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Determination of 2-carboxy thiazolidine-4-carboxylic acid in biological fluids by ion-exchange chromatography

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Abstract: A column ion-exchange chromatographic method for the determination of 2-carboxy thiazolidine-4-carboxylic acid (TDCA)† in blood and urine is described. After elimination of endogenous thiols, alkaline hydrolysis of the compound yields cysteine which is determined spectrophotometrically. The described method is specific for intact TDCA. Using a 3-ml aliquot of sample it allows the linear determination of this hepatoprotective drug from 5 μg TDCA ml^{-1} with a detection limit of 3 μg ml^{-1}. The method was applied to study the time course of plasma levels and urinary excretion. An *in vitro* metabolism study showed that TDCA has a potential as a cysteine donor for glutathione synthesis.

Keywords: *2-Carboxy thiazolidine-4-carboxylic acid; ion-exchange chromatography; pharmacokinetics; metabolism.*

Introduction

Thiazolidine-4-carboxylic acid (TCA), a cyclic cysteine derivative, has been reported as a hepatoprotective drug [1–3] and marketed since 1964. It is metabolized to cysteine by liver proline oxidase [4]. Its toxicity in children and in overdose situations [5] has led to the synthesis of 2-substituted derivatives such as 2-methyl thiazolidine-4-carboxylic acid (MTCA) which is less toxic and more effective than the parent compound [6]. The stoichiometric reaction between glyoxylic acid and cysteine yields 2-carboxy thiazolidine-4-carboxylic acid (TDCA), the arginine salt of which has been marketed as a hepatoprotective and detoxicant drug.

Although TDCA shows very low toxicity (LD_{50} = 2 g kg^{-1}) after oral administration to rats and mice, pharmacokinetic and metabolism studies would require that a specific analytical method be developed.

The major aim of this paper was to develop a selective analytical method for the determination of intact TDCA in biological fluids.

In addition the urinary excretion and plasma concentration of TDCA was monitored with time and the fate of the compound addressed.

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†Tidiacic arginine salt (DCI): active ingredient of Tiadilon marketed in France.

Experimental

Reagents

Phosphate buffer (pH 6.9) was prepared by addition of 100 ml of $Na_2HPO_4 \cdot 12H_2O$ solution (170 g l^{-1}) to 200 ml of distilled water. The pH of this solution was then brought to 6.9 by addition of KH_2PO_4 solution (78 g l^{-1}). The thiol reagent was prepared by dissolving 40 mg of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma) in 100 ml phosphate buffer pH 6.9. This reagent was stable for at least 1 month in the refrigerator.

Anionic-exchange column. Dowex 1 × 8 100–200 mesh (Cl^-) resin was conditioned by treatment with 2 M NH_4OH and 2 M HCl and rinsed with distilled water after the application of each reagent. The above cycle was repeated about 10 times. Then 6.5 M HCOOH was added and the column was washed with distilled water until neutrality. Dowex 1 ($HCOO^-$), 5 ml, was poured into a 40 × 0.7 cm column equipped with a plug of quartz wool. The anionic resin was regenerated by washing with distilled water (with the aid of a pump) until pH 3. Then 20 ml of 13 M HCOOH were added and the column was washed with distilled water until neutrality.

Cationic-exchange column. AG 50 W × 8 200–400 mesh (H^+) resin was conditioned with 2 M NaOH and 2 M HCl, a water rinse following each treatment. 3.5 ml of cationic resin (H^+) was poured into a 10 × 0.7 cm column equipped with a plug of quartz wool.

The cationic resin was regenerated by washing with distilled water until neutrality then 10 ml of 2 M HCl were added and the column was washed once more with distilled water until neutrality.

Animals — treatment

Male Wistar rats (330 ± 20 g) were divided into three groups of three rats each. The rats were starved for 12 h before the intragastric administration of TDCA at three different doses 45, 22 and 2.7 mg kg^{-1}. Urine samples were collected for 48 h. Some experiments were made on a human male volunteer with a single oral dose of TDCA arginine salt corresponding to 2.7 mg TDCA kg^{-1} which represents the mean daily therapeutic dosage.

For the determination of plasma levels eight groups of three male Wistar rats (330 ± 20 g) each received intragastrically a single dose of TDCA (22 mg kg^{-1}). At various times after administration, the animals were killed by decapitation, and blood samples were drawn into heparinized tubes. Plasma was separated by centrifugation.

Incubations

Hepatocytes were isolated by the method of Berry and Friend [7] with minor modifications.

