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Note

Reversed-phase ion-pair high-performance liquid chromatographic determination of 2-carboxythiazolidine-4-carboxylic acid in plasma

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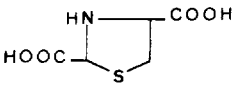
Thiazolidine-4-carboxylic acid, a cyclic cysteine derivative, has been reported as a hepatoprotective drug [1–3] as it is metabolized to cysteine by liver proline oxidase [4]. However, its toxicity toward children and in overdose situations [5] has led to the synthesis of 2-substituted derivatives such as 2-methylthiazolidine-4-carboxylic acid, which is less toxic and more effective than the parent compound [6]. At ambient temperature in acidic aqueous solution the reaction of equimolar amounts of glyoxylic acid and cysteine yields 2-carboxythiazolidine-4-carboxylic acid (I, Fig. 1), the arginine salt of which has been marketed in France as a hepatoprotective and detoxicant drug approved for the treatment of hepatic diseases such as hepatitis and cirrhosis. Moreover, the use of the drug led to an improvement in the precirrhosis and dyspepsia status [7,8].

A specific spectrophotometric method has recently been developed for the study of metabolism and urinary excretion [9] after a high oral dosage of the drug. However, the method involves time-consuming sample preparation steps



(I)

Fig 1 Structure of 2-carboxythiazolidine-4-carboxylic acid

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and an inadequate sensitivity for studying the time course of the drug in plasma after single oral ingestion at the therapeutic dosage. Hence there is a need for a simple method to monitor the levels of I in plasma from both therapeutic and pharmacokinetic points of view. In this paper, a more rapid and sensitive method is described, based on ion-pair high-performance liquid chromatography (HPLC) after sample clean-up by ion-exchange column chromatography.

EXPERIMENTAL

Reagents

All chemicals were purchased from Merck (Darmstadt, F R G). Hydrochloric acid, trichloroacetic acid and ammonium acetate were of analytical-reagent grade, acetonitrile and water were of LiChrosolv quality and tetrabutylammonium hydrogensulphate was of LiChropur quality.

Dowex 50W-X8 (H^+) resin (200–400 mesh) was obtained from Fluka (Buchs, Switzerland) and conditioned with 2 *M* sodium hydroxide and 2 *M* hydrochloric acid; a water rinse followed each treatment The above cycle was repeated five times, then 2 ml of conditioned Dowex resin (H^+) were poured into a 5 cm×0 7 cm I.D. column equipped with a funnel and a plug of quartz-wool.

The cationic resin was regenerated by washing with distilled water, then 10 ml of 2 *M* hydrochloric acid were added and the column was washed with distilled water until the eluate was neutral.

Chromatography

The chromatographic system consisted of a Waters Assoc. (Milford, MA, U S.A) M45 solvent delivery system and a Kontron (Milan, Italy) 722 LC UV detector set at 210 nm. The detector output was quantitated by a Milton-Roy (Riviera Beach, FL, U.S.A.) CI 10B integrator–printer. Chromatographic experiments were performed on a LiChrosorb RP-8 (7 μm) analytical column (250 mm×4 mm I.D.) (Merck) protected by a precolumn equipped with a 10 mm×4 mm cartridge packed with LiChrosorb RP-18 (10 μm) (Merck).

The mobile phase was a 5 95 (v/v) mixture of acetonitrile and 0.001 *M* disodium hydrogenphosphate solution containing 680 mg/l tetrabutylammonium hydrogensulphate with the pH adjusted to 6.5 with 2 *M* sodium hydroxide. The mobile phase was filtered through a 0.45-μm nylon filter (SM 200 06 N; Sartorius, Gottingen, F.R.G.) and degassed under vacuum before use.

All experiments were performed isocratically at a flow-rate of 1.5 ml/min at ambient temperature (18–24°C). The void volume was determined by injection of methanol and measurement of the time from injection to the top of the peak

Standard solutions

A stock solution of 1 mg/ml I was prepared in distilled water and stored at 4°C. Working standard solutions containing 10 and 20 μg/ml I were prepared daily by sequential dilutions of the stock solution with distilled water.

