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Note

Determination of tenilsetam in human plasma and urine by high-performance liquid chromatography

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Tenilsetam (CAS 997), (±)-3-(2-thienyl)-2-piperazinone (I, Fig. 1) is a new potential nootropic agent which has shown improvement in impaired cognitive functions in animal models [1–3] and increased vigilance in geriatric patients [4]. An assay was required to determine the parent drug in plasma and urine samples from human volunteer trials so that the pharmacokinetic parameters of the compound could be estimated. High-performance liquid chromatography (HPLC) using UV detection provided a simple methodology to routinely perform these determinations.

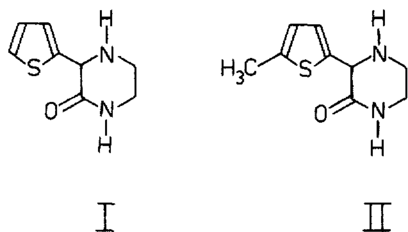

Fig. 1. Structures of tenilsetam (I) and the internal standard (II).

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EXPERIMENTAL

Chemicals and reagents

Tenilsetam and the internal standard (II, Fig. 1) were obtained from Cassella (Frankfurt, F.R.G.). Acetonitrile (far UV grade), ammonium acetate (HPLC grade), dichloromethane (AR) and sodium hydroxide (AR) were all purchased from Fisons (Loughborough, U.K.). Dichloromethane was re-distilled before use.

Chromatographic instrumentation

The liquid chromatograph consisted of an isocratic pump (Constametric III, Milton Roy, Stone, U.K.), an autosampler (WISP 712, Waters Assoc., Harrow, U.K.) and a UV absorption detector (Spectromonitor D, Milton Roy, Stone, U.K.). The analytical column was 20 cm$\times$4.6 mm I.D. stainless steel packed with Spherisorb C6 reversed-phase silica material (5 μm particle size; Phase Separations, Queensferry, U.K.) and was used in conjunction with a 1.5 cm$\times$3.2 mm I.D. guard column packed with RP2 (7 μm particle size; Newguard, Brownlee, Anachem, U.K.). The detector was operated at a wavelength of 236 nm with a 0.5-s time constant. On-line data processing of the detector signal was by a data system (3350X, Hewlett-Packard, Winnersh, U.K.) with the A/D converter operating at 2 Hz.

The mobile phase was prepared by mixing together 0.01 *M* ammonium acetate (900 ml) and acetonitrile (100 ml). This was degassed before use with helium and pumped through the column at a flow-rate of 1.0 ml/min at ambient temperature.

Preparation of plasma samples

An aqueous solution of the internal standard (1 μg in 50 μl) was pipetted into an 8-ml screw-cap glass test tube. The plasma sample (0.5 ml) was added and thoroughly mixed using a vortex mixer. Dichloromethane (5 ml) and 1 *M* sodium hydroxide (0.5 ml) were added and the plasma extracted for 5 min on a rotary inversion mixer at 20 rpm. The phases were separated by centrifugation at 2000 *g* for 5 min and then the upper plasma phase was aspirated and discarded. Tubes containing emulsified extracts were placed on a vortex mixer for a few seconds and were centrifuged again before any remaining plasma was aspirated. The organic phase was transferred to a 10-ml tapered glass test tube which was placed in an aluminium heating block maintained at 40–50°C. The solvent was evaporated to dryness under a gentle stream of nitrogen. HPLC mobile phase (100 μl) was added and the residues were dissolved with the aid of a vortex mixer. An aliquot (50 μl) of this solution was injected onto the chromatograph.

Preparation of urine samples

These were processed in the same way as the plasma samples except that 10 μg of internal standard were added to each sample and the volume of the aliquot injected onto the chromatograph was reduced to 10 μl.

Calibration

Aqueous solutions of tenilsetam were added to blank plasma and urine to produce concentrations of 2 and 10 μg/ml, respectively. Each matrix was subdivided and stored at −20°C. The response factor was determined for each batch of samples by analysing six of the calibration samples in parallel with the unknowns. The day-to-day and within-day variations in the response factor were used to monitor assay performance. A check on linearity was made by also including quality control samples containing known amounts of tenilsetam which covered the working ranges of the assay. Blank samples were also included to estimate possible background interference.