Incubations were carried out in a shaking water bath with 50 oscillations min^{-1} at 37°C for 2 h using 10 ml of cell suspension (4 × 10^7 cells) in Hank's medium. After incubation the cells were separated by centrifugation for 10 s on a tabletop microfuge and washed once by resuspension in Hank's solution followed by a second centrifugation. The cell pellet was resuspended with 4 ml Hank's solution and treated by addition of 0.5 ml trichloroacetic acid (0.7 g ml^{-1}). The proteins were removed by centrifugation at 4°C. Non-protein sulphhydryl levels were determined on a 0.5 ml aliquot of the supernatant with DTNB reagent.

Procedure

The collected urine and plasma samples were adjusted to pH 6, and a 3-ml aliquot was drained into the anionic column at a flow rate of 0.5 ml min^{-1}. The column was successively washed with 10 ml of distilled water, 10 ml of 1.45 M HCOOH and 10 ml of distilled water. These washings were discarded. The cationic column, equipped with a 20 ml funnel was placed in series with the anionic column which was eluted with 10 ml of saturated oxalic acid solution followed by a 10 ml distilled water rinse. The anionic column was removed and the cationic was washed with 10 ml of 0.1 M HCl and then eluted with 10 ml of 2 M NH_3 solution. This latter eluent containing TDCA was recovered and concentrated to about 2 ml by rotary evaporation under reduced pressure at 45°C. The resulting volume was accurately measured and two 0.5 ml aliquots were poured into two glass tubes with screw stoppers. 0.5 ml of 10 M sodium hydroxide solution was added to each tube which was stoppered and placed in an oven at 50°C for 30 min. After cooling, 2 ml of phosphate buffer (pH 6.9) and 0.4 ml of 7.1 M H_3PO_4 was rapidly added giving a pH in the 6.8–7.0 range. If necessary the pH was adjusted to 6.9 by dropwise addition of 10 M NaOH or 7.1 M H_3PO_4. Then 0.5 ml of DTNB reagent was added.

The blank for urine or plasma determination was made without alkaline hydrolysis by mixing a 0.5 ml aliquot of the sample from the concentration step with 2.9 ml of phosphate buffer pH 6.9 and 0.5 ml of DTNB reagent.

The absorbance at $\lambda = 412$ nm was recorded against the blank within 10 min. TDCA concentration was obtained from a standard curve in the range 2.5–40 μg TDCA in 0.5 ml samples.

Results and Discussion

Owing to the high solubility of TDCA in water even in a strongly acidic medium (2 mg ml^{-1} at pH 1.1) and its insolubility in organic solvents, a method based on liquid–liquid extraction cannot be used for the determination of microamounts of TDCA in biological fluids.

In order to determine the level of TDCA in urine samples, attempts were carried out using high-performance liquid chromatography (HPLC) with an anion-exchange column and aqueous eluent. All these experiments were unsuccessful since TDCA absorbs only in the 210 nm zone where numerous urinary compounds also absorb strongly. Moreover, with aqueous eluent in the 1.5–7.5 pH range, TDCA gives two peaks, the relative intensities of which vary with the pH of the eluent.

Derivatization of the carboxylic groups using 4-bromomethyl-7-methoxycoumarin [8] was also a failure.

N-benzoylation of TDCA required the synthesis of the dimethyl ester intermediate and did not give quantitative recovery of TDCA in biological samples.

As 2-substituted thiazolodine 4-carboxylic acid can be hydrolysed in alkaline medium into cysteine and the corresponding aldehyde [9], the determination of glyoxylic acid was attempted but without success since the strong alkalinity required for hydrolysis leads to the dismutation of the aldehyde. Therefore we were led to determine the cysteine moiety. However, as thiols, disulphides and thioesters occur naturally in biological fluids, their preliminary elimination was necessary. Each step of the TDCA determination was studied in water and optimized in order to increase the specificity and the sensitivity of the procedure.

Buffering the sample to pH 6.0 led to strong binding of TDCA by Dowex 1 resin whereas endogenous compounds such as cystine and cysteine passed straight through the column and were drained with the subsequent washings.

Foreign compounds with structures closely related to TDCA such as proline, thioproline and 2-methylthioproline were eluted with 1.45 M formic acid ($pK = 3.77$) since they are only weakly bound, whereas TDCA remained fixed on the Dowex 1. Numerous trials were carried out in order to elute the strongly bound TDCA as a single fraction.

