plasma, using urine from a non-medicated volunteer. The calibration ranges were 0.05–20 μg/ml for I and 0.5–200 μl/ml for III.

Plasma and urine standards were divided into small portions of ca. 2.5 ml and stored deep frozen (−20°C) until needed for analysis. The stock solutions were found to be stable for at least four weeks in a refrigerator at +5°C. The stability of the working solutions was not controlled. They were made fresh from the stock solutions whenever needed. A stock solution of the internal standard was prepared by dissolution of 10 mg of V in 10 ml of methanol. Then 80 and 200 μl of this solution for plasma and urine, respectively, were diluted to 10 ml with doubly distilled water and used for the addition to plasma and urine samples.

Calibration and calculation

Calibration curves were obtained from six or seven spiked plasma or urine standards covering the expected concentration ranges of the unknowns. Standards and unknowns were extracted together as described above. A least-squares linear regression of the peak-height ratios of the substance/internal standard versus substance concentrations was calculated, using a weighting factor of $1/y^2$. With this equation the concentrations of the unknowns were calculated from the measured peak height of each substance divided by the peak height of the internal standard. On-line data storage and calculations were carried out by a computing integrator using, in part, our own user software [5].

RESULTS AND DISCUSSION

Sample preparation

Extraction of spiked human plasma samples at different aqueous-phase pH values with various organic solvents clearly indicated that sufficiently high recovery rates for moclobemide and its three metabolites II–IV, as well as clean extracts, could be obtained only above pH 10. This result was partly predetermined by the basic properties of moclobemide ($pK_a = 6.2$) and its metabolite II. However, the extraction of the N-oxide metabolite (III) was found to be the most critical. Its recovery was found to be highly dependent not only on the type of organic solvent (dichloromethane being much more effective than chloroform, *n*-butyl chloride or *tert.*-butyl methyl ester), but also on the salt concentration of the aqueous phase. A high salt concentration of sodium phosphate was necessary to achieve satisfactory recovery for this compound.

The use of Extrelut 1 glass columns filled with kieselguhr further simplified and slightly enhanced the speed of the sample preparation procedure compared with the classical extraction. In addition, the more effective liquid–liquid partition process taking place within the Extrelut glass columns enhanced the recovery of metabolite III by 15–20%, whereas the recoveries of the other substances were not affected. Chromatograms of blank plasma extracts did not reflect any marked difference in the amounts of coextracted endogenous substances when

TABLE I

RECOVERY DATA FOR PLASMA ($n=5$)

Moclobemide (I)			Metabolite II			Metabolite III			Metabolite IV		
Added (ng/ml)	Recovery (%)	C.V. (%)	Added (ng/ml)	Recovery (%)	C.V. (%)	Added (ng/ml)	Recovery (%)	C.V. (%)	Added (ng/ml)	Recovery (%)	C.V. (%)
250	76	3.4	50	85	2.2	25	70	4.5	125	81	3.9
500	84	0.8	100	70	0.6	50	74	4.4	250	86	1.0
1000	88	0.7	400	89	0.9	100	74	2.6	500	90	1.9
2500	85	1.1	1500	82	0.8	250	72	2.1	1250	87	1.6
Mean	83	1.5		82	1.1		73	3.4		86	2.1

the two modes of extraction were compared. Recoveries were determined under optimized conditions by repeated analysis of spiked plasma and urine samples with the internal standard being added to the final extract. Mean recoveries in plasma of 83, 82, 73 and 86% were found for I, II, III and IV, respectively. For urine, the recoveries of I and III increased to 92 and 89%, possibly owing to the lower amount of protein coagulating within the extraction column compared with plasma (for details cf. Tables I and II).

