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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PIRENZEPINE IN PLASMA

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SUMMARY

A high-performance liquid chromatographic method for the determination of pirenzepine in human plasma is reported using imipramine as an internal standard The assay has a lower limit of detection of 2 5 ng/ml The calibration function is found to be linear in the range from 5 ng/ml up to at least 100 ng/ml Two sets of chromatographic conditions are described, which provide different chromatographic selectivities for the separation of the compounds of interest from other material present in a sample

INTRODUCTION

Pirenzepine (Fig. 1, I) (Gastrozepin®, Dr Karl Thomae GmbH) is an anticholinergic agent, which inhibits gastric secretion [1] During the development of the drug, the pharmacokinetics and the metabolism of pirenzepine were investigated using liquid scintillation counting for the determination of plasma concentrations after administration of radiolabelled pirenzepine [2] A radioimmunoassay (RIA) has been employed for the measurement of pirenzepine in serum and cerebrospinal fluid after administration of therapeutic doses to healthy volunteers [3] The use of these sensitive (and selective, in the case of RIA) techniques was called for, since the mean maximum plasma concentration after 25 mg of pirenzepine given as a single oral dose to volunteers was found to be 25 ng/ml, whereas 24 h after dosing a mean concentration of 7 5 ng/ml was reported [4]

To our knowledge, only one high-performance liquid chromatographic (HPLC) method for the determination of pirenzepine has been published until now [5] This method, however, is more suited for the analysis of dosage

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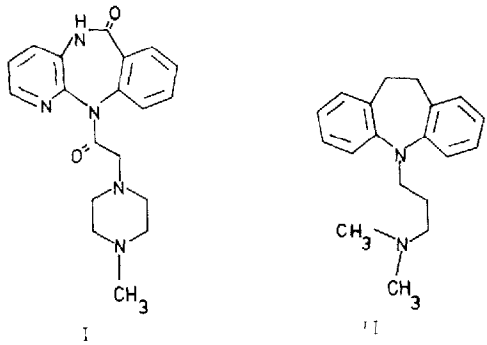

Fig 1 Chemical structures of pirenzipine (I) and imipramine (II)

forms, since it exhibits a limit of detection as high as 1 μg/ml, and is therefore not applicable for the determination of pirenzepine concentrations in the therapeutic range Additionally, an internal standard was not used in the work cited above.

A second publication mentions the HPLC separation of pirenzepine and ranitidine, but a fully developed analytical method is not described [6]. We report here an HPLC assay that is sensitive enough to allow the measurement of pirenzepine in therapeutic concentrations

EXPERIMENTAL

Materials

Pirenzepine was purchased from Karl O Helm (Hamburg, F R G) as the dihydrochloride salt Imipramine (Fig 1, II) was chosen for use as an internal standard and was delivered by Sigma Chemie (Taufkirchen, F.R G) Sodium bicarbonate, dichloromethane and phosphoric acid were obtained from Merck (Darmstadt, F R G) Acetonitrile was received from Riedel de Haen (Seelze, F R G) Triethylamine (97—99% purity) was from Aldrich Chemie (Steinheim, F R.G.). All chemicals were of analytical grade if not stated otherwise Human plasma was obtained from a local blood bank Bovine serum was purchased from Behring (Marburg, F.R.G) Purified water (resistance 18 MΩ) was produced by a Millipore-Q reagent water system (Millipore—Waters, Eschborn, F.R.G.).

Chromatographic apparatus

The chromatographic system consisted of a Model 6000A pump, a WISP 710B automatic sample processor, a system controller and a Data Module integrator, all from Millipore—Waters The UV detector was a Spectroflow 773 variable-wavelength instrument from Kratos (Karlsruhe, F R G) The detector was operated at a wavelength of 280 nm and the range was set at 0 002 a u f s

Chromatographic conditions

Two sets of chromatographic conditions were developed. The first set comprised a 25 cm × 4 6 mm I D Altex Ultrasphere silica 5-μm column (Beckman Instruments, Munich, F.R.G) or a 30 cm × 4 6 mm I D μPorasil silica 10-μm

column (Millipore—Waters) and a presaturator column containing μPorasil (10 μm), which was placed between the pump and the injector The mobile phase used on the silica columns was 0 014 *M* triethylamine in water (adjusted to pH 10 5 with phosphoric acid)—acetonitrile (40 60).

