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Note

Determination of tacrine hydrochloride in human serum by chloroform extraction, reversed-phase high-performance liquid chromatography and fluorimetric detection

D.R FORSYTH, J.M. FORD, C.A. TRUMAN, C.J.C. ROBERTS* and G.K. WILCOCK

University Department of Medicine, Bristol Royal Infirmary and Department of Care of the Elderly, Frenchay Hospital, Bristol BS2 8HW (U.K.)

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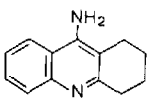
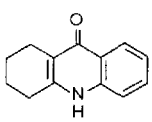
Tacrine hydrochloride (THA) is a centrally acting reversible cholinesterase inhibitor, which has been used in the management of anti-cholinergic overdoses and it is currently under investigation as a potential therapy in Alzheimer's disease [1–3]. This is a progressive and fatal dementia characterised by loss of short-term memory and intellectual failure. Its aetiology is possibly related to depletion of central cholinergic activity. It is important that a sensitive and precise assay be available for the quantification of this drug so that dosing requirements can be assessed. Its chemical structure is shown in Fig. 1. The sensitivities of two high-performance liquid chromotagraphic (HPLC) methods using ultraviolet detection described by Yago et al. [4] and Hsieh et al. [5] were found to be inadequate for clinical use. In our hands the fluorimetric method described by Park



THA, 9-amino-1 2,3,4-tetrahydroacridine



INTERNAL STANDARD, 1,2,3,4-tetrahydro-9-acridanone

Fig. 1. Chemical structures of THA and internal standard.

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et al. [6] yielded non-reproducible results at low concentrations due to similar excitation and emission wavelengths. Therefore an assay using a dual monochromator fluorescence detector and incorporating a novel internal standard has been developed.

EXPERIMENTAL

Apparatus

A Perkin Elmer MPF3 fluorescence spectrophotometer (Beaconsfield, U.K.) was used to determine the optimal excitation and emission wavelengths for the preliminary scan of THA. A Shimadzu liquid chromatography pump was used with a Shimadzu RF-535 fluorescence HPLC monitor (Dyson Instruments, Hetton, U.K.). The sensitivity selector switch of this dual monochromator instrument was set at the high position (100 times more sensitive than low position) with the excitation wavelength at 330 nm and the emission wavelength at 365 nm. A Pye Unicam CDP4 (Philips Scientific, Cambridge, U.K.) computing integrator was used at attenuation 8 (=8mV full scale), peak width 6, peak threshold 200 with a chart speed of 0.5 cm/min. A Pye Unicam PU8251 pen recorder was used to determine the signal-to-noise ratio, using the 10-mV setting at a chart speed of 300 mm/h. THA samples were injected through a Rheodyne 7125 valve (Dyson Instruments) fitted with a 100-μl loop. The chromatography was performed on a 25 cm$\times$0.5 cm I.D. Shandon Hypersil ODS column (HPLC Technology, Macclesfield, U.K.) with 5 μm particle size packing. A 5 cm$\times$0.5 cm I.D. Shandon Hypersil ODS solvent conditioning column with 5 μm particle size packing was placed between the pump and the injection valve. A range of SMI micro/pettors (Alpha Laboratories, Eastleigh, U.K.) was used throughout the analysis and were regularly calibrated and checked for accuracy. The mobile phase was a mixture of methanol–distilled water (49.5:49.5, v/v) with 1% (v/v) triethylamine adjusted to pH 5 with Analar orthophosphoric acid (15.9 *M*). The filtered mobile phase was constantly degassed with helium during analysis. The flow-rate was set at 1.5 ml/min at a temperature of 19–21°C.

Reagents

1,2,3,4-Tetrahydro-9-acridanone was selected as internal standard (I.S.) because of its stability in methanol and close chemical similarity to THA (Fig.1). THA and internal standard were obtained from Aldrich (Gillingham, U.K.). Triethylamine and Tris base were obtained from Sigma (Poole, U.K.). HPLC-grade methanol, Analar chloroform, Analar sodium sulphate and Analar orthophosphoric acid were obtained from BDH (Poole, U.K.). Analytical reversed-phase Hypersil columns and pre-columns were obtained from HPLC Technology. All glassware was cleaned in 30% (v/v) hydrochloric acid and washed thoroughly with distilled water before use.

