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Determination of cycloserine in human plasma by high-performance liquid chromatography with fluorescence detection, using derivatization with *p*-benzoquinone

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Abstract

A new method for determining cycloserine in plasma samples is described. This method is based on the derivatization of cycloserine with *p*-benzoquinone, a reaction that takes place at the same time as the process of plasma deproteinization due to the presence of ethanol as solvent in the solution of the derivatization reagent. Four derivatives are obtained from this reaction. The main derivative is well correlated with the cycloserine concentration. The ratio between the volumes of the plasma sample and the reagent solution is 1:2 for a *p*-benzoquinone concentration of 1000 μg/mL. Elution from a C_{18} column was isocratic, using a mobile phase containing (v/v) 85% aqueous 0.1% formic acid solution, and 15% (v/v) of a mixture of methanol and acetonitrile (1:1), with a flow-rate of 1 mL/min, at 25°C. Determinations by fluorescence detection were achieved with excitation at 381 nm and emission at 450 nm, with a detection limit of 10 ng/mL for an injection volume of 5 μL. This method was validated and applied to the determination of cycloserine in blood plasma samples of several healthy volunteers.

Keywords: Derivatization, LC; Cycloserine; *p*-Benzoquinone

1. Introduction

Cycloserine (4-amino-3-isoxazolidinone) is a broad-spectrum antibiotic that is produced by a strain of *Streptomyces orchidaceous* and has also been synthesized. Its main application is the treatment of active pulmonary and extra-pulmonary tuberculosis (including renal disease) [1]. In addition to its activity as a broad-spectrum antibiotic, it also exhibits the ability to increase the level of the inhibitory neurotransmitter γ-aminobutyric acid in vivo and to inhibit the pyridoxal 5′-phosphate-dependent enzyme γ-aminobutyric acid aminotransferase in vitro [2]. After oral administration, cycloserine is readily absorbed from the gastrointestinal tract, with peak blood levels of several μg/mL occurring in 4–8 h. Taking into account the fact that this drug has a limited potential for absorbing UV radiation, the best potential for determining this drug in human plasma is given by the possibility of its chemical derivatization, either to improve its absorption properties, or to convert it into a fluorescent form.

According to the literature there are only a few

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methods for the determination of cycloserine in various samples. A spectro-fluorimetric method reported in the literature is based on the reaction between cycloserine and *p*-benzoquinone [3]. The derivative absorbs UV radiation with a maximum at 381 nm. According to the authors, this method also has fluorescent properties, with a maximum emission at 502 nm for excitation at 381 nm. Calibration graphs were rectilinear in the range 4–20 μg/mL for spectrometric determination and 0.04–0.2 μg/mL for the fluorescent method. This method has been applied to the determination of cycloserine in capsules, and it was assumed that it could also be applied to biological fluids.

In another report, plasma was mixed with 0.2 *M* sodium borate buffer (pH 9.75), 6-aminohexanoic acid (as internal standard) and 5-methoxyindol-3-yl acetic acid (as internal standard) and centrifuged. The supernatant solution was analyzed by HPLC–DAD-FLD [4].

Powdered tablets containing cycloserine were dissolved in water [5] and the solution obtained after filtration was derivatized with 9-methoxyacridine in acetonitrile at 60°C for 1 h. The mixture was cooled, and after 15 min the absorbance was measured at 438 nm. The detection limit was 0.3 μg/mL.

Cycloserine forms a complex with chloranil in aqueous solution at pH 9, which has an absorption maximum at 348 nm. The method has been applied to the determination of cycloserine from capsules [6].

In another method, the content of powdered tablets is dissolved in water, stirred, and then filtered [7]. A portion of the solution is treated with chloramine B, and the excess of the reagent is determined by titration, after reaction with H_2SO_4 and KI.

Two methods using second-derivative ultraviolet spectrometry and HPLC analysis with fluorescence detection [8] are based on derivatization with the 9-chloro-10-methylacridinium triflate (trifluoromethanesulphonate) to yield a reaction product that absorbs at 361 nm and fluoresces at 475 nm (excitation at 257 nm). The detection limit of this method reaches 0.15 μg/mL.

