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Determination of the polar drug dimiracetam in human plasma and serum by column-switching high-performance liquid chromatography

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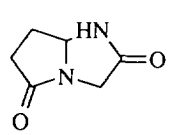
Abstract

A sensitive and fully automated column-switching HPLC method was developed for the determination of a novel cognition enhancer, dimiracetam, in human plasma and serum. A sample of plasma was ultrafiltered and then directly injected onto a first μBondapak C_{18} column. The sample was eluted with water. The portion of the eluate (heart-cut) from this column containing the drug was selected and loaded on a second μBondapak C_{18} column and eluted with water. During the elution of the second column, the first column was automatically rinsed with water–acetonitrile (50:50, v/v) and then equilibrated for a new injection. The total analysis time for each sample was 35 min. This corresponded to a 30% decrease in analysis time compared with the time that would have been needed with a simple run with two μBondapak C_{18} columns assembled in series. Ultraviolet detection at 200 nm was used to monitor the eluate. The method was fully validated over the quantitation range 27 ng/ml–15 μg/ml. The applicability of the method was demonstrated by analysis of serum samples from a study performed in human healthy volunteers.

1. Introduction

Dimiracetam is a new compound [1] belonging to the pharmacological class of cognition enhancers, selected among a series of bicyclic lactams. It contains both the 2-pyrrolidone and 4-imidazolidinone moieties, already recognised as important structural features for cognition enhancing activity; moreover it contains the backbone of piracetam [2] and oxiracetam [3] with the acetamide side chain restricted in a folded conformation. When given orally it is 10–30 times more potent than the reference drug oxiracetam. The molecular structure of dimiracetam is shown in Fig. 1. It is a very polar



(I)

Fig. 1. Structure of dimiracetam.

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compound of small molecular mass (M_r 140.1), its solubility in water is 0.3 g/ml and the partition coefficient in *n*-octanol–water is 0.035. Its UV absorption is due to the amide moiety with the maximum at ca. 200 nm, a wavelength at which many endogenous plasma components absorb.

The high-performance liquid chromatographic (HPLC) method in the present study uses two μBondapak C_{18} columns and only water as eluent, operating at 200 nm without an internal standard and with minimum sample handling. The total analysis time for elution of dimiracetam and reconditioning of the clean-up column after the water–acetonitrile rinse is 35 min for each sample. Since dimiracetam is virtually unmetabolized [4], only the parent compound had to be determined.

2. Experimental

2.1. Reagents and chemicals

Water (HPLC grade) was obtained from a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Acetonitrile (HPLC grade) was supplied by Merck (Darmstadt, Germany). Dichloromethane, orthophosphoric acid 85% (w/v), 1 *M* sulphuric acid, and sodium phosphate were purchased from Carlo Erba (Milan, Italy). Triethylamine and trifluoroacetic acid were supplied by Pierce (Rockford, IL, USA). Ultrafree-MC filters, 10 000 NMWL, polysulfone membrane, were from Millipore. Micro test-tubes of 1.5-ml capacity were from Eppendorf (Hamburg, Germany).

2.2. Standard solutions and calibration standards

A stock solution of dimiracetam was prepared in water at a concentration of 750 μg/ml. It was prepared weekly and stored at +4°C. Stability checks showed it to be stable for at least one month. Aliquots of the stock solution were diluted with water to give appropriate working standard solutions. Calibration standards were prepared by adding 10-μl volumes of the corresponding working standard solution to 0.5 ml of drug-free human plasma to give a concentration range of 0.023–15 μg/ml.

2.3. Apparatus

A schematic representation of the column-switching system is given in Fig. 2.