Assay validation

A series of plasma samples and a series of urine samples containing known amounts of tenilsetam at eleven different concentrations were prepared by adding aqueous solutions of tenilsetam to the blank matrix. The samples were subdivided and stored deep frozen (−20°C) in polystyrene tubes and then each series was analysed by the assay described on six separate occasions.

The quality control samples, which were analysed with every batch of unknowns to monitor the assay performance, also provided data on the storage stability of tenilsetam in the two matrices.

RESULTS AND DISCUSSION

Assay performance

Data from the analysis of the two series of validation samples were used to evaluate the plasma and urine assays. Measurements made at four of the eleven concentration levels are summarized in Tables I and II.

The minimum day-to-day precision of the plasma assay over the range 10–100 ng/ml was ±3.3 ng/ml and above this (0.1–25 μg/ml) averaged 1.7% of the measured mean values. Day-to-day precision of the urine measurements averaged 2.2% over the working range of the assay (0.1–250 μg/ml).

Estimates of the within-day precision were made from the calibration samples and averaged 1.8% for plasma and 2.1% for urine ($n=6$).

The measured plasma concentrations were within 2 ng/ml of the amount added over the range 10–100 ng/ml and were within an average of 1.2% of the amount added over the rest of the working range. Urine concentrations were estimated to be within 0.02 μg/ml of the amount added between 0.1 and 1 μg/ml and within 3.0% of the amount added between 1 and 250 μg/ml.

TABLE I

RECOVERY OF TENILSETAM AFTER ADDITION TO PLASMA

Concentration added (μg/ml)	Concentration found (mean ± S.D., $n=6$) (μg/ml)	Mean recovery (%)	Coefficient of variation (%)
0	0 ± 0		
0.0246	0.0265 ± 0.0019	107.7	7.1
0.237	0.248 ± 0.005	104.6	2.2
2.45	2.44 ± 0.002	99.7	0.9
24.4	24.2 ± 0.3	99.3	1.4

TABLE II

RECOVERY OF TENILSETAM AFTER ADDITION TO URINE

Concentration added (μg/ml)	Concentration found (mean ± S.D., $n=6$) (μg/ml)	Mean recovery (%)	Coefficient of variation (%)
0	0.020 ± 0.015		
0.099	0.114 ± 0.002	115.2	1.8
0.973	0.998 ± 0.024	102.6	2.5
9.72	10.0 ± 0.2	103.0	2.3
99.0	102 ± 2	103.2	1.6

The limit of detection (defined as twice the minimum standard deviation) is 6 ng/ml for plasma and 0.02 μg/ml for urine. In routine use the minima of the working ranges (defined as the point at which the bias exceeds 20% of the measured value) are 10 ng/ml for plasma and 0.1 μg/ml for urine.

Selectivity

Endogenous background material in plasma or urine extracts were well separated from tenilsetam and the internal standard on the HPLC system described (Figs. 2 and 3). Caffeine, theobromine and theophylline, arising from normal dietary intake, could potentially interfere as they had similar retention times to the analytes. Theobromine and theophylline were not fully resolved from tenilsetam but were not extracted under the alkaline conditions chosen, whereas tenilsetam extracts with equal efficiency over the pH range 5–10. Caffeine was still present in the extracts but is resolved from the internal standard (Fig. 2) and only causes problems if present in high concentrations. This was not normally the case as volunteers were on a caffeine-restricted diet in the majority of studies analysed.