Chromatographic conditions

First attempts to develop a reversed-phase HPLC system for the separation of moclobemide and its metabolites were unsuccessful because badly tailing peaks were observed for moclobemide and its metabolites II and III, which were found to be related to the stationary phases used. Such non-ideal interactions of organic substances with reversed-phase materials, which have already been reported in several publications, especially for basic drugs [6], have been eliminated in some cases by the addition of a small amount of an amine, e.g. triethylamine, to the mobile phase [7]. In fact, a content of 0.1% triethylamine in the mobile phase strongly improved the peak symmetry for moclobemide and its basic metabolite (II) but not for the N-oxide metabolite (III). More than twenty commercially available reversed-phase materials were tested, with the result that only two

TABLE II

RECOVERY DATA FOR URINE ($n=3$)

Moclobemide (I)			Metabolite III		
Added (μg/ml)	Recovery (%)	C.V. (%)	Added (μg/ml)	Recovery (%)	C.V. (%)
0.2	89	5.8	2	92	0.7
0.5	94	6.0	5	88	2.3
2	93	0.6	20	88	1.3
10	92	0.7	100	88	0.7
Mean	92	3.3		89	1.3

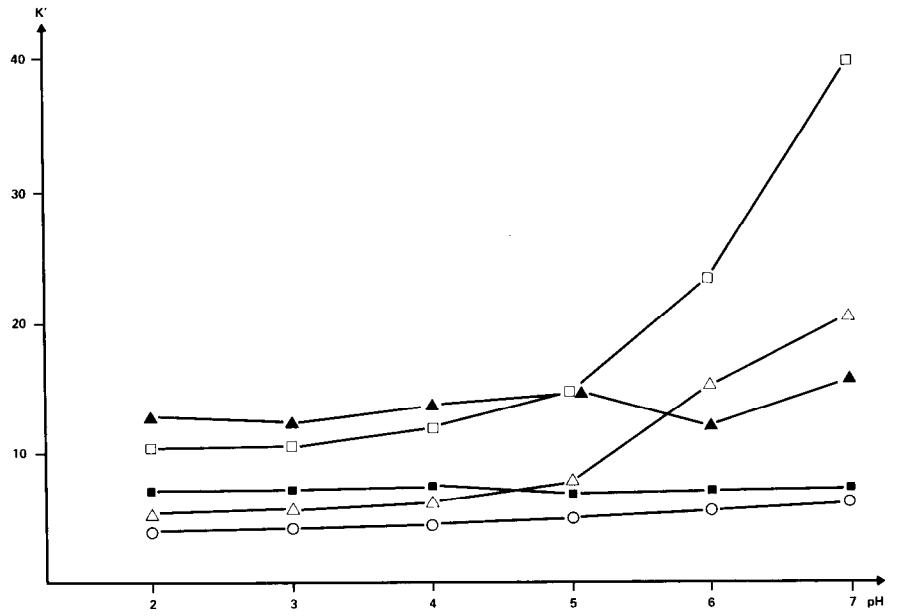

Fig. 2. Influence of the pH of the mobile phase on the k' values of moclobemide (△), its metabolites II (○), III (■), and IV (▲), and the internal standard (□).

materials, μBondapak C_{18} and Spherisorb C_6, were sufficiently deactivated to enable symmetrical peaks to be obtained for all compounds, even without the addition of an amine modifier to the mobile phase. Of these two materials the 5-μm Spherisorb C_6 phase was preferred because of its lower particle size, resulting in ca. 30% lower detection limits, and its improved separation efficiency compared with the 10-μm μBondapak material. In addition, an influence of the mobile phase pH on the peak symmetry of the studied compounds was evident since, despite the use of the two optimized stationary phases, peak tailing occurred above an eluent pH of 4 for metabolite III, and above pH 6 for moclobemide and metabolite II. Another effect dependent on the pH of the mobile phase was the marked increase of the capacity factors of moclobemide and the internal standard, starting above pH 5 (Fig. 2), which has already been reported for other basic drugs [7]. The reasons for these observed effects appear to be complex, with contributions from protonation equilibria of residual silanol groups [8] as well as those of the basic substances [9] possibly playing important roles.

