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Development of a liquid chromatographic method for the determination of related substances and assay of D-cycloserine

Murali Pendela, Sanja Dragovic, Lien Bockx, Jos Hoogmartens, Ann Van Schepdael, Erwin Adams*

Laboratorium voor Farmaceutische Analyse, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, O & N 2, PB 923, Herestraat 49, B-3000 Leuven, Belgium

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ABSTRACT

D-cycloserine or D-4-amino-3-isoxazolidinone is an antibiotic produced by *Streptomyces garyphalus* and *Streptomyces orchidaceus*. D-Cycloserine is used in the second line treatment of tuberculosis and is often used in developing countries. Therefore, expensive high-tech techniques are not recommended for analysis. Here, a liquid chromatography method with ultraviolet detection (LC–UV) is described using a base deactivated column (Hypersil BDS column; 25 cm × 4.6 mm I.D.) kept at 45 °C. The gradient method uses mobile phases containing acetonitrile (ACN), 20 mM sodium octane sulphonate (SOS), 0.2 M potassium dihydrogen phosphate buffer pH 2.8, water: A: (4:70:10:16 v/v/v/v) and B: (17:70:10:3 v/v/v/v). The method proved to be robust, linear, repeatable, sensitive, selective and easy to perform. For the related substances test 50 μl of a 0.5 mg/ml D-cycloserine solution is injected. For assay, a concentration of 0.1 mg/ml is proposed to avoid overloading of the detector.

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1. Introduction

D-cycloserine or D-4-amino-3-isoxazolidinone (Fig. 1a) is a broad spectrum antibiotic, produced by *Streptomyces garyphalus* and *Streptomyces orchidaceus*. This drug can also be obtained by synthesis. D-Cycloserine is an analogue of the amino acid D-alanine and so it inhibits the enzymes D-alanine racemase and D-alanine synthetase [1–4]. In general it is more effective against Gram-positive than against Gram-negative bacteria [5]. The most important property of D-cycloserine is the inhibition of the growth of *Mycobacterium tuberculosis*. However, it is seldom used in the management of this disease due to toxicity associated with effective dosages. Nevertheless, the worldwide resurgence of tuberculosis, the emergence of multiply drug-resistant tuberculosis and the problematic use of available drugs required to treat these infections, have resulted in the application of D-cycloserine as a second line drug for the treatment of tuberculosis [6].

Only a few LC methods have been described for the determination of D-cycloserine [7–9]. All these methods used reversed-phase LC with UV detection. They were tried out to check their performance towards the determination of D-cycloserine and its related substances.

In *The United States Pharmacopeia* (USP) a reversed-phase isocratic LC method is described [7]. Using the USP method, no stable baseline could be achieved. Changing the column, using other equipment or other mobile phase components did not solve the noisy baseline problem. Also, the USP method is described as an assay procedure and there is no method for related substances.

A reversed-phase LC method using *p*-benzoquinone for derivatization combined with fluorescence detection is described by David *et al.* for the determination of cycloserine in blood plasma samples [8]. It gives good sensitivity and it can also be applied to the determination of cycloserine in drug products (capsules and tablets) and in biological fluids. However, the dimer does not provide a fluorescent product and so cannot be detected. According to this, UV detection should be adopted for monitoring the dimer and other cycloserine impurities. The dimer is the primary degradation product of cycloserine, in both solid state and solution (Fig. 1b) [5]. Due to the disadvantages and complexity related to the derivatization procedure, this method was not further studied here.

Burge *et al.* described a gradient LC method, using cycloserine for the evaluation of polar-embedded and polar-endcapped C12–C18 stationary phases [9]. Initially their aim was to develop a related substances method to support both the manufacturing of bulk drug starting from D-serine and analysis of final capsule formulations. Although they observed a suppressed response for the cycloserine dimer with many of the columns, this problem was not encountered in our investigation using a Hypersil BDS column. The D-cycloserine

* Corresponding author. Tel.: +32 16 323444; fax: +32 16 323448.
E-mail address: Erwin.Adams@pharm.kuleuven.be (E. Adams).