The HPLC system consisted of the following components: a solvent delivery system of two pumps (A and B) (Model 510, Waters Assoc., Milford, MA, USA), connected to an automated gradient controller (AGC) (Model 680, Waters) delivering the mobile phase to the clean-up column, and an autosampler (AS) (Model 1050, Hewlett-Packard, Avondale, PA, USA) for sample injection. The nitrogen-actuated valve (V1) for column switching consisted of a pneumatic actuator (Model 5701, Rheodyne, Cotati, CA, USA), a solenoid valve kit (Model 7163, Rheodyne), a six-port valve (Model 7010, Rheodyne) and was controlled by the external time events of the AGC.

The solvent was delivered to the analytical column by pump (C) (Model 510, Waters). Detection was performed with a UV-Vis detector (UV) (Model Spectromonitor 3200, LDC Analytical, Thermo Instrument System, Riviera Beach, FL, USA) operating at 200 nm, connected with a manually switched six-port valve (V2) (Model 7120, Rheodyne) to the clean-up column (during the heart-cut adjustment) or to the analytical column (during analysis). The computing integrator was a Hewlett-Packard Model 3396A.

2.4. Columns and mobile phases

Both the clean-up and analytical columns were μBondapak C_{18} (300 × 3.9 mm I.D., Waters Assoc.). The apparatus operated as a fully automated system, using water (eluents A and C) to elute the analyte from both columns and water–acetonitrile (50:50, v/v, eluent B) to rinse the clean-up column after each injection. Water was delivered by pumps A and C and water–acetonitrile (50:50, v/v) by pump B. The following