The elution of TDCA by using 6 M HCOOH solution does not allow its recovery as a single fraction. Therefore a saturated solution of oxalic acid ($pK_1 = 1.25$) was used as eluent. In these conditions 20% of the TDCA was found in the 10 ml oxalic acid fraction and 80% was recovered in the subsequent 10-ml water washings. The oxalic acid present in this fraction had to be removed before the alkaline hydrolysis of TDCA since sodium oxalate precipitation leads to losses of cysteine recovery. The use of a strong cationic column has proved satisfactory since TDCA is strongly bound by this column, owing to the protonation of the ring NH at pH 1, while oxalic acid passes straight through the column. The last traces of oxalic acid were removed by washing with 0.1 M HCl solution.

The TDCA was eluted with 2 M NH_3. The collected eluent contains TDCA and trace amounts of cysteine and cystine from TDCA hydrolysis by the 2 M NH_3.

After the concentration step carried out as described in the procedure section, the concentrated solution was submitted to alkaline hydrolysis which was optimized by studying three parameters: the sodium hydroxide concentration (Fig. 1) and the influence of time and temperature (Fig. 2).

The sulphhydryl compound formed by hydrolysis of TDCA was characterized as cysteine by HPLC after derivatization with DTNB reagent [10].

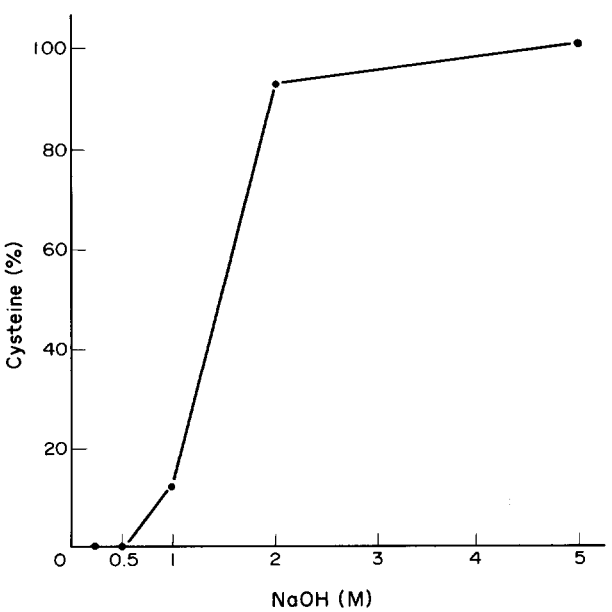

Figure 1
Influence of NaOH concentration on cysteine recovery (50°C — 30 min).

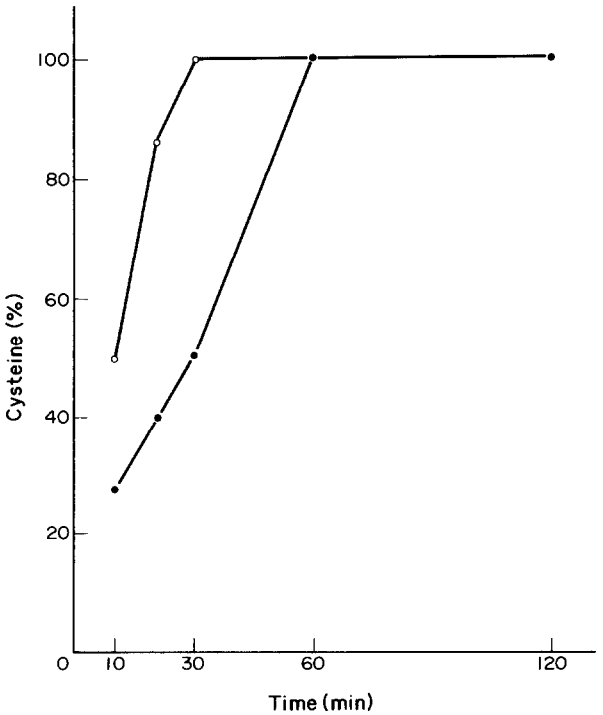

Figure 2
Influence of temperature and time on cysteine recovery (5 M NaOH). ●, 20°C; ○, 50°C.

Using the described procedure for TDCA hydrolysis compounds such as cystine, thioproline and 2-methyl thioproline are also decomposed into cysteine. In the strong alkaline medium used for hydrolysis, the oxidation of cysteine into cystine does not occur but cysteine was partially decomposed into H_2S at higher temperature (100°C) giving non-reproducible recovery.

With the aim of applying this procedure to biological fluid samples, recoveries were determined from drug-free rat plasma and urine spiked with TDCA. The results are summarized in Table 1 showing good recovery from urine samples. For serum samples recoveries were lower at about 70% in the usual pharmacological working range of TDCA.