The second set of chromatographic conditions employed a 25 cm × 4 6 mm I D Nucleosil SA ion-exchange 10-μm column (Knauer, Bad Homburg, F R G.) In this case, the mobile phase was 0.1 *M* triethylamine in water (adjusted to pH 3 0 with phosphoric acid)—acetonitrile (45 55) A presaturator column was not used with this mobile phase. The mobile phase was degassed before use under vacuum by sonification. In all experiments, the HPLC pump was operated at a flow-rate of 1 5 ml/min and the columns were kept at room temperature.

Preparation of plasma samples

Samples were stored at −20°C prior to analysis To 1 ml of plasma, 0 1 ml of 1 *M* carbonate buffer (pH 9.0) was added followed by 75 μl of a solution containing 2 μl/ml imipramine in water. The sample was then extracted for 10 min into 5 ml of dichloromethane on a reciprocating shaker After 10 min centrifugation at 4000 *g*, 4 5 ml of the organic layer were pipetted into a clean tube The solvent was then evaporated under a gentle stream of nitrogen at room temperature The residue was finally redissolved in 100 μl of the appropriate mobile phase and 40—60 μl of the sample were injected into the HPLC system

Calibration and calibration samples

Standard solutions were prepared containing 1, 0 5 and 0 1 μg/ml pirenzepine in water, respectively. When stored at 4°C, these solutions can be used for up to four weeks Appropriate aliquots of the standard solutions were added to human plasma or bovine serum to give 1-ml samples containing 5—100 ng/ml pirenzepine Additionally, a blank sample and a reagent blank (which was not spiked with the internal standard) were worked up in each series. The calibration samples were then processed as described above.

From the measured peak-area ratios and the corresponding spiked concentrations, the calibration function was calculated by means of least-squares regression analysis. A complete series of calibration samples was prepared and evaluated on each day of analysis.

RESULTS

The assay method reported in this paper has now been used for the analysis of several hundred plasma samples from clinical trials, under both sets of chromatographic conditions.

Fig. 2 shows three typical chromatograms of samples taken at various time points during a clinical trial The pirenzepine peaks in Fig 2B and C correspond to 35 and 8 ng/ml, respectively The chromatograms demonstrate that the work-up procedure results in sufficiently clean samples with no interfering endogenous material present in the region of interest It can further be seen that the method provides the analytical sensitivity that is necessary to follow-up a 24-h sampling protocol after single-dose administration of pirenzepine