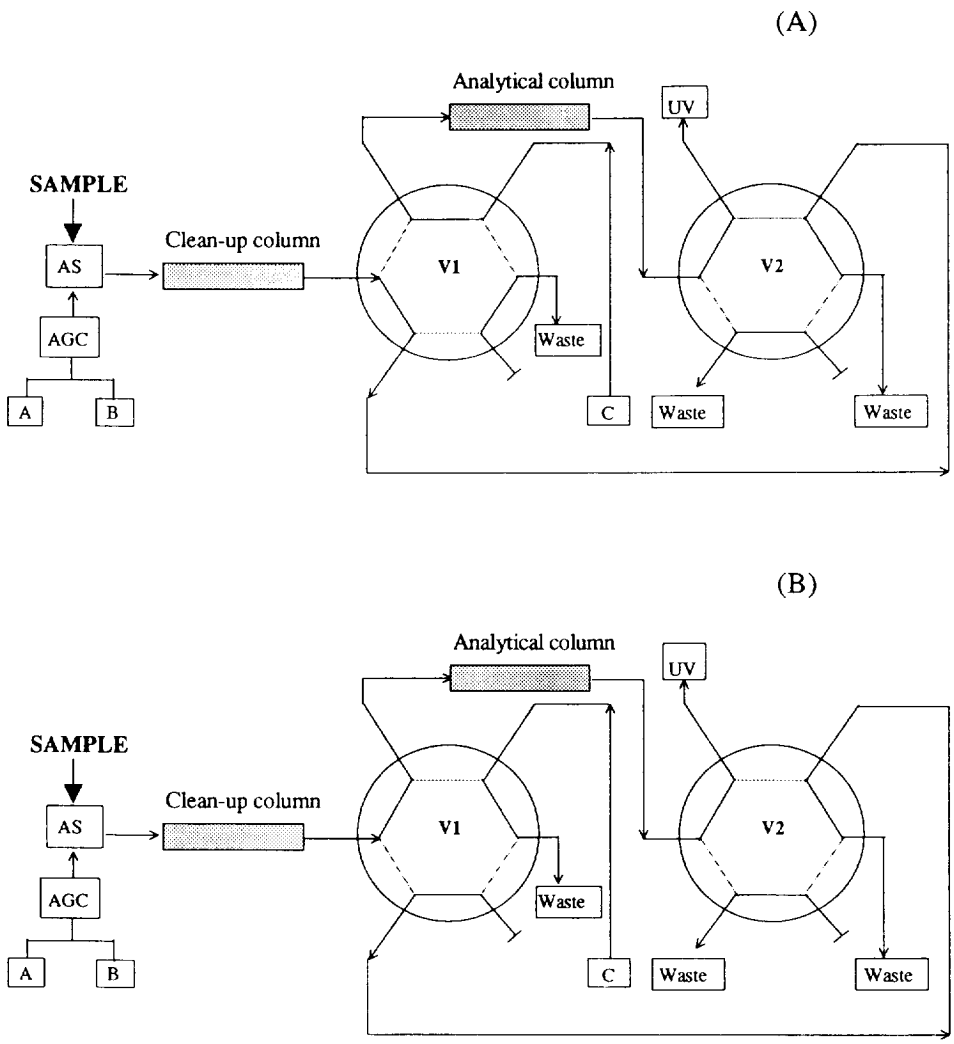

Fig. 2. Schematic representation of the column-switching system for the determination of dimiracetam. For further details see text. (A) Sample application and analytical step; (B) heart-cut.

gradient system was used for the clean-up column: 100% A for 12 min; then after changing from 0 to 50% B within 1 min, the clean-up column was rinsed with 50% B for 3 min; the reconditioning with 100% A lasted for 15 min. The flow-rates of the mobile phases were 1 ml/min for both columns.

2.5. Column-switching conditions

The sample was injected on the clean-up column by the autosampler, using water as eluent at a flow-rate of 1 ml/min. The retention time of dimiracetam on this column was ca. 10.2 min (Fig. 3A).

The heart-cut of the eluate was selected between ca. 9 and 11 min after injection (it had to be precisely adjusted as recommended in section 2.6) and loaded onto the analytical column. Also the analytical step was performed with water as mobile phase at a flow-rate of 1 ml/min. The overall retention time through the two-column system was about 20.5 min (Fig. 3B). During the analysis on the analytical column, the first col-

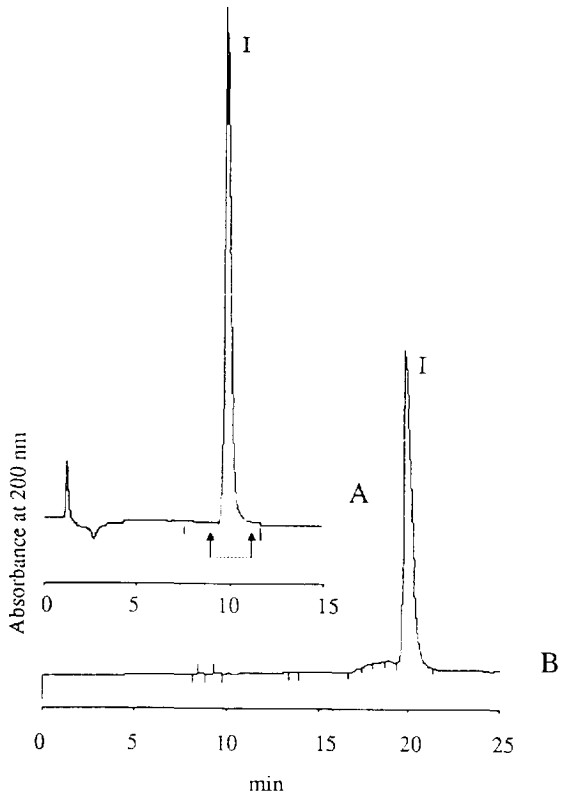

Fig. 3. Chromatogram of dimiracetam standard (A) on the clean-up column (heart-cut adjustment mode), (B) on the column-switching system (analysis mode). Ordinate UV absorption at 200 nm (mAU).

umn was automatically rinsed with water–acetonitrile (50:50, v/v) and then reequilibrated for a new injection.

Two operation modes could be used:

–heart-cut adjustment mode, with valves V1 and V2 connecting the eluate of the clean-up column directly to the detector.

–analysis mode, in which valve V1 was time-actuated after injection so that the eluate of the clean-up column was sent to waste (0–9 min) (loading step, Fig. 2A), to the analytical column (9–12 min) (heart-cut, Fig. 2B), and then again discharged (12 min–end run) (analytical step, Fig. 2A). Valve V2 directed the eluate of the analytical column to the detector throughout the analytical run.