Procedure

Blood samples from rat or man were collected in heparinized containers and centrifuged at 2300 *g* for 10 min at 4°C to separate plasma The proteins in 5-ml aliquots of plasma were precipitated with 0 5 ml of trichloroacetic acid solution (65%, w/v) and the sample was centrifuged at 15 000 *g* for 15 min at 4°C The supernatant was drained into the cationic column at a flow-rate of 0.5 ml/min. The column was then washed with 10 ml of 0 05 *M* hydrochloric acid with application of vacuum The elution of I was performed with 1 ml of 2 *M* ammonium acetate solution followed by 5 ml of distilled water under vacuum The two eluates were combined and dried by rotary evaporation under reduced pressure at 40°C The residue was dissolved in 0.5 ml of distilled water before immediate injection of a 50-μl aliquot of the solution into the HPLC column Quantitation was performed by recording the ratio of the peak area versus that of calibration standards of I directly injected into the HPLC column. For every set of five injections two standards containing the same concentration of I were injected. The concentrations of the standards were chosen in the same range as those of the unknown sample.

RESULTS AND DISCUSSION

In order to achieve a reliable and rapid determination of I, the aim of this work was to optimize the separation of I from endogenous compounds causing interference, especially at the detection wavelength of 210 nm.

Clean-up procedure

Interference from endogenous plasma constituents was minimized by protein precipitation before the extraction step. Trichloroacetic acid was chosen in preference to perchloric acid because during the concentration step trace amounts of the latter would oxidize I. The use of a strong cationic column has proved satisfactory as I is strongly retained in acidic conditions (pH 0.6) owing to the complete protonation of the ring NH ($pK_B \approx 6$).

Previous experiments using 2 *M* ammonia solution as eluent were unsuccessful as at pH values higher than 9 compound I is partially decomposed into cysteine/cystine. Accordingly, 2 *M* ammonium acetate solution (pH 7) was used as the eluent.

In order to determine the recovery of I from the column, 5-ml aliquots of three sample types (distilled water, deproteinized plasma and normal plasma) were identically spiked with two different amounts of I (5 and 20 μg).

Four replicates of each standard were acidified, extracted and analysed. The extraction recovery of I from each sample type was determined by comparing the peak area obtained with that resulting from direct injection of a known amount of I. When distilled water or previously deproteinized plasma was spiked with I, the recoveries averaged 90% and were independent of the concentrations of I in the range considered.

When normal plasma was spiked with I before deproteinization the mean recovery yield was lower, averaging 80% The observed 10% decrease was related to the partial adsorption of I by the protein precipitate In addition, no significant decrease in recovery was observed during the concentration step at 40°C

Chromatographic performance

Because of the strong hydrophilic and ionizable character of the drug, an isocratic ion-pair chromatographic system using quaternary ammonium ion in the mobile phase was considered to be the most suitable. Tetrabutylammonium hydrogensulphate was chosen because of its strong lipophilicity and the low UV background absorbance at 210 nm of sulphate ion, which is also known to be a poor ion-pair reagent compared with other mineral anions [10]

Preliminary experiments carried out by injection of drug-free plasma extracts showed that the capacity factors (k') of the endogenous compounds (except that of trichloroacetic acid) were not changed significantly by modifying either the pH or the concentration of the ion-pair reagent, whereas increasing the concentration of the organic modifier in the mobile phase decreased the k' values of the endogenous compounds These preliminary results suggested that the retention of the endogenous compounds did not involve an ion-pair mechanism

In order to achieve a good separation of I from endogenous compounds and taking into account our previous observations, we investigated the effectiveness of varying the chromatographic parameters to increase the retention of the drug versus that of endogenous compounds However, the k' value of I had to be brought to a reasonable magnitude to avoid broadening of the peak and subsequent low sensitivity as the absence of a strong chromophore in I leads to a molar absorptivity (ϵ) of only 2000 l mol^{-1} cm^{-1} at 210 nm

As I contains three ionizable groups ($pK_A \approx 1.2$, $pK_A \approx 4$, $pK_B \approx 6$), it is present as a zwitterion in the pH range 2–5 Therefore, a progressive increase in the retention of I could be predicted by increasing the pH The expected increase in k' with increase in pH is illustrated in Fig. 2A It should be related to the increase in the net negative charge of I with increasing pH. However, at pH >6.5 a counterbalancing effect occurs owing to an increase in the ionic strength. Therefore, the pH of the mobile phase was set at 6.5.