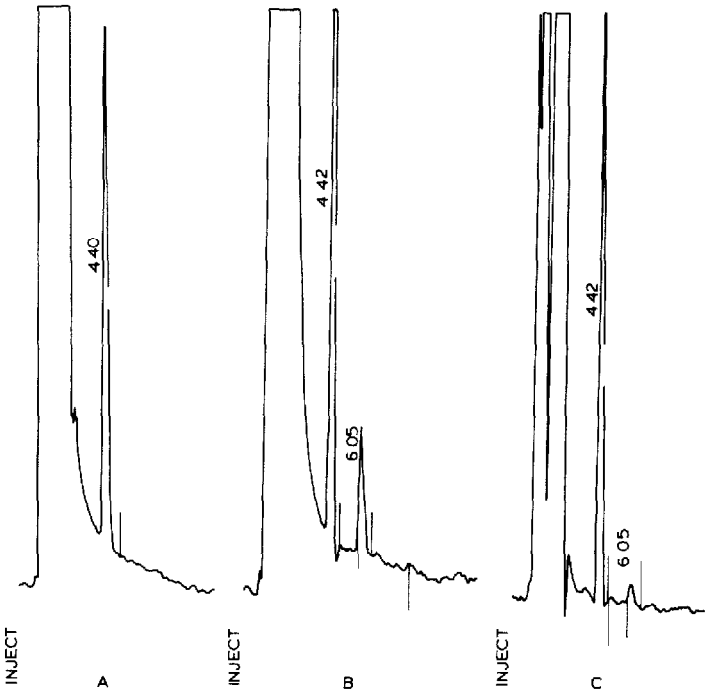

Fig 2 Chromatograms of plasma samples (A) Predose, (B) 3 h and (C) 24 h after a single oral dose of 50 mg of pirenzepine Peak at 4 4 min is the internal standard Chromatographic conditions μPorasil column, for mobile phase, see text

The limit of detection of the method was estimated during the development of the assay. In order to do so, plasma was repeatedly spiked with decreasing concentrations of pirenzepine and subsequently analysed.

Fig 3 depicts one series of results The signal corresponding to 2 5 ng/ml can be clearly distinguished from the blank A calibration sample containing 5 ng/ml was routinely quantitated, thus establishing the lower end of the calibration range. Consequently, under the conditions reported, the limit of quantitation was accepted to be 5 ng/ml, whereas the limit of detection was < 2 5 ng/ml

Due to the high pH value of the mobile phase, the column life of the normal-phase columns was sub-average when the system was operated continuously without intermittent purge steps In consequence, we developed the second chromatographic system. For this purpose, an ion-exchange column was chosen, since early results had indicated unsatisfactory chromatography on reversed-phase materials showing broad front peaks at the sensitivity range of 0.002 a u.f.s. used in our experiments

Fig 4 demonstrates the separation and sensitivity that were achieved using the ion-exchange material The pirenzepine peak in Fig 4B corresponds to 15 ng/ml

With both sets of chromatographic conditions, precise and accurate determinations were possible Peak-area ratios of the calibration samples measured over the entire calibration range on both chromatographic systems showed no statistically significant differences between the systems The calibration

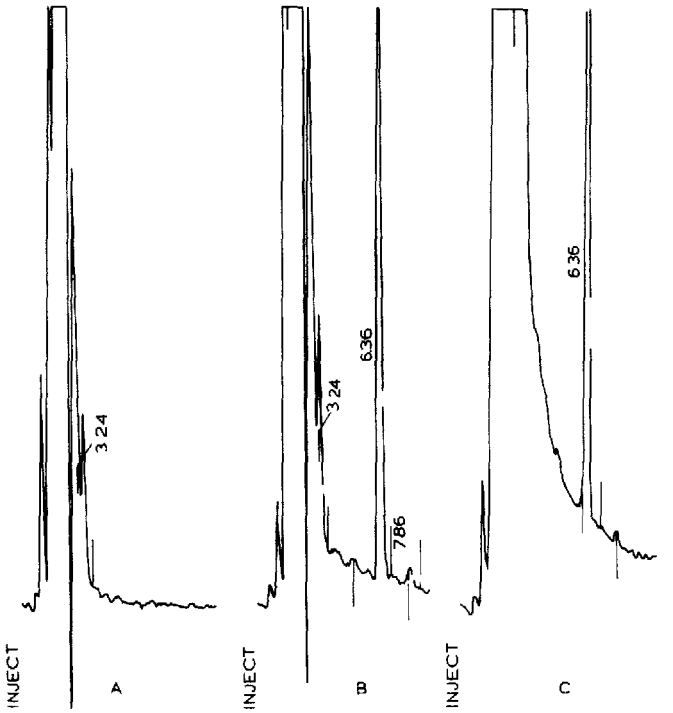

Fig 3 Chromatograms of plasma spiked with pirenzepine (A) Blank plasma, (B) plasma spiked with 5 ng/ml pirenzepine; (C) plasma spiked with 2 5 ng/ml pirenzepine. Peak at 6 4 min is the internal standard, peak at 7 86 min is pirenzepine Chromatographic conditons Altex Ultrasphere Si column, for mobile phase, see text