Recently, cycloserine and a large group of antimycocaterial drugs (amikacin, kanamycin, ofloxacin, *p*-aminosalicylic acid, prothionamide, pyrazinamide, and thambutol) were assessed in serum samples by reversed-phase HPLC, gas–liquid chromatography and fluorescent polarization immunoassay [9].

In this paper a new HPLC–FLD method of determining cycloserine in human plasma samples is described, based on a derivatization procedure with *p*-benzoquinone [3]. An attempt was made to give a new interpretation of the mechanism that takes place during this reaction.

2. Experimental

2.1. Chromatographic system

A Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Deutschland GmbH) equipped with a HP diode-array (G1315A) and a HP fluorescence detector (G1321A) were used. A XDB C-18 (double encapped octadecyl silica gel) column, 250 mm in length, 4.6 mm I.D. and 5 μm particle size was used. Elution was isocratic, using a mobile phase containing 85% (v/v) aqueous 0.1% formic acid solution and 15% (v/v) of a mixture of methanol and acetonitrile (1:1), with a flow-rate of 1 mL/min, at 25°C. Injection was performed automatically using a HP G1329A autosampler with an injection volume of 5 μL. Chromatograms were monitored by DAD at 240±2 nm (reference wavelength 360 nm) and by FLD at the following parameters: excitation at 381±2 nm and emission at 450±4 nm.

2.2. Chemicals and reagents

All solvents and *p*-benzoquinone were of HPLC grade and purchased from Merck (Darmstadt, Germany). Cycloserine was from Help S.A. Pharmaceuticals (Greece).

A solution of 1000 μg/mL *p*-benzoquinone in ethanol was used for the derivatization of plasma samples.

2.3. Sample preparation

The sample preparation procedure is very simple, consisting of two operations:

- 1 mL plasma sample is mixed with 2 mL of a solution of *p*-benzoquinone (1000 μg/mL) in ethanol;
- after 15 min the sample is centrifuged (1500 *g*) and 5 μL of the supernatant is injected onto the chromatographic column.

The role of the ethanol used as a solvent for *p*-benzoquinone is considerable in the process of deproteinization of the plasma sample. A ratio of 1:2 between the volumes of the plasma sample and the reagent solution is favored for proper deproteinization, such that the loading effect of the matrix upon the chromatographic column is minimized, but dilution of the samples is not excessive. Unlike the methods for the determination of other drugs in plasma samples based on a derivatization procedure, this method avoids the necessity of extracting the derivatives into a non-miscible solvent, which may be time-consuming and a source of errors [10–12].

This method was applied with very good results to plasma samples for a bioequivalence study of Helpocerin (250 mg per tablet), produced by Help S.A. Pharmaceuticals. Cycloserine produced by Lilly was used as the reference compound. This drug was administered as tablets to 18 healthy volunteers. Venous blood samples were collected pre-dose (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24 and 48 h post-dose.

3. Results and discussion

3.1. Reaction mechanism

Cycloserine only weakly absorbs UV radiation, and for this reason a derivatization procedure is necessary to allow the determination of low concentrations of this drug in plasma samples. Derivatization with *p*-benzoquinone is based on an addition reaction between the analyte and the derivatization reagent with the possibility of obtaining two derivatives (**I** and **II**) [13], but still another reaction is also possible with an equilibrium between the condensation compound (**III**) and its mezomeric form (**IV**), as shown in Fig. 1. A similar aromatization mechanism involved in the inactivation of the pyridoxal 5′-phosphate-dependent enzyme by cycloserine has recently been proposed using HPLC and UV–Vis spectrometric measurements [14].