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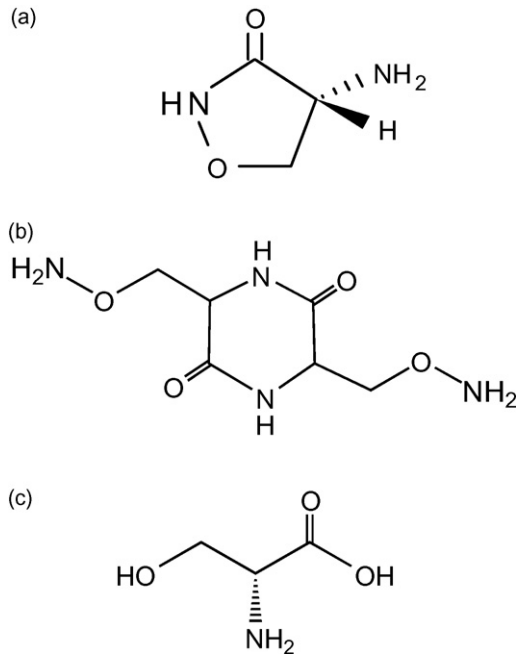

Fig. 1. Chemical structure of: (a) D-cycloserine, (b) D-cycloserine dimer and (c) D-serine.

peak was eluted near the solvent peak. The D-cycloserine peak was well separated, but several small peaks were not well separated from the solvent peak.

Because none of the methods tested gave satisfactory results for the determination of related substances, the development of a new LC–UV method for D-cycloserine was initiated. Since D-cycloserine is often used in developing countries to treat tuberculosis, relatively simple or classical techniques are recommended for analysis. In this study, a LC method using UV detection for the analysis of D-cycloserine and related substances was developed and validated.

2. Experimental

2.1. Instrumentation

The liquid chromatographic system from Dionex Corporation (Sunnyvale, CA, USA) consisted of a P680 HPLC pump, an ASI-100 automated sample injector and UVD 170U detector. A Hypersil BDS C_{18} column (250 mm × 4.6 mm I.D.) 5 μm, 120 Å from Thermo Electron Corporation (Dreieich, Germany) was used as stationary phase. The temperature of the column, immersed in a water bath, was maintained using a Julabo EM heating circulator (Seelbach, Germany). Chromeleon software (Dionex) was connected to the detector to record the signals.

2.2. Reagents and samples

Acetonitrile (ACN) HPLC gradient grade was purchased from Fisher Scientific (Leicester, UK). Sodium octane sulfonate (SOS), potassium dihydrogen phosphate and phosphoric acid (85wt.%., analytical grade) were from Acros Organics (Geel, Belgium). Demineralised water was further purified in the laboratory through Milli-Q water purification system (Millipore, Milford, MA, USA). Cycloserine raw material, cycloserine working standard, cycloserine capsules (250 mg), D-cycloserine dimer, D-cycloserine condensation product (structure is not available) and D-serine (Fig. 1c) were made available by the World Health Organisation (WHO) (Geneva, Switzerland).

2.3. Sample preparation

25.0 mg of D-cycloserine powder was accurately weighed in a 50.0 ml volumetric flask. It was dissolved and made up to volume using mobile phase A. This solution was kept at 4 °C to reduce degradation of D-cycloserine.

3. Results and discussion

3.1. Method development

The method described by Burge *et al.* was used as starting point for further method development. This method uses a gradient with mobile phase A: 20 mM SOS adjusted to pH 2.5 with phosphoric acid and B: ACN. In the described gradient method, the concentration of ACN reaches 30% in 10 min. In the mean time, the amount of SOS decreases. This way a combined effect of continuously varying concentrations of ACN and SOS was experienced. Based on this method it was tried to develop an isocratic method. D-Cycloserine was eluted within 3 min when a mobile phase with more than 10% of ACN was used. So, a mobile phase containing 10% of ACN was selected for further experiments.