Selectivity

Owing to the high number of metabolites identified in plasma and urine by metabolism studies [10], special interest was directed to the evaluation of possible interferences arising from other coextracted metabolites. A total of thirteen additional metabolites synthesized as pure substances were tested with respect to their extractability under the conditions described and their chromatographic retention. It was found that none of these metabolites interfered because either

they were not extracted or they had different retention times. In addition, corresponding peaks of three to five unidentified metabolites, which appeared in the chromatograms of plasma and urine samples with retention times between 5 and 12 min, were clearly separated from the peaks of interest as demonstrated in Figs. 3B and 4B.

Limit of quantitation

For 0.5-ml plasma samples under the work-up conditions described, quantitation limits of 20, 20, 25 and 30 ng/ml were obtained for moclobemide and its metabolites II, III and IV, corresponding to coefficients of variation (C.V.) of 12–15%. The quantitation limits in urine for moclobemide and metabolite III were 75 and 150 ng/ml, owing to the lower sample volume (0.2 ml) and the higher volume of the final extract.

Linearity

Linear regression lines were obtained for the concentration ranges 30–3000 ng/ml for I, 30–250 ng/ml for II and III 30–1250 ng/ml for IV in plasma, within which the concentrations of each substance were usually found following i.v. or p.o. administration of the putative therapeutic doses (100–300 mg). The use of a weighting factor of $1/y^2$ improved the fit of the regression line, particularly in the low concentration range. For urine the linearity of the calibration line was proved within the concentration ranges 0.05–20 μg/ml for moclobemide and 0.5–200 μg/ml for metabolite III.

Precision

The inter-assay precision of the method was calculated from repeated analyses of quality-control samples on different days. The results (Tables III and IV) clearly demonstrate the good reproducibility of the method, even for low concentrations of the four substances in plasma and urine. Clinical samples of various concentrations analysed in duplicate or even more, indicated good agreement between the C.V. calculated for spiked and "real" samples.

Stability

The stability of spiked plasma samples after 24 h storage at room temperature (+20°C), after six and nine months storage at −20°C was evaluated. Freshly spiked plasma samples were analysed the same day as stored plasma samples of the same concentration. The peak-height ratios of each substance/internal standard were determined, and the geometric means of both sets of samples compared according to an established method [11]. In general, no relevant decrease (more than 10%) of concentrations was detected for any of the substances under the conditions described.

Application

The method has been applied to the analysis of more than 3000 plasma and several hundred urine samples in order to evaluate the pharmacokinetics of moclobemide and its metabolites in humans and animals. Figs. 3 and 4 show