2.6. Heart-cut adjustment

The retention time on the clean-up column had to be checked to adjust the fraction of eluate to be loaded onto the analytical column. This was done manually every 20 injected samples as follows: an aqueous solution of dimiracetam at a concentration of 2 μg/ml was prepared and 100 μl was injected onto the clean-up column, sending the eluate directly to the detector by proper switching of valves V1 and V2. The retention time of dimiracetam on the clean-up column was then checked (see Fig. 3A). A 0.1-min delay in the retention time, due to the dead volume of the system from valve V1 to the detector, was considered negligible in selecting the start of the heart-cut. The retention time may slightly vary from one column to another and also over the time course of use of the column. The accepted range of variation was: 9.7–11.5 min. The heart-cut of the eluate was generally selected from 1 min before the retention time of dimiracetam and lasted for 2 min. A volume of 100 μl of the aqueous solution used for heart-cut adjustment was injected every 10 samples in the analysis mode to control the heart-cut by comparing the reproducibility of the peak area of dimiracetam.

2.7. Sample preparation

Frozen samples were allowed to thaw at room temperature and were then homogenised on a vortex-mixer. A 400-μl aliquot of human plasma or serum was transferred into an Ultrafree-MC filter unit and then centrifuged at 15 000 g for 10 min. Aliquots of 100 μl of the ultrafiltered solution were then injected for analysis.

2.8. Calibration

For calculation of the calibration curve, three separate replicates of each of six different concentrations of dimiracetam in human plasma were performed. The best linear relationship between concentration and response was determined by a least-squares linear regression ($y = a + bx$, where y is the peak area of dimiracetam

and x is the concentration in μg/ml) and linearity was tested by analysis of variance.

Five human plasma standards, covering the expected concentration range, were carried through the procedure routinely together with the unknown samples on each analysis day.

3. Results and discussion

3.1. Chromatography

As dimiracetam is a very polar compound, the correct choice of an appropriate stationary phase for both the clean-up and the analytical columns was very important. The use of conventional normal-phase packings, frequently used for the separation of polar compounds [5], was not feasible because the use of apolar eluents was incompatible with the plasma and serum matrices and because the extraction of the drug from biological samples was not possible without complicated and time-consuming handling. On the other hand, owing to its high hydrophilicity, dimiracetam was expected to be poorly retained on columns containing reversed-phase materials.

Nevertheless, a large number of columns containing medium polarity materials (amino, diol, cyano) and reversed-phase packings [C_1, C_2, C_8, C_{18}, poly(styrene–divinylbenzene)], produced by several manufacturers were tested. The eluents used were different mixtures of water–acetonitrile, and phosphate buffers at various pH values. Dimiracetam was poorly retained on medium polarity stationary phases and attempts to increase the retention time on the amino column by addition of dichloromethane or sulphuric acid to the eluent as reported for oxiracetam [6], were unsuccessful.

As regards reversed-phase packings, dimiracetam showed poor retention, even with water as eluent, on almost all tested columns, with exception of μBondapak C_{18}. The influence of different water–acetonitrile mixtures on the retention of dimiracetam was then investigated on several reversed-phase packings (C_1, TMS Zorbax; C_2, Spheri 10; C_{18}, μBondapak). The data obtained are graphically represented in Fig. 4. The highest retention was obtained with μBondapak C_{18}. As can be seen, the retention of dimiracetam was increased by very low and very high acetonitrile percentages, while at intermediate percentages the retention times were short. This could be explained by the solvophobic and silanophilic interactions [7–9] of dimiracetam. With no organic modifier present, the driving force for the interaction between the analyte and the aliphatic moiety of the stationary phase is caused by the tendency of water to decrease the non-polar surface of the molecules exposed to the solvent. The chromatographic process consists in the reversible association between the analyte and the hydrophobic ligand on the stationary phase and therefore is ruled by the hydrophobic effect. With very high acetonitrile contents the predominant interaction occurs between the analyte and the accessible silanol groups at the surface of the alkyl-silica bonded phase; in this case the silanophilic interaction is the driving force for the retention.