A change in pH (from pH 2.5 to pH 6.0) had no significant influence on the separation of the degradation products. However, a lower pH is necessary to obtain protonation of the amino group in the D-cycloserine molecule. The peaks are much sharper at a pH below 3.0 and therefore pH 2.8 was retained. The mobile phase was further adapted by replacing the phosphoric acid by a 0.2 M potassium phosphate buffer pH 2.8. The effect of the volume of 20 mM SOS in the mobile phase was tested. At lower concentrations of SOS, D-cycloserine was splitted into two peaks. This could be attributed to an insufficient amount of ion pairing agent since this phenomenon disappeared upon adding more. A good peak shape was obtained with 70% of 20 mM SOS. This led to the following mobile phase composition: ACN–20 mM SOS–phosphate buffer–H_2O (10:70:10:10, v/v/v/v).

When injecting a fresh sample, nearly no impurities could be detected. Due to high price and availability of the reference substances, it was decided to develop the method using a degraded sample. Degradation peaks were formed by conservation at room temperature for 7 days. For a better separation of the degradation products, the flow rate was decreased from the initially used 1.5 ml/min to 1.0 ml/min. In addition, due to the interference of the negative solvent peak with the degradation products, the solvent was changed from water to mobile phase. However, these conditions were not giving a satisfactory separation of the degradation products. So, it was necessary to further optimize the chromatographic conditions in order to separate the degradation products. The concentration of SOS and the flow rate were kept constant, while the ACN concentration was decreased to 4% to increase the elution of the first eluted polar compounds. The effect of temperature was examined from 25 °C to 50 °C with increments of 5 °C. A degraded solution (room temperature for 7 days) as well as the 3 reference impurities (dimer, condensation product and D-serine) was used to evaluate the selectivity. The combination of a column temperature of 45 °C and an ACN concentration of 4% in the mobile phase showed the best separation of the peaks.

In the final step, a gradient was added to improve the sensitivity for the late eluted peaks. Several gradient profiles were tried out and the final conditions are shown in Table 1. A chromatogram of the three impurities spiked into the fresh D-cycloserine solution obtained with the final conditions is depicted in Fig. 2. The dimer was eluted after D-cycloserine as two peaks which are probably due to isomerism. The remaining two impurities were eluted before the main peak. Overlay of typical chromatograms of fresh D-cycloserine sample and 7 days old degraded solution is shown in Fig. 3a and b.

Table 1
Final chromatographic conditions for the D-cycloserine analysis

Flow rate	1.0 ml/min
Injection volume	50 μl
Column	Hypersil BDS C_{18}, 250 × 4.6 mm, 5 μm
Temperature	45 °C
Detection	UV detection at 219 nm
Sample preparation	Related substances: 0.5 mg/ml of D-cycloserine in mobile phase A Assay: 0.1 mg/ml of D-cycloserine in mobile phase A
Mobile phase	Mobile phase: ACN: 20mM SOS: 0.2M KH_2PO_4 pH 2.8: H_2O A: (4:70:10:16, v/v/v/v) B: (17:70:10:3, v/v/v/v)

Gradient program

Time	%A	%B
0.0–16.0	100	0
16.0–18.0	0	100 gradient
18.0–22.0	0	100 gradient
22.0–24.0	100	0
24.0–30.0	100	0

3.2. Sample stability

Stability experiments on a 0.5 mg/ml D-cycloserine sample were performed to determine the conditions in which the sample solution should be kept. In these experiments the same sample was injected every 2 h for 24 h. The decrease of the peak area of D-cycloserine was calculated. The peak area of the sample which was kept at room temperature over this time period decreased linearly with 9.3%, while the peak area of the sample kept at 4 °C was stable.

3.3. System suitability test

In an official method, a system suitability test (SST) is proposed to ensure the selectivity of the chosen chromatographic system. The resolution is one of the parameters which are used in assessing the column performance [10].