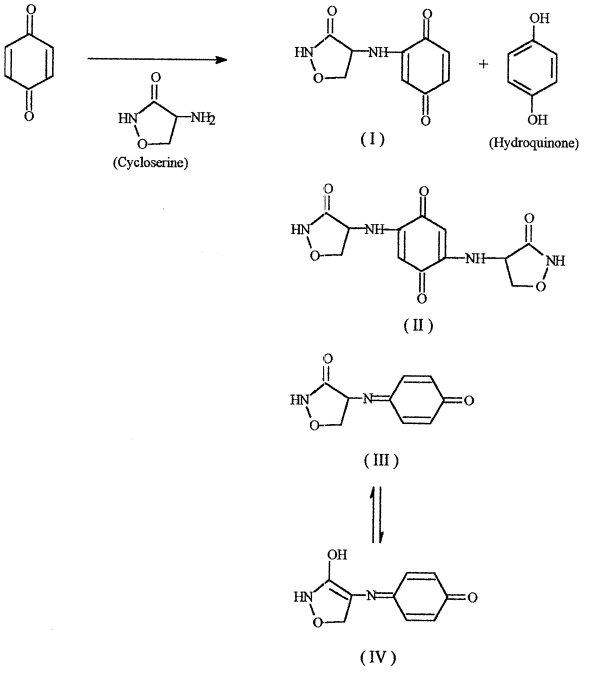

Fig. 1. Derivatization reactions between cycloserine and *p*-benzoquinone.

3.2. Optimization of the FLD measurements

A chromatogram monitored by FLD using an excitation wavelength of 381 nm and an emission wavelength of 502 nm exhibits four expected chromatographic peaks of different intensities, as can be seen from Fig. 2a. If the emission wavelength is changed to 450 nm according to the three-dimensional fluorescence spectrum of the most intense peak, the chromatogram will show only two signals, corresponding to the second and fourth peak in the previous chromatogram (Fig. 2b). It is worth mentioning that blank plasma subjected to the same derivatization procedure exhibits no chromatographic peaks in FLD. The interference of any species found in the plasma matrix is completely eliminated by this method.

A possible source of interference in this method is serine, which can be found as a trace component in

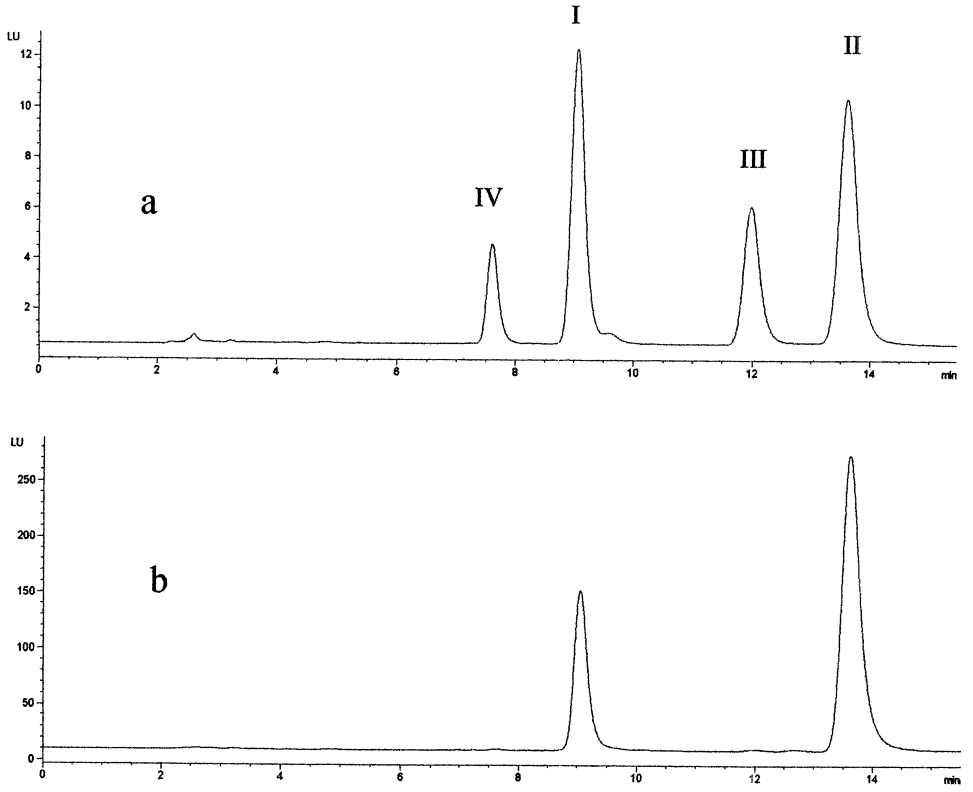

Fig. 2. Two HPLC–FLD chromatograms of 10 μg/mL cycloserine in a plasma sample derivatized with *p*-benzoquinone. Fluorescence parameters: (a) excitation 381 nm, emission 502 nm; (b) excitation 381 nm, emission 450 nm. Retention times for derivatives are assigned as: **IV**, 7.70 min; **I**, 9.05 min; **III**, 11.90 min; **II**, 13.60 min.

plasma samples or can result from the hydrolysis of cycloserine. However, in the reaction with *p*-benzoquinone it gives rise to a single derivative that has a different retention time (6.05 min), and exhibits poor fluorescence. Hydroquinone, the major by-product of the derivatization process, and *p*-benzoquinone are not observable in FLD, but they can be monitored by DAD using a wavelength of 240 nm.