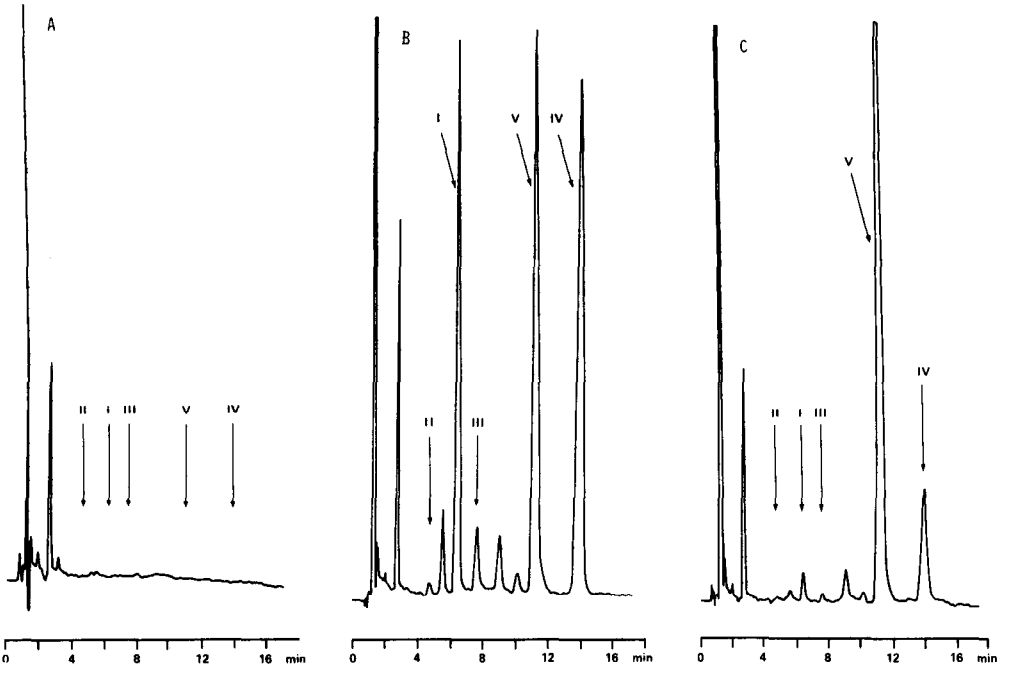

Fig. 3. Chromatograms of human plasma. (A) Before drug administration; (B) 4 h after the administration of a 400-mg oral dose of moclobemide; the concentrations are 1130 ng/ml for I, 223 ng/ml for III and 2280 ng/ml for IV; (C) 12 h after administration of a 400-mg oral dose of moclobemide; the concentrations are 51.6 ng/ml for I, 22.9 ng/ml for III and 398 ng/ml for IV.

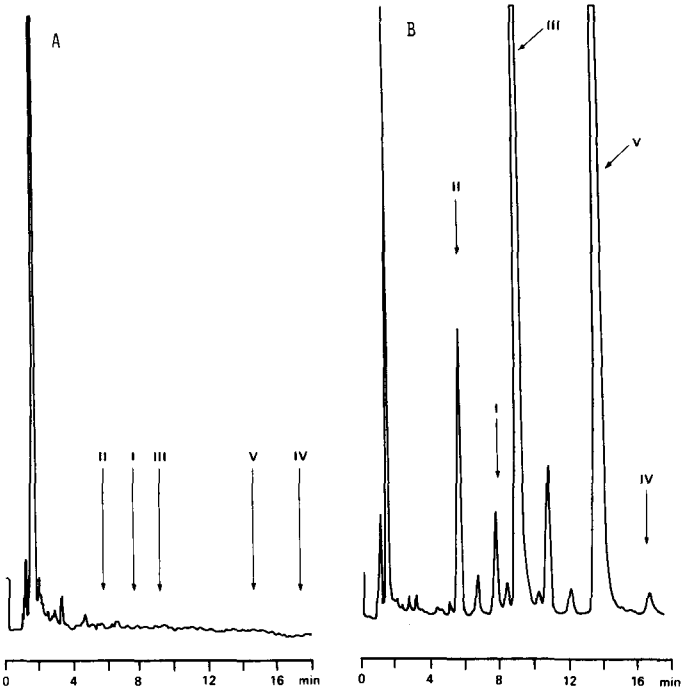

Fig. 4. Chromatograms of human urine. (A) Before drug administration; (B) urine collected 2–4 h after the administration of a 150-mg i.v. dose of moclobemide containing 1.05 μg/ml I and 16.1 μg/ml III.

TABLE III

INTER-ASSAY REPRODUCIBILITY FOR PLASMA

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	n	Difference between found and added concentration
Moclobemide (I)	50	49.7	7.9	5	−0.6
	200	202	3.3	5	+1.0
	750	781	1.9	5	+4.1
	1500	1534	1.0	5	+2.3
Metabolite II	50	50.2	3.8	5	+0.4
	200	201	4.7	5	+0.5
	750	790	3.3	5	+5.3
	1500	1542	2.1	5	+2.8
Metabolite III	50	48.9	5.4	5	−2.2
	200	200	4.1	5	0
	750	770	2.7	5	+2.7
	1500	1520	3.9	5	+1.3
Metabolite IV	50	52.0	13.5	16	+4.0
	200	195	6.9	9	−2.5
	500	511	3.5	19	+2.2
	1000	1055	3.4	9	+5.5

representative chromatograms of human plasma and urine samples obtained at different times during a pharmacokinetic trial. The plasma concentration–time curves for moclobemide (I) and its metabolites III and IV demonstrate that the plasma levels of these substances could be followed for more than four half-lives, which was sufficient to enable the calculation of pharmacokinetic parameters (Fig. 5), whereas the concentration levels of the metabolite II in human plasma were too low for this purpose.