The influence of the concentration of tetrabutylammonium on the retention of I is illustrated in Fig. 2B, showing a decrease in retention above 3 m*M*

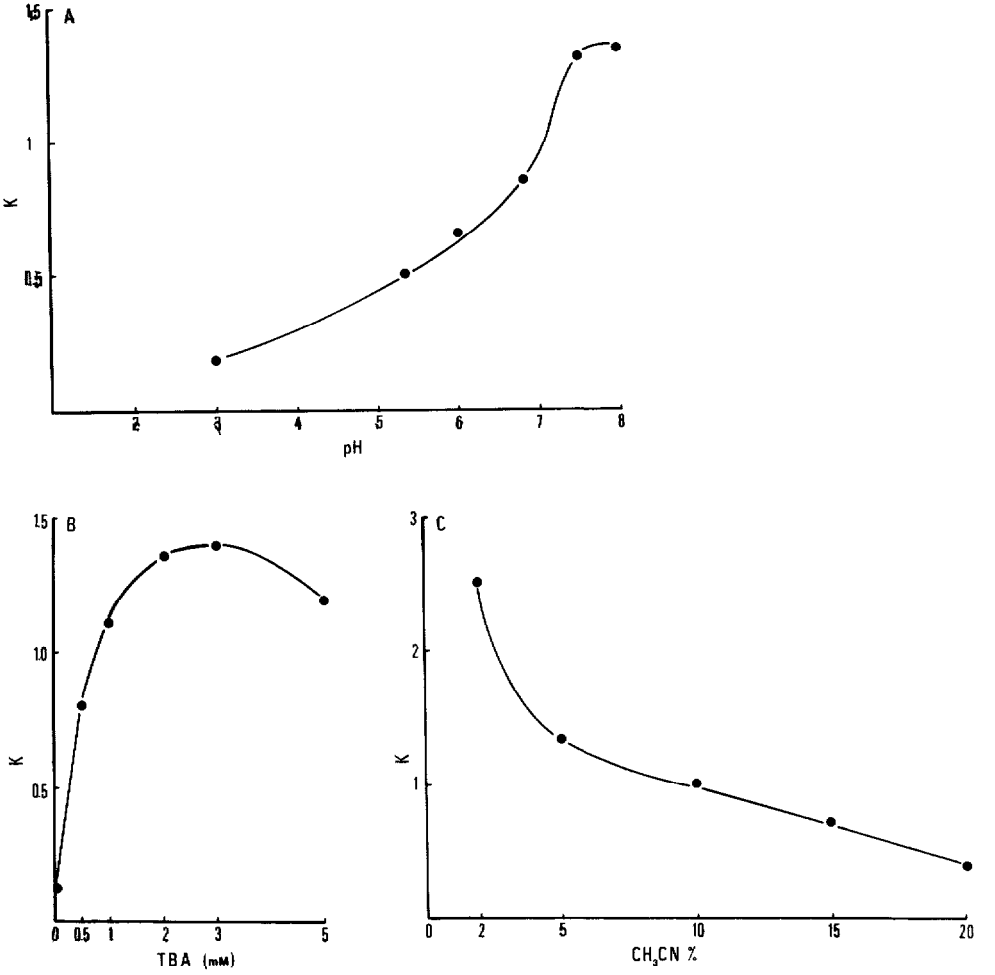

Fig 2 (A) Capacity factor of I (mean values, $n=3$) as a function of pH Mobile phase acetonitrile–1 m*M* disodium hydrogenphosphate buffer (5 95, v/v) containing 2 m*M* tetrabutylammonium Titrated to the appropriate pH with 5 *M* sodium hydroxide Flow-rate, 1 5 ml/min (B) Capacity factor of I (mean values, $n=3$) as a function of ion pair tetrabutylammonium (TBA) concentration Mobile phase acetonitrile–1 m*M* disodium hydrogenphosphate buffer (5 95, v/v), pH 6 5 Flow-rate, 1 5 ml/min (C) Capacity factor of I (mean values, $n=3$) as a function of percentage (v/v) of acetonitrile (CH_3CN) in the mobile phase containing 1 m*M* disodium hydrogenphosphate buffer–2 m*M* tetrabutylammonium, pH 6 5 Flow-rate, 1 5 ml/min

tetrabutylammonium. This result has been reported previously by others [11–14] and could be related to an increase in the amount of sodium sulphate leading in turn to an increase in ionic strength. As shown in Fig. 2C, a change in the proportion of acetonitrile in the mobile phase caused a relatively large change in the retention of I. A low percentage (5%) of acetonitrile was used in

the mobile phase because a higher percentage resulted in incomplete separation of I from endogenous compounds and a lower percentage (2%) caused a longer retention and lower sensitivity

Fig. 3 shows typical chromatograms obtained by injection of extracts from drug-free human plasma and spiked human plasma

Using a 5-ml aliquot of plasma, the calibration graph was linear over the concentration range 0 4–50 μg/ml I. In plasma samples, the limit of detection was 0 2 μg/ml based on a signal-to-noise ratio of 3 Using the described procedure, the concentration of drug that could be determined with 10% precision was 0.4 μg/ml The accuracy and intra-assay precision of the method were evaluated by simultaneously assaying four replicate 5-ml plasma standards containing 4 and 1 μg/ml I The coefficients of variation were 4 7 and 12%, respectively For all points, the observed mean recovery was 80% of the prepared value.