Interference from other drugs was not investigated as the samples for anal-

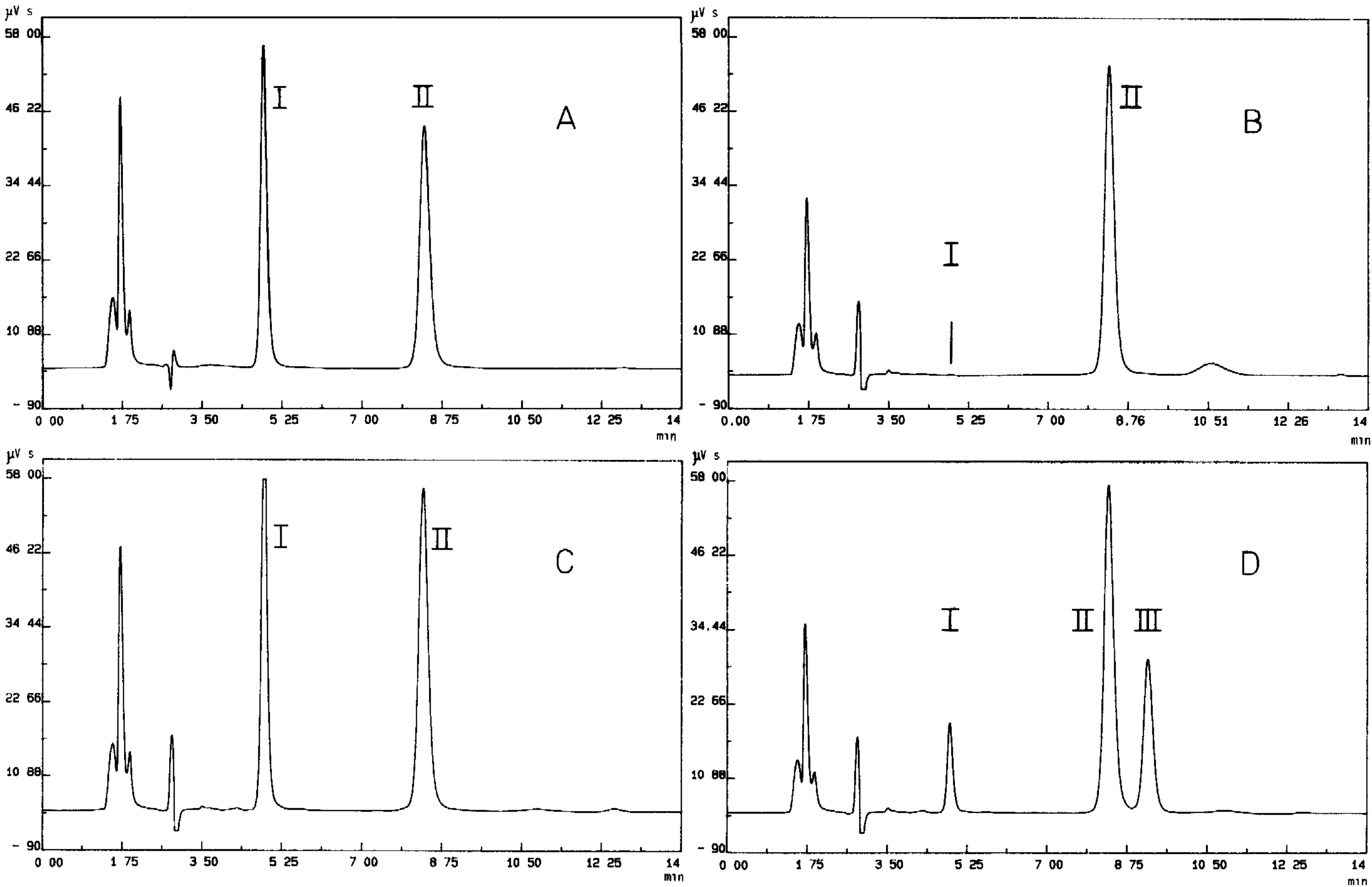

Fig. 2 Representative chromatograms of plasma extracts showing tenilsetam (I), internal standard (II) and caffeine (III). (A) Calibration sample containing 2 $\mu g/ml$ tenilsetam and the internal standard; (B) pre-dose sample from a volunteer; (C) sample taken 24 h after oral administration of 150 mg tenilsetam containing 1.72 $\mu g/ml$ I; (D) sample taken 72 h after oral administration of 150 mg tenilsetam containing 0.391 $\mu g/ml$ I.