3.3. pH dependence

Because pH plays a major role in this procedure, or can influence the hydrolysis reaction of cycloserine, it is necessary to find the optimum conditions for the derivatization method as a function of the pH of the reaction medium. For this purpose, solutions of 100 ppm cycloserine, each in different borate buffer solutions, were prepared: pH 7.5, 8.1, 9 and 10. An acidic medium is not appropriate for such a derivatization due to the hydrolysis of cycloserine to serine. The maximum value of the peak area is reached at pH 7.5, which is very close to the pH of blood samples. For this reason, in the final procedure, buffering of the plasma samples can be eliminated to avoid dilution of the plasma.

3.4. Dependence of the reagent concentration

The derivatization procedure is influenced by the reagent concentration in different ways for the two chromatographic peaks monitored in FLD, for excitation at 380 nm and emission at 450 nm. The conclusion that can be drawn from Fig. 3 is that the highest values for these peaks are: the peak at t_R = 13.60 min has a maximum for 1000 μg/mL *p*-benzoquinone, while the maximum of the peak

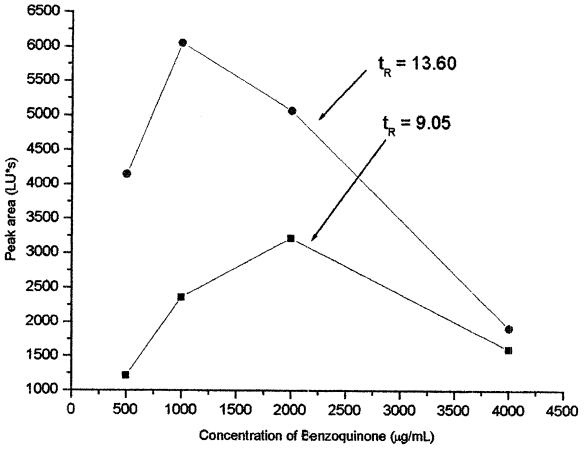

Fig. 3. Dependence of the peak areas of derivatives **I** and **II** on the concentration of *p*-benzoquinone.

situated at $t_R = 9.05$ min is obtained for 2000 μg/mL *p*-benzoquinone. Nevertheless, an optimum value of 1000 μg/mL for the derivatization reagent was established for the final procedure, due to the fact that, for quantitative determinations, only the chromatographic peak at 13.60 min was used. Another conclusion drawn from Fig. 3 is related to the mechanism of the derivatization process: a high value of the reagent concentration involves a high probability of obtaining derivative **I**, assigned to the peak at $t_R = 9.05$ min. In the case of derivative **II**, the probability of obtaining this compound is higher for smaller values of the reagent concentration. This compound can be assigned to the peak situated at $t_R = 13.60$ min.

The attribution of the other two derivatives, **III** and **IV**, can be achieved by means of their UV spectra, and are specified in Fig. 2.

3.5. Time dependence of the derivatization reaction

In spite of the complexity of the reactions that occur during this derivatization process, time proved to be less significant for the kinetics of the method. The time dependence of the peak area for **II** led to the conclusion that a reaction time of more than 15 min does not influence the analytical results. For the final procedure, the optimum value of this parameter was established to be 15 min, taken from the start of the reaction and sample centrifugation. Samples resulting from derivatization are stable for at least 24 h, with less than 1.86% change over this time period.

3.6. Calibration curve

The calibration curve for this method was obtained for spiked plasma samples in the concentration range 0.25–10 μg/mL. The best linearity was obtained for peak **II** with calibration curve equation:

$$\text{Peak area (LU*s)} = (243.92 \pm 0.2)*\text{concentration} - (7.667 \pm 1.3),$$

$r = 0.998$, $s_d = 75.27$

3.7. Validation

The validation procedure focussed on the following aspects: recovery of cycloserine from plasma samples, inter- and intra-day precision, time stability of the derivatives (discussed in Section 3.5), and detection and quantification limits of cycloserine in plasma samples.