Preparation of THA standards and internal standard

Normal human serum from the Blood Transfusion Unit at Southmead Hospital Bristol was extracted using the following method and found to be free of any

co-eluting peaks. This serum was spiked with varying amounts of THA giving final concentrations of 1, 2, 5, 10 and 20 ng/ml. An identical range of THA standards in distilled water was also prepared. The full range of THA serum standards was extracted in duplicate, the mean peak areas were compared with those for the non-extracted THA water standards and recovery was assessed. Prior to analysis 25 μl of the internal standard stock solution (1000 ng/ml) were added to each sample. Quantitation was based on the peak-area ratio of THA in the sample to I.S. A full range of spiked serum standards from 1 to 20 ng/ml was used to calibrate the CDP4 computing integrator and the results were stored in memory. The patients' extracted serum could then be reported directly in ng/ml.

Patients' samples

Venous blood samples from patients with Alzheimer's disease were taken at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16 and 24 h after a 25- and a 50-mg single oral dose of THA separated by an interval of three days. The clotted blood was centrifuged at 1000 *g* for 10 min at 19–21 °C and the serum separated. No inhibitors were added and samples were stored at −20 °C until analysis. The patients selected were all drug-free and involved in a clinical trial of THA in the management of Alzheimer's disease. Approval for the study had been obtained from Frenchay Health District Ethical Committee.

Assay validation

Intra-assay variation was assessed at 1, 5 and 20 ng/ml by simultaneously extracting ten samples for each of these concentrations. Analysis was then performed in the same assay. Inter-assay variation was assessed at 5 ng/ml by extraction on ten consecutive days. The standard curve was constructed on a second occasion from spiked serum standard samples that had been prepared six weeks previously and stored frozen at −20 °C.

Extraction procedure

The serum samples were defrosted overnight at 4 °C and then gently mixed at 19–21 °C. Duplicate 1-ml aliquots of serum were mixed with 25 μl of I.S. and 500 μl of 1.5 *M* Tris–HCl buffer (pH 10). The samples were then extracted twice with 5 ml chloroform by vigorous mixing on a vortex mixer for 45 s. After centrifugation at 1000 *g* for 10 min at 19–21 °C the lower organic layers were carefully removed, combined and mixed with a small quantity of sodium sulphate and recentrifuged at 1000 *g* for 5 min at 19–21 °C. The clear upper organic layer was decanted and evaporated to dryness at 40 °C under a gentle stream of compressed air. The dried samples were dissolved in 1 ml of mobile phase for HPLC or stored at −20 °C for future analysis. To check the validity of the standard curve stored in the integrator memory a THA-spiked serum standard was extracted with each set of patient samples. Patient 0-h serum samples were extracted to exclude interfering substances.

Pharmacokinetics

A linear equation was fitted by least-squares linear regression to the terminal phase of the plot of the natural log of the serum drug concentration against time.

Elimination half-life was then calculated by dividing the natural logarithm of 2 by the rate constant so obtained. Area under the curve extrapolated to infinity was calculated by the trapezoidal rule up to the end of the sampling time plus a terminal portion calculated from the elimination rate constant.

RESULTS

A typical example of a chromatogram is shown in Fig. 2. From this it can be seen that all the peaks of interest are resolved within 6 min and the retention times for THA and I.S. are 3.5 and 4.7 min, respectively. An unknown peak (U) was observed at 2.1 min which was not present in any patient's serum before administration of THA or in the samples spiked with THA or I.S. but was present in all patients' serum after THA administration. Another small peak of unknown identity was noticed in some serum samples at approximately 1.9 min and when present it was resolved and integrated separately from the unknown at 2.1 min.

The serum concentrations of THA in the same patient plotted against time are shown in Fig. 3. No THA was detectable at the 0.5-h time point after the 50-mg dose. It can be seen that within the limits of the sampling procedure the maximum concentration after the 25-mg dose occurred at 30 min and after the 50-mg dose at 90 min. There was a subsequent linear decay in serum concentrations with both doses. The elimination half-life was 1.2 h after the 25-mg dose and 1.6 h after the 50-mg dose. The area under the curve extrapolated to infinity was 7.5 ng/ml·h for the 25-mg dose and 27.3 ng/ml·h for the 50-mg dose.