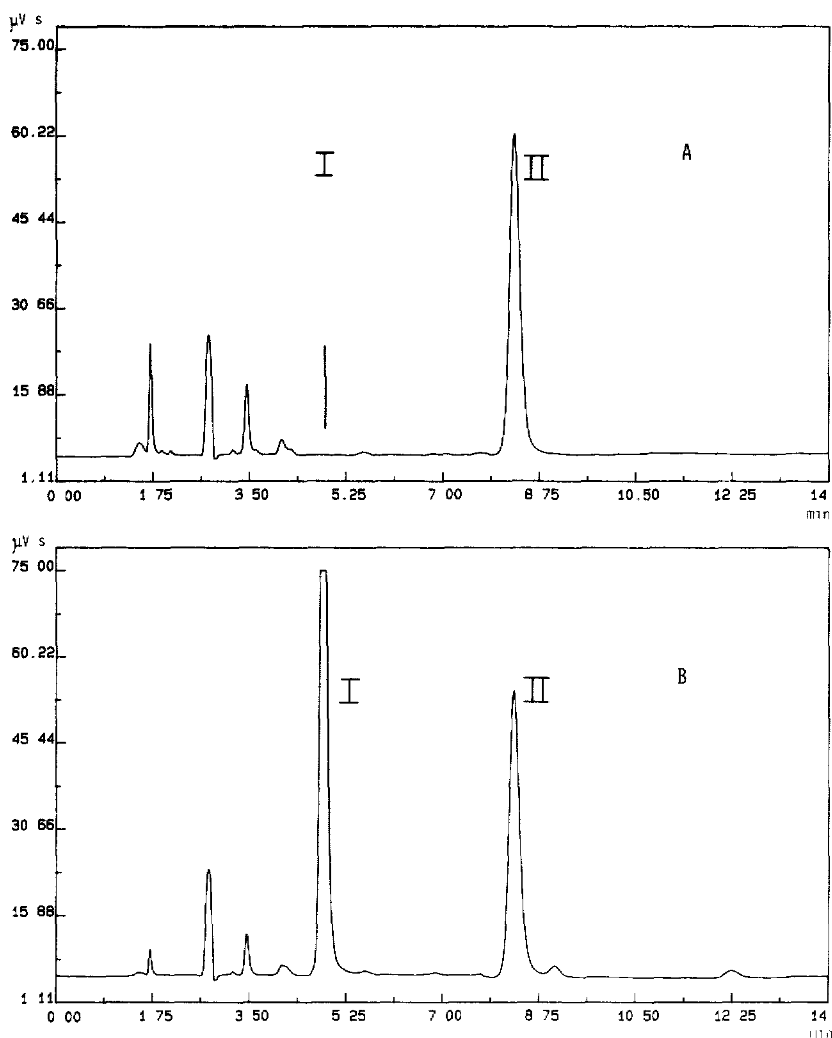

Fig. 3. Representative chromatograms of urine extracts. (A) Pre-dose sample from a volunteer; (B) sample collected 24–36 h after oral administration of 150 mg tenilsetam containing 18 2 µg/ml tenilsetam (I) and 10 µg/ml internal standard (II).

ysis were obtained from pharmacokinetic trials in which tenilsetam was administered alone.

Application

The assay has been applied to samples resulting from several pharmacokinetic studies and has been found to have sufficient sensitivity for both plasma and urine determinations in these studies. In one such trial six volunteers each received a single oral administration of tenilsetam (150 mg) in capsule form