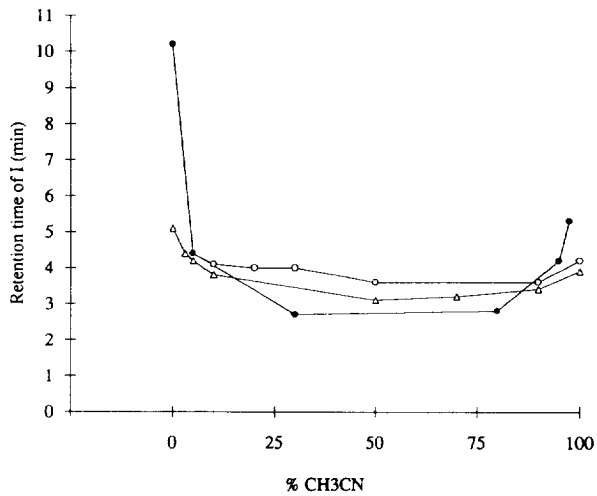

Fig. 4. Influence of acetonitrile content (% in water) in the mobile phase on the retention of dimiracetam on reversed-phase materials: (○) C_1, TMS Zorbax; (△) C_2, Spheri 10; (●) C_{18}, μBondapak.

Based on the above and taking into account the compatibility with the biological matrix, we choose water as eluent. However, rinsing of the clean-up column with acetonitrile after the elu-

tion of dimiracetam was required in order to clean the column from the retained plasma or serum components.

On the other hand, the reconditioning step is also important, because even traces of acetonitrile in the eluent caused a dramatic decrease in the retention time of dimiracetam. With the column-switching system used the clean-up column could be rinsed with a suitable eluent and reconditioned with water for 15 min while analysis was being performed on the second column, thus substantially shortening the analysis times.

3.2. Selectivity

Blank human plasma samples from different sources and human serum samples from sixteen different volunteers were tested. Fig. 5 shows the complex chromatographic profile of blank plasma on the clean-up column (A) and the result obtained after heart-cutting (B). Thus the column-switching system used permitted a good separation of dimiracetam from the endogenous components of the biological matrices.

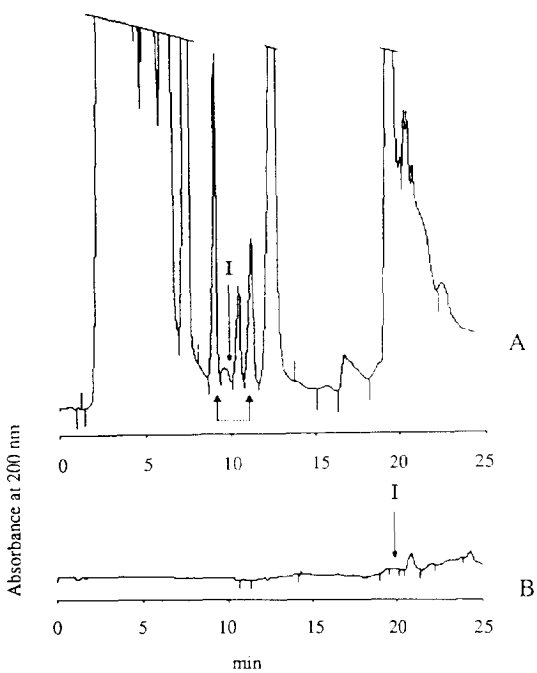

Fig. 5. Chromatographic profile of a blank plasma sample (A) on the clean-up column, (B) on the column-switching system (the arrows indicate the position where dimiracetam elutes). Ordinate UV absorption at 200 nm (mAU).