Recovery from spiked plasma samples was estimated over a wide range of cycloserine concentrations against spiked water samples containing the same cycloserine concentration. The recovery values were very high (Table 1), and almost constant with concentration, demonstrating that, in the presence of ethanol, this compound is almost entirely desorbed from the proteins contained in human plasma.

Inter- and intra-day precision in human plasma samples were assessed by performing five replicate analyses of spiked plasma samples with different concentrations of cycloserine within an interval of 6 days (Table 1).

The limit of detection (LOD) was computed [15] for a signal-to-noise ratio of 3, while for the limit of quantification (LOQ) this ratio was taken as 10. The LOD and LOQ values were estimated to be 10 and 33.3 ng/mL, respectively. A chromatogram showing the peak signal assigned to derivative **II** versus a blank plasma sample is given in Fig. 4.

The plasma cycloserine concentrations for one healthy volunteer receiving a single dose are given in Fig. 5.

Table 1
Values of the main parameters used in the validation procedure

Cycloserine conc. (μg/mL)	Recovery ($100C_{found}/C_{theor.}$)	Intra-day precision (RSD%) ($n = 5$)	Inter-day precision (RSD%) ($n = 5$)
0.25	98.2	4.22	5.54
1.0	98.3	4.61	5.08
2.5	98.7	3.87	4.33
5.0	98.5	3.46	4.42
10.0	99.1	3.67	4.35

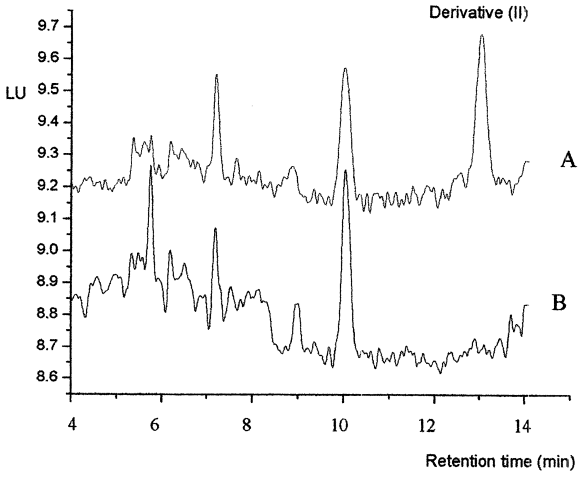

Fig. 4. Two overlapping chromatograms showing the chromatographic peak assigned to derivative **II** close to the LOQ. (A) A spiked plasma sample with cycloserine (50 ng/mL). (B) A blank plasma sample (peak area 7.04 LU*s; $t_R = 13.60$ min).

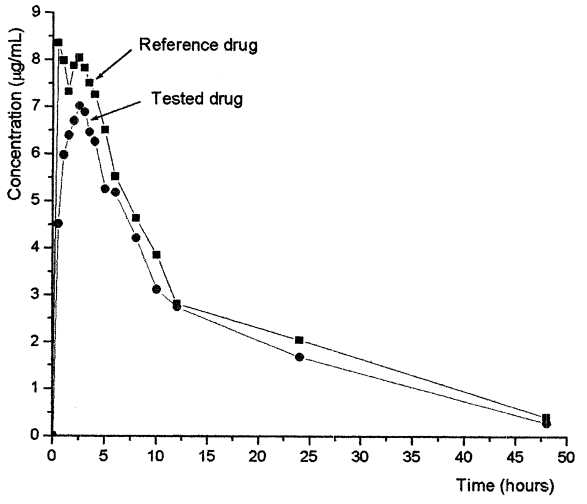

Fig. 5. Concentration vs. time profile for cycloserine in a plasma sample from one volunteer.

4. Conclusions

Cycloserine in plasma samples can be determined successfully by HPLC–FLD using a derivatization procedure with *p*-benzoquinone. Four derivatives are obtained from this reaction. The best linearity between peak area and cycloserine concentration was observed for the derivative eluting in the last chromatographic peak. This method does not need any special operation to concentrate the analyte in the plasma matrix and the deproteinization process takes place during the derivatization procedure by using ethanol as solvent for *p*-benzoquinone.

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