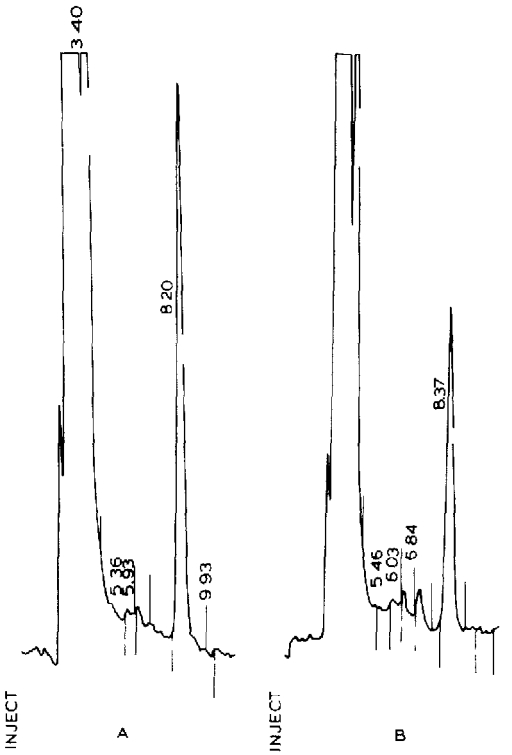

Fig 4 Chromatograms of plasma samples (A) Predose, (B) 8 h after a single oral dose of 50 mg of pirenzepine Peaks at 8 20 and 8 37 min are the internal standard, peak at 6 8 min is pirenzepine Chromatographic conditions Nucleosil-SA column, for mobile phase, see text

function ($y = ax + b$, where y is peak-area ratio and x is concentration) had a slope a of 6 112 and an intercept b of −0 009 on the basis of 150 calibration samples. The correlation coefficient for this set was calculated to be 0.991, thus indicating a linear relationship between concentration and peak-area ratio over the calibration range The coefficients of variation ranged from 25% at 5 ng/ml to 8% at 100 ng/ml ($n \geqslant 17$ at each concentration level). The accuracy of the determinations was monitored by means of control samples of known concentrations, which were stored and analysed together with the test samples The average bias within the calculated results of these control samples was found to be −8% at 5 ng/ml, +10% at 10 ng/ml and −4% at 100 ng/ml

DISCUSSION

Our objective was the development of an HPLC assay for pirenzepine that would reliably measure pirenzepine in samples taken up to 24 h after administration of a single dose of 25 mg We knew from the literature that such an assay should require a limit of quantitation of at least 7.5 ng/ml and that an upper limit of 100 ng/ml in the calibration range would meet our needs [4] Furthermore, the assay method should be simple and allow a sufficient number of samples to be analysed in the laboratory.

A number of solvents were tested for the liquid—liquid extraction step that was used for clean-up and concentrating Most of them showed good extraction recoveries of ca 80%, which were similar for both substances of interest Finally, we chose dichloromethane for our method, because this solvent gave cleaner extracts compared to others, e g chloroform. This fact became increasingly important when our experiments demonstrated that pirenzepine and imipramine could not be re-extracted with satisfactory yield into small volumes of acid

A sufficiently high pH value is required for the successful extraction of both compounds from aqueous systems into organic solvents Since the use of carbonate buffer resulted in acceptable recoveries and good phase separation, possible alternatives were not investigated

It is possible to further increase the recoveries up to ca 95% by extracting twice with 3 ml of solvent each. Since increased peak areas were found for both compounds after this modification, an increased analytical sensitivity may be achieved in this way

During a survey on stationary phases for HPLC at an early phase of this work, we found that pirenzepine and imipramine could be separated on silica columns and ion-exchange columns without interferences from co-extracted endogenous materials The development of the final mobile phase for the silica column was a simple task, after we had accepted that a pH > 10 in the aqueous part of the mobile phase is inevitable Under these conditions, the retention times of both compounds are decreased with decreasing percentage of organic solvent in the mobile phase

The situation on the ion-exchange column is a more complex one Fig 5 shows the retention times of both compounds as a function of pH and the aqueous constituent of the mobile phase Depending on the percentage of the aqueous buffer in the mobile phase, three different regions can be defined.