3.3. Linearity

Under the conditions described, the calibration graph was linear (at the $p = 0.05$ significance level) over the concentration range 0.023–15 μg/ml.

A typical equation of the calibration line obtained for dimiracetam is: $y = 0.07560 + 0.81877x$.

3.4. Accuracy, precision and lower limit of quantitation (LLQ)

Accuracy and precision of the method were determined by replicate analysis of blank human plasma spiked with five different concentrations of dimiracetam within the range 27 ng/ml (expected LLQ)–15 μg/ml. Six replicates of each concentration were analysed on each of three separate days.

The results obtained are shown in Table 1. The average within-run precision, expressed as average of the within-run C.V. for each concentration, excluding the LLQ was $<8.9\%$ and was 18.0% at the LLQ. The between-run precision, expressed as C.V. of the within-run mean concentrations for each concentration, excluding the LLQ, was $<12.4\%$ and was 16.8% at the LLQ. The average accuracy, expressed as average of the accuracy values for each run, determined for each concentration, including the LLQ, ranged from 95.7% to 112.6%. The LLQ, established on the basis of these reproducibility results was 27 ng/ml.

3.5. Stability

Dimiracetam was stable in human serum and plasma kept frozen at −20°C for 10 months; no degradation was observed after three repeated freeze–thaw cycles. After ultrafiltration, serum samples were stable on the autosampler plate for at least 18 h.

Table 1
Accuracy and precision data for dimiracetam

Nominal concentration (μg/ml)		Found concentration[a] (μg/ml)			Average within-run precision[b]	Between-run precision[c]	Average accuracy[d]
		Day 1	Day 2	Day 3			
14.464	Mean	13.771	15.075	14.055			
	C.V.	9.0%	3.3%	4.2%	5.5%	4.8%	
	Accuracy	95.2%	104.2%	97.1%			98.9%
7.232	Mean	6.693	7.523	7.324			
	C.V.	6.3%	3.9%	1.5%	3.9%	6.0%	
	Accuracy	92.5%	104.0%	101.3%			99.3%
0.108	Mean	0.113	0.106	0.091			
	C.V.	6.7%	11.3%	8.8%	8.9%	10.3%	
	Accuracy	104.6%	98.1%	84.3%			95.7%
0.054	Mean	0.058	0.048	0.061			
	C.V.	10.3%	8.3%	6.1%	8.2%	12.4%	
	Accuracy	107.4%	88.9%	113.0%			103.1%
0.027	Mean	0.031	0.035	0.025			
	C.V.	22.5%	11.4%	20.0%	18.0%	16.8%	
	Accuracy	115.6%	129.6%	92.6%			112.6%

[a] Six replicates were performed.
[b] Average within-run precision: $[(C.V._{day\ 1} + C.V._{day\ 2} + C.V._{day\ 3})/3] \cdot 100$.
[c] Between-run precision: $(s_{determined\ from\ the\ within\text{-}run\ means}/average_{within\text{-}run\ means}) \cdot 100$.
[d] Average accuracy: $[(accuracy_{day\ 1} + accuracy_{day\ 2} + accuracy_{day\ 3})/3] \cdot 100$.

3.6. Recovery

The absolute recovery from human plasma and serum was assessed by calculating dimiracetam concentrations in spiked plasma and serum samples, covering the complete quantitation range relative to a calibration curve prepared in water. The results are reported in Table 2. The recovery can be considered quantitative ($>95.8\%$ for both plasma and serum) and constant (C.V. $<3.5\%$) over the total quantitation range. These results together with the selectivity results allowed to consider the two blank media equivalent, and also to avoid the use of an internal standard.

3.7. Application to clinical samples

The method was applied to the analysis of serum samples from a study performed on human healthy volunteers treated with different single oral doses of dimiracetam (50, 100, 200, 400 and 800 mg). A representative chromatogram obtained from a subject given a single dose of 50 mg is reported in Fig. 6. Serum profiles of representative subjects for each dosage are shown in Fig. 7. The method was sensitive enough to measure the dimiracetam concentrations up to 24 h post-dose.