THA fluorescence response was linear from 1 to 20 ng/ml in the extracted spiked serum standards ($r=0.9998$; $n=5$; $y=-0.144+5.363x$). The intra-assay

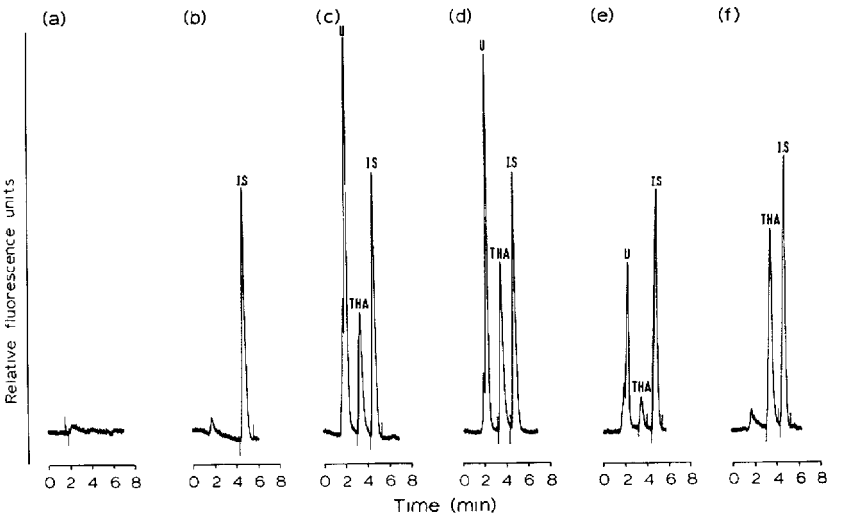

Fig. 2. Chromatograms from a patient (a) extracted 0-h serum, (b) extracted 0-h serum plus I.S., (c) extracted 1-h serum containing 2.8 ng/ml THA after a single 25-mg oral dose plus I.S., (d) extracted 1-h serum containing 4.3 ng/ml THA after a single 50-mg oral dose plus I.S., (e) extracted 10-h serum containing 0.5 ng/ml THA plus I.S. after a single 50-mg oral dose and (f) extracted human serum spiked with 5 ng/ml THA plus I.S. U = unknown possible metabolite of THA.

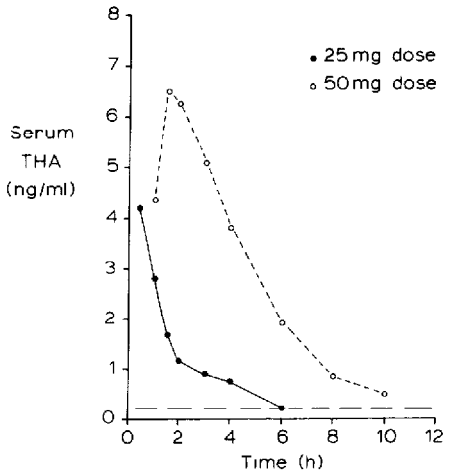

Fig. 3. Serum concentration-time curve in a single patient following two single oral doses.

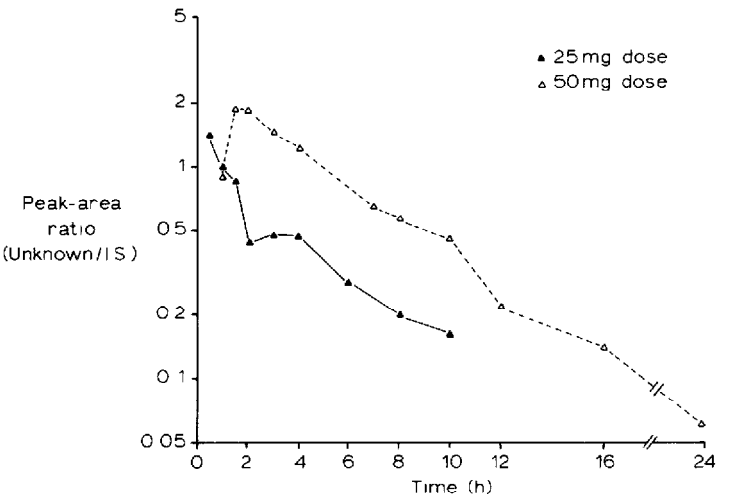

Fig. 4. Plot of peak-area ratio of unknown (U) against time following two single oral doses of THA.