Following the described procedure, linearity was observed in the range 5–70 μg TDCA ml^{-1} in urine samples and between 5–50 μg TDCA ml^{-1} for plasma. By using a 3-ml aliquot of both samples the limit of detection was 3 μg ml^{-1}. During the course of this study replicate analyses of the same sample were carried out giving a relative standard deviation of ±10%. At the present time we are working to increase the sensitivity of the method for the analysis of plasma samples.

Plasma and urine levels

The proposed method has been applied to the specific determination of intact TDCA in plasma following a single oral administration (22 mg kg^{-1}). These results were compared with those from radioactive measurements carried out elsewhere (unpublished data) using a ^{14}C-labelled thiazolidine ring in the same experimental conditions.

Table 1
Recovery of TDCA in 3-ml spiked samples

Urine*		Plasma†	
TDCA present ($\mu g\ ml^{-1}$)	TDCA found ($\mu g\ ml^{-1}$)	TDCA present ($\mu g\ ml^{-1}$)	TDCA found ($\mu g\ ml^{-1}$)
10	10.3 ± 1.36	5	3.7 ± 0.86
16.6	17.3 ± 1.81	13.3	9.3 ± 0.60
33.3	33.0 ± 3.11	66.6	56.6 ± 0.86
50	50.0 ± 1.46		

*Values are means ± SD (n = 10).
†Values are means ±SD (n = 7).

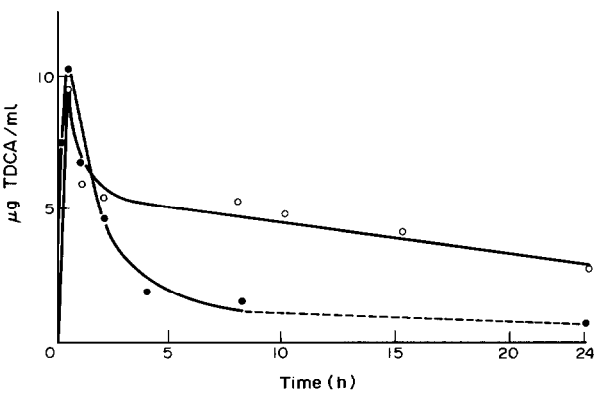

Figure 3
Time course for TDCA plasma levels following a single oral administration (22 mg kg^{-1}). ●, Proposed method; ○, radioactive measurements. Mean data from three rats. With 3–6 ml aliquots of plasma.

As shown in Fig. 3, within 2 h after oral administration, the concentrations determined by the two methods were of the same order of magnitude. Then radioactive measurements showed only a slight decrease with time which was not confirmed by the specific method. This was presumably due to the presence of radioactive TDCA metabolite(s) which were not taken into account by the specific method.

These results are undoubtedly in agreement with the study of the protein bound radioactivity which increased from 5% after 1 h to 45% within 2 h, and reached 85% beyond 4 h after oral administration.

In all events, the levels of intact TDCA in the blood were low corresponding to about 2% of the administered dose. As shown in Fig. 4 the oral administration of high doses of TDCA led to strong urinary excretion of the compound in the unchanged form, corresponding to about 50% of the administered dose. Using mean daily dosage, 10% of the administered TDCA was excreted in the urine in both the rat and in man. In all cases the excretion was rapid corresponding to 70% within the first 4 h and 90% over 8 h following oral administration. The excretion can be estimated as complete 24 h after administration of a single dose.

Following a single oral dose (22 mg kg^{-1}) radioactivity measurements in urine samples confirmed the percentages shown in Fig. 4, this supports the urinary excretion of unchanged TDCA.

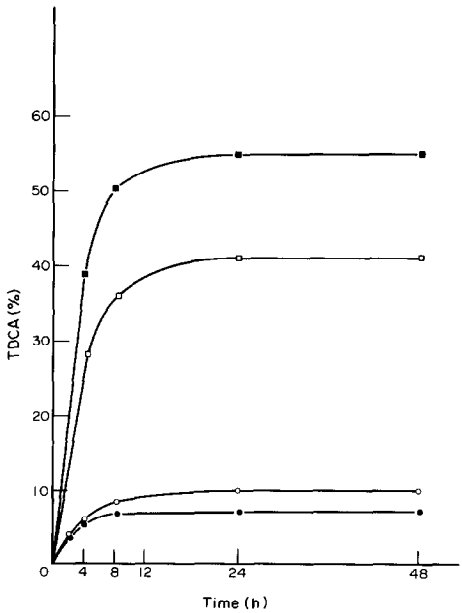

Figure 4
Accumulative percentage of dose of TDCA excreted in urine following a single oral administration. ●, Man (2.7 mg kg^{-1}); ○, rat (2.7 mg kg^{-1}); □, rat (22 mg kg^{-1}); ■, rat (45 mg kg^{-1}).