TABLE IV

INTER-ASSAY REPRODUCIBILITY FOR URINE

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	n	Difference between found and added concentration
Moclobemide (I)	0.2	0.21	8.2	19	+5.0
	1	1.06	4.5	21	+6.0
	2.5	2.65	3.6	19	+6.0
	5	5.34	3.1	21	+6.8
Metabolite III	2	2.0	5.5	18	0
	10	9.6	3.2	19	−4.0
	25	24.5	4.9	20	−2.0
	50	50.2	3.9	21	+0.4

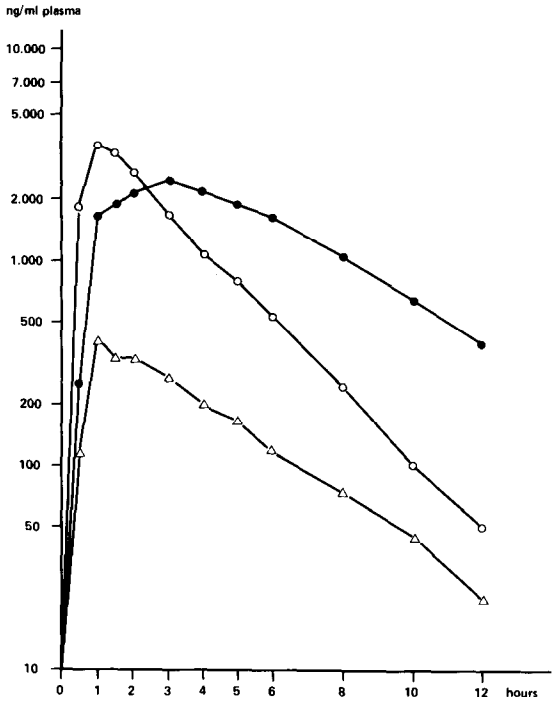

Fig. 5. Plasma concentration-time curve for moclobemide (○) and its metabolites III (△) and IV (●), following the administration of a single 400-mg oral dose of moclobemide to a healthy volunteer.

REFERENCES

1 M. Da Prada, R. Kettler, H.H. Keller and W.E. Haefely, Mod. Probl. Pharmacopsychiatry, 19 (1983) 231-245.
2 J. Raaflaub, P. Haefelfinger and K.-H. Trautmann, Arzneim.-Forsch., 34 (1984) 80-82.
3 R. Jauch, 1982, personal communication.
4 K.P. Maguire, T.R. Norman, B.M. Davies and G.D. Burrows, J. Chromatogr., 278 (1983) 429-433.
5 U. Timm, D. Dell and D.A. Ord, Instrum. Comput., 3 (1985) 29-35.
6 B.-A. Persson, S.-O. Jansson, M.-L. Johansson and P.-O. Lagerström, J. Chromatogr., 316 (1984) 291-300.
7 J.S. Kiel, S.L. Morgan and R.K. Abramson, J. Chromatogr., 320 (1985) 313-323.
8 K.E. Bij, C. Horvath, W.E. Melander and A. Nahum, J. Chromatogr., 203 (1981) 65-84.
9 K. Sugden, G.B. Cox and C.R. Loscombe, J. Chromatogr., 149 (1978) 377-390.
10 R. Jauch, 1982, personal communication.
11 U. Timm, M. Wall and D. Dell, J. Pharm. Sci., 74 (1985) 972-977.