coefficients of variation for spiked serum standards containing 1, 5 and 20 ng/ml were 8.5% ($n=10$), 2.2% ($n=10$) and 3.0% ($n=10$), respectively. The inter-assay coefficient of variation for a spiked serum standard containing 5 ng/ml was 5.0% ($n=10$). The recovery of THA from spiked serum was $71.1 \pm 8.9\%$ (mean $\pm$ S.D.; $n=5$). The recovery of internal standard extracted from serum was similar: $73.1 \pm 3.3\%$ (mean $\pm$ S.D.; $n=5$). The limit of detection of the assay at a signal-to-noise ratio of 2.5:1 was 0.2 ng/ml.

There was no statistically significant difference in the peak-area ratios of the spiked serum standards after six weeks storage at $-20°C$ ($p>0.2$; $r=0.9956$; $n=5$; $y=-0.434+5.579x$).

Fig. 4 shows the rise and decay in the concentration of the unknown peak (U) expressed as a peak-area ratio to the I.S. It can be seen that within the limits of

the sampling procedure the maximum concentration after the 25-mg dose occurred at 30 min, that for the 50-mg dose was at 90 min and there was a subsequent linear decay in serum concentrations with both doses. The half-life of this decay was 2.5 h after the 25-mg dose and 4.3 h after the 50-mg dose. The area under the curves were 4.4 and 11.1 arbitrary units.

DISCUSSION

This method has been shown to provide a sensitive and reliable assay for detecting and quantitating THA after oral administration of standard doses to patients with Alzheimer's disease. It has also detected an unknown substance present in patients' serum only after administration of THA which is possibly a metabolite. The extraction procedure proved simple and effective and the whole assay could be achieved on easily obtained equipment.

The first assay methods described for THA used HPLC with UV detection [4,5]. However, these methods were lacking in sensitivity for clincial use, since the limit of detection was in the region of 10–100 ng/ml. To overcome this, a later method [6] used a filter fluorimetric detector and achieved a detection limit of 2 ng/ml on a spiked plasma sample. However, this sensitivity was still insufficient to calculate the pharmacokinetics of THA at the doses being administered to patients. As can be seen from Fig. 3 the concentration of THA in the patient's serum had fallen below the detection limit quoted by Park et al. [6] of 2 ng/ml within 2 h of the 25-mg dose and at 4–5 h after the 50-mg dose. The increased sensitivity of the present method over the method of Park et al. [6] might be attributed to the fact that a dual monochromator fluorescence detector can select the exact excitation and emission wavelengths of THA which are close. The intra- and inter-assay coefficients of variation were well below the figures of 10.3 and 10.6%, respectively, quoted by Park et al. [6]. This study has provided alternative methodology for studying the kinetics of THA in human serum.

The unknown peak (U) present in the serum of patients who had taken THA in this study has been noticed previously. Park et al. [6] found a peak with a retention time of 4.2 min which they tentatively identified as a metabolic product. However, they did not describe its time course. In this study the rise and decay of the unknown with time has been shown. The early maximum serum concentration of the unknown appears to coincide with that of the parent drug. It is possible therefore that this represents a metabolite formed either in the gut or in the liver by presystemic metabolism. If that proves to be the case it would account for the wide variability in THA concentrations observed between patients in current and previous studies. Identification of the unknown substance is clearly important for the further development of this line of treatment.

Thus this assay has been shown to be suitable for further clinical pharmacological research on THA. If the drug proves useful in the management of Alzheimer's disease and subject to in vitro interference studies, the HPLC–fluorimetric detection method might provide the basis for therapeutic monitoring.

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