For this experiment, a 0.025 mg/ml sample of D-cycloserine was degraded in mobile phase A at 60 °C for 30 min as well as in basic and acidic conditions. Degradation of the sample at 60 °C did not show any difference compared to a fresh one. In basic or acidic conditions, degradation took place (main peak decreased), but impurities were not noticed because the huge solvent peak interfered with the degradation products. When the heating temperature of the solution in mobile phase A was increased from 60 °C to 100 °C for 30 min, sample degradation was observed: the D-cycloserine peak decreased and a few impurities were formed. A typical chromatogram is shown in Fig. 3c. The resolution between the main peak and the large degradation peak at 3.2 min (SST peak) was 40. The degradation experiment was repeated three times and it was found to be repeatable.

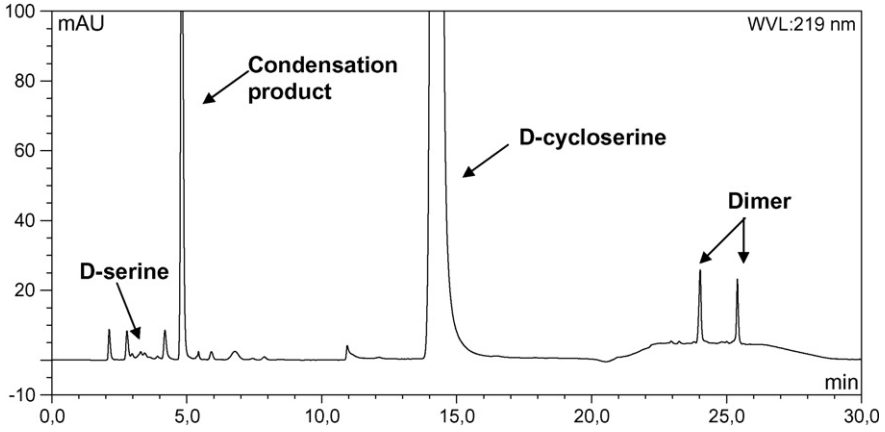

Fig. 2. Chromatogram of a mixture containing D-serine, condensation product, D-cycloserine and dimer.

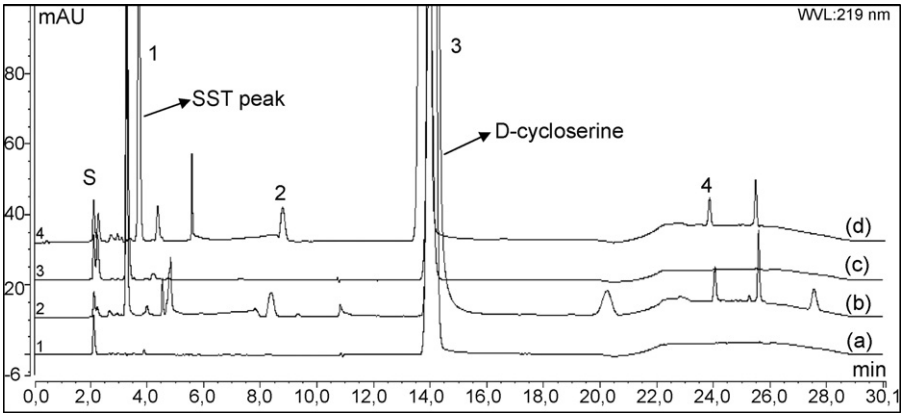

Fig. 3. Overlay of typical chromatograms obtained with the final conditions: (a) fresh D-cycloserine sample, (b) 7 days old degraded D-cycloserine solution, (c) chromatogram of a 0.025 mg/ml D-cycloserine in mobile phase A, degraded at 100 °C for 30 min for system suitability test, (d) typical chromatogram of 4 days old degraded D-cycloserine sample for robustness study. The peak numbers (1–4) refer to the robustness test (S, solvent peak).

Table 2
Lower, nominal and higher levels for