However the sum of plasma and urine levels does not account for the total amount administered. Radioactive measurements carried out on the organs contained 30% of the administered dose. The highest percentages were found in the liver and kidneys, while a small fraction (4%) of labelled TDCA was eliminated in the faeces. This low faecal elimination is in favour of the fast and virtually complete absorption of the unchanged form of TDCA by the gastrointestinal tract. Moreover, pulmonary elimination corresponded to 5% of the dose within 72 h. Thus 72 h after a single oral dose (22 mg TDCA kg^{-1}) radioactive measurement accounts for about 90% of the TDCA administered.

The *in vitro* fate of TDCA was addressed using incubation with isolated rat hepatocytes in Hank's medium (pH 7.4). A preliminary study dealing with the cellular uptake of TDCA was carried out for comparison with MTCA. In both cases the percentage of the unaltered form of each compound was determined (Table 2).

The results show that TDCA has a higher chemical stability than MTCA since this latter undergoes partial non-enzymatic time-dependent dissociation into cysteine and acetaldehyde in Hank's solution. In contrast TDCA is quantitatively recovered in its

Table 2
Percentages of intact MTCA and TDCA after incubation

	Hank's medium*	Hank's medium + hepatocytes†	
		Extracellular	Intracellular
MTCA	80 ± 5	10 ± 5	0
TDCA	100 ± 5	50 ± 10	0

1.2 μmol of MTCA and TDCA was incubated in 10 ml Hank's medium for 2 h at 37°C.
*Without hepatocytes.
†In the presence of 4×10^7 hepatocytes.

unaltered form. In the presence of hepatocytes MTCA is almost quantitatively metabolized while about half of the TDCA remains intact in the extracellular medium. The low lipophilic nature of TDCA (partition coefficient between *N*-octanol and water 0.12) and the fact that the compound is always present as the ionized form ($pK_1 \approx 1.4$) should not promote rapid uptake by hepatocytes.

These *in vitro* results should be considered together with the *in vivo* elimination of $^{14}CO_2$. The percentage of the administered dose eliminated as CO_2 reached 52.8% for MTCA within the first 4 h after intraperitoneal administration [6]. Following intravenous administration of TDCA this percentage was 3.5% within the first 6 h and reached only 13% after 72 h.

During the course of TDCA metabolism by isolated rat hepatocytes, changes in intracellular non-protein sulphhydryl (NPSH) levels were determined as described by Beutler [11] with some modifications. Although this procedure for the estimation of the sulphhydryl content is not specific for reduced glutathione (GSH) it effectively measures the level of glutathione since the content of other acid-soluble thiols in rat hepatocytes is very low [12].

For incubations carried out in the absence of TDCA, the NPSH content of hepatocytes expressed as glutathione decreased from 28 ± 2 nmol/10^6 cells to 20 ± 2 nmol/10^6 cells after 2 h of incubation.

These results showed a significant loss of GSH during the course of incubation in a medium free from sulphur donors. They are in accordance with previous data from the literature [11–13]. In contrast, after the addition of TDCA (1.2 μmol) to 10 ml incubation medium, no evidence was obtained for intracellular GSH depletion. A slight increase of GSH was even observed since the GSH content reached 30 ± 2 nmol/10^6 cells after 2 h of incubation.

The data show that isolated rat hepatocytes can maintain their intracellular GSH level only when they have access to cysteine precursors in the medium. Moreover, it was impossible to demonstrate any intracellular increase of free cysteine after 2 h of incubation. As TDCA was not decomposed non-enzymatically into cysteine in the incubation medium, its access to the cells in this form can be ruled out. Thus, it can be expected that TDCA is taken up by the cells just as it is. Then the cysteine formed by cleavage of the thioazolidine ring is rapidly used for glutathione synthesis. Because glutathione synthesis is regulated by a feedback mechanism [16, 17] the observed depletion should markedly stimulate GSH synthesis. The availability of cysteine becomes the rate-limiting step of GSH synthesis [18]. Thus the presence in the medium of a non-toxic and effective intracellular cysteine delivery compound such as TDCA leads to restoration of the normal hepatic glutathione content.

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