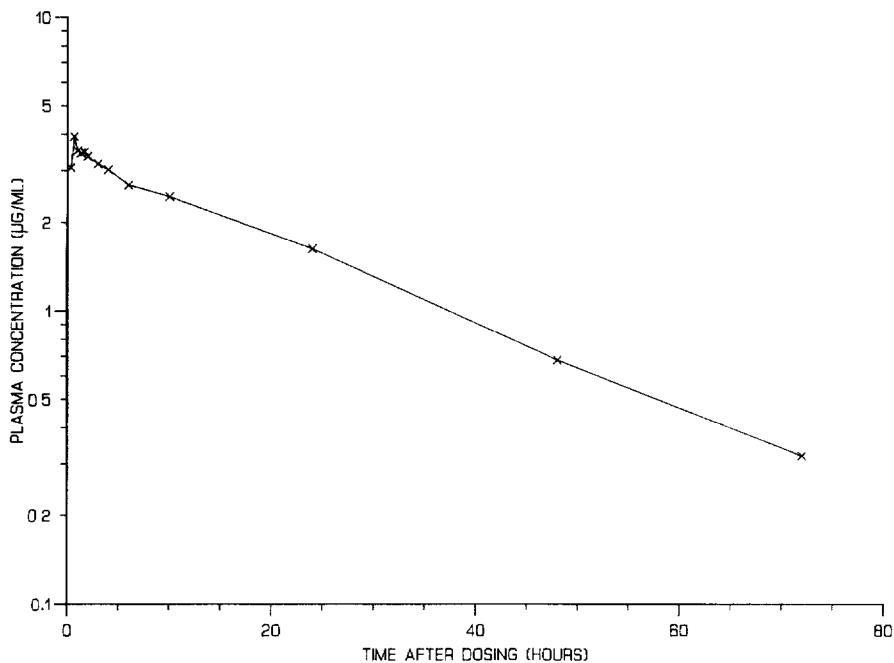

Fig. 4. Mean plasma profile of six volunteers after oral administration of tenilsetam (150 mg).

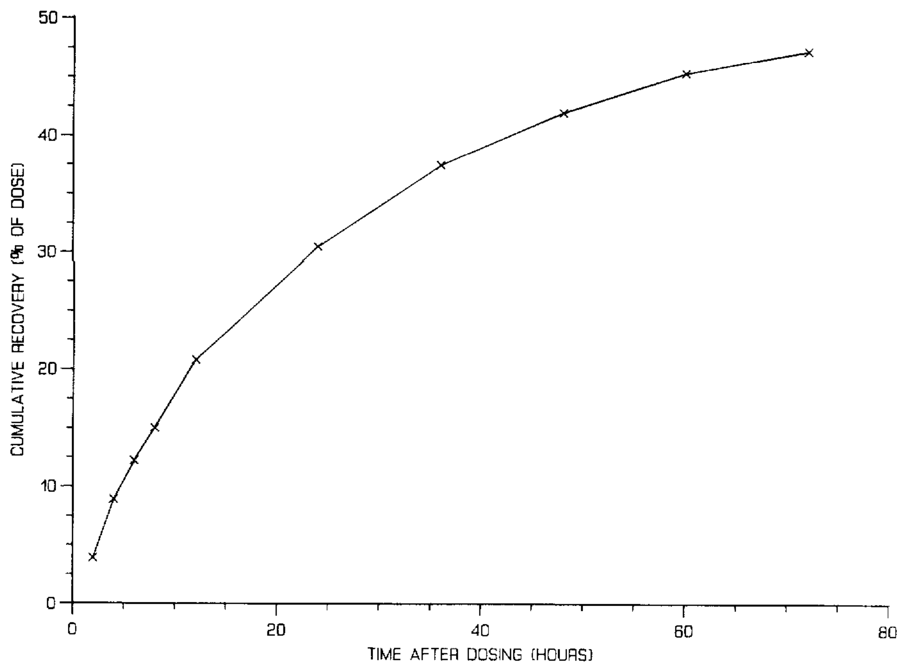

Fig. 5. Mean urinary recoveries from six volunteers after oral administration of tenilsetam (150 mg).

and blood samples were taken over the following 72 h. The mean plasma profile of these volunteers is shown in Fig. 4 and has a maximum value of 3.93 ± 0.64 μg/ml at 40 min after administration. Urine samples were also collected during this period and Fig. 5 shows the mean cumulative recovery from these subjects. Urine concentrations found in the samples reached a maximum of approximately 70 μg/ml with $47.3 \pm 3.4\%$ of the dose recovered over 72 h.

ACKNOWLEDGEMENT

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