4. Conclusions

A procedure involving direct injection of ultrafiltered human plasma or serum in an automated column-switching system was developed for the determination of dimiracetam. Since dimiracetam is a polar compound, the choice of a satisfactory chromatographic system for its determination in biological fluids was complex. The developed method combined sample clean-up and chromatographic separation in one process,

Table 2
Recovery data of dimiracetam from plasma and serum

Nominal concentration (μg/ml)	Plasma		Serum	
	Found concentration (μg/ml)	Recovery (%)	Found concentration (μg/ml)	Recovery (%)
0.026	0.024	92.3	0.025	96.2
0.212	0.215	101.4	0.209	98.6
1.060	1.008	95.1	1.003	94.6
10.598	10.031	94.6	10.186	96.1
14.964	14.283	95.4	14.283	95.4
Mean (%)		95.8		96.2
C.V. (%)		3.5		1.6
n		5		5

using only water as eluent and with short analysis times, allowing a considerable saving of both chemicals and time. As the method requested minimum sample pretreatment and the chromatographic apparatus was automated, at least 30 samples could be analysed in a 24-h period.

Dimiracetam could be quantified over the range 27 ng/ml–15 μg/ml in human plasma and serum with good linearity, recovery, selectivity, precision and accuracy. The method was sensitive enough to measure the plasma and serum concentrations after oral administration of dimiracetam to human volunteers in dose-range

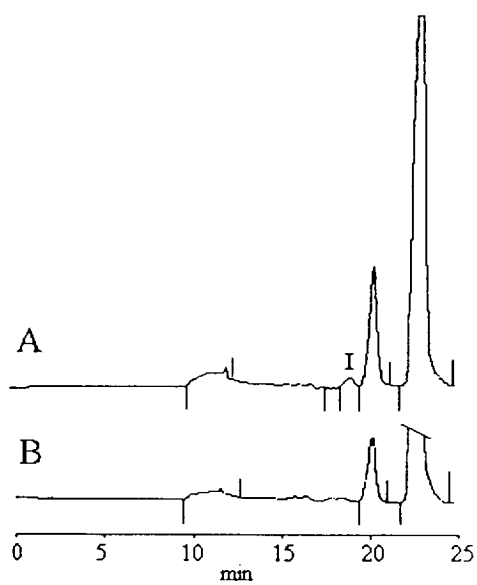

Fig. 6. Chromatograms of (A) a 24-h serum sample following a single 50-mg oral dose of dimiracetam, concentration 90 ng/ml; (B) corresponding pre-dose sample. Ordinate UV absorption at 200 nm (mAU).

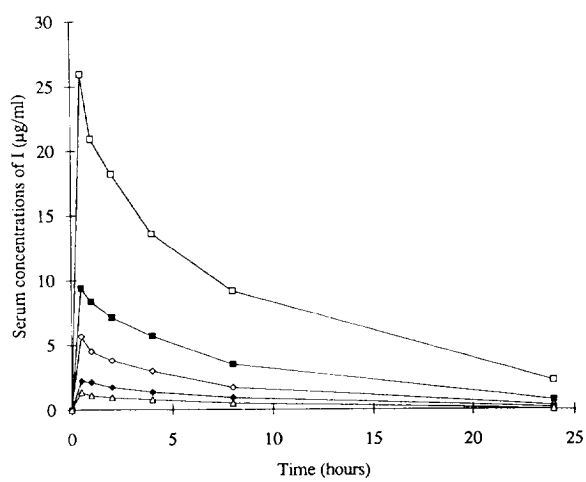

Fig. 7. Serum profiles of dimiracetam in healthy volunteers following oral doses of (△) 50, (◆) 100, (◇) 200, (■) 400 and (□) 800 mg.

finding studies. More than 250 serum samples were analysed by the present method.

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