Using the described procedure, structurally related compounds were tested for potential interference with the quantification of the drug. L-Proline showed a lower retention ($k' = 0\ 4$) than I whereas 2-methylthiazolidinecarboxylic acid was strongly retained ($k' = 2\ 2$) Its must be noted that the procedure cannot be used for the determination of the drug in urine samples owing to the large number of endogenous UV-absorbing compounds that are not removed by the described clean-up procedure

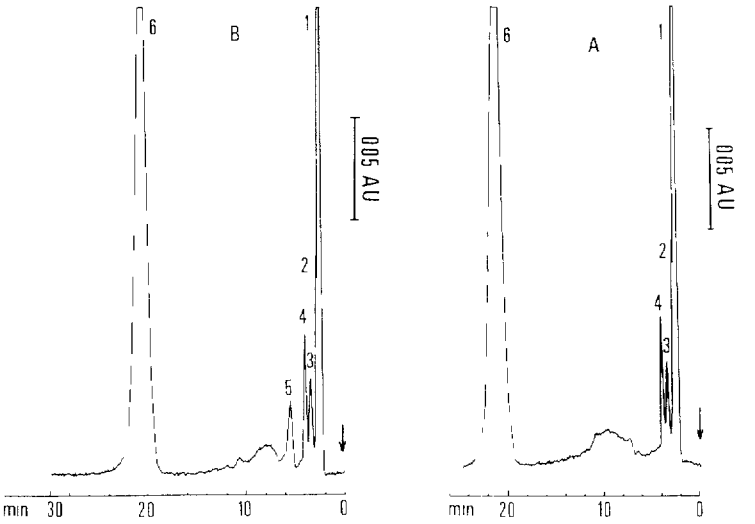

Fig 3 Chromatograms of extracts from (A) drug-free human plasma and (B) plasma spiked with 1 μg/ml I Peaks 1 = chloride ion, 2,3 = unknowns, 4 = unknown (originating from distilled water), 5 = drug, 6 = trichloroacetate ion

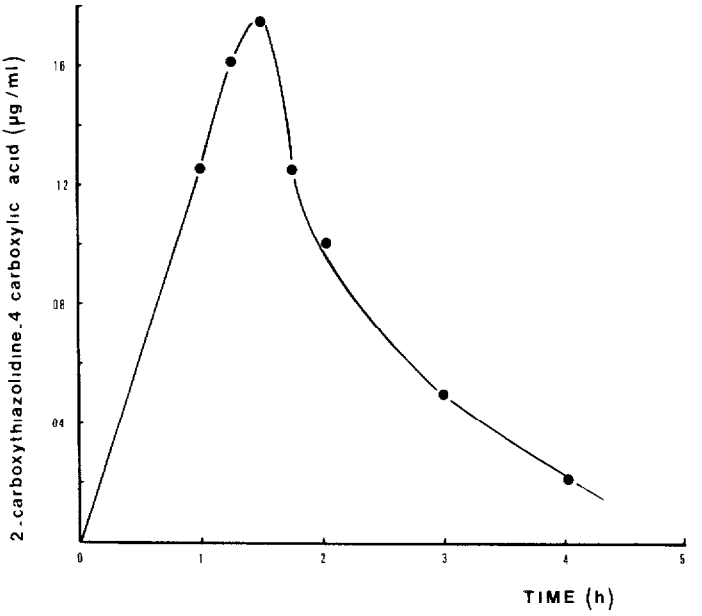

Fig 4 Plasma concentration–time profiles of 2-carboxy-4-thiazolidinecarboxylic acid after a single oral administration of the drug (150 mg) to two healthy male volunteers

Application

The method was developed for monitoring the time course of plasma drug levels In a single dose, 150 mg of the drug were given orally to two healthy male volunteers. The resulting mean plasma concentration curve is shown in Fig 4

CONCLUSION

An isocratic HPLC method was established for monitoring the hepatoprotective drug 2-carboxythiazolidine-4-carboxylic acid in plasma in both therapeutic and pharmacokinetic studies The method involves a simple solid-phase extraction procedure The drug was retained as an ion pair with tetrabutylammonium and determined by UV absorbance detection at 210 nm. The range of the assay (0 2–50 μg/ml) spans the plasma concentrations following single oral therapy

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