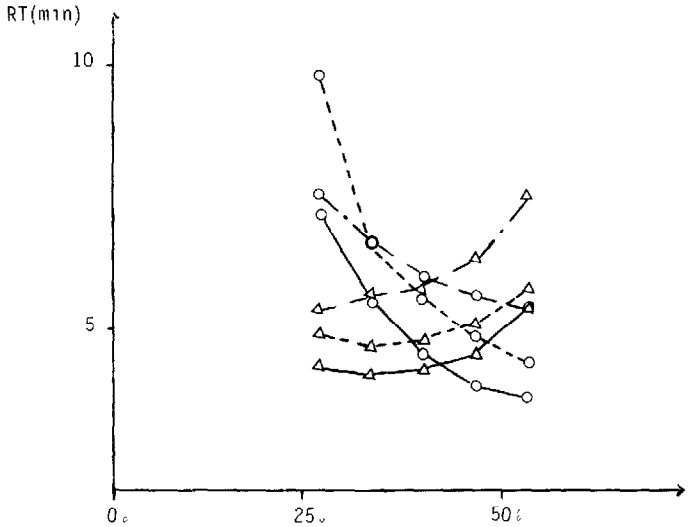

Fig 5 Retention time (RT) as a function of pH and mobile phase composition Abscissa percentage of buffer (0 1 *M* triethylamine) at pH 2 0 (—), pH 5 0 (- - -) and pH 7 0 (— • — • —), acetonitrile was used to make up to 100% Ordinate retention time (○) Pirenzepine, (△) imipramine, the internal standard

At low contents, a separation can be achieved with imipramine eluting first; at higher contents, the separation is pertained with a reversed eluting order, between these two regions, both compounds elute together with little or no separation Thus, the chromatography of pirenzepine and imipramine on an ion-exchange column displayed features of theoretical interest The data strongly suggest a non-uniform mechanism of separation Mechanisms that are possibly operative in the chromatography of tricyclic amines with solvents containing alkylamines as modifiers have been discussed recently [7] We chose the option to use a mobile phase with a high percentage of aqueous buffer, because this resulted in narrower peaks with a better shape for pirenzepine

Both chromatographic systems can be used with good results in routine applications Therefore, the question of which system a user should employ in order to achieve separation free from interferences by endogenous or xenobiotic compounds will entirely depend on a specific clinical situation, with possible influences also from co-medication and disease state We have not yet investigated systematically the influence of possible interferences by other medications

In conclusion, we have developed a sensitive HPLC assay method for the determination of pirenzepine in the low nanogram range, which permits the processing of ca 50 samples per day by one person

REFERENCES

1 R Hammer, C P Bernie, N J Birdsall, A S Burgen and E C Hulme, Nature, 90 (1980) 283
2 R Hammer, G Bozler, A Zimmer and F W Koss, Therapiewoche, 27 (1977) 1575
3 B H Jaup and Ch Blomstrand, Scand J Gastroenterol , Suppl , 15 (Suppl 66) (1980) 35
4 R Hammer, G Bozler, G Heinzel and F W Koss, Arzneim -Forsch , 27 (1977) 928
5 S A Bobhair, J Liq Chromatogr , 7 (1984) 2401
6 J P Desager and M Sclavous, Chromatographia, 15 (1982) 451
7 J S Kiel, S L Morgan and R K Abramson, J Chromatogr , 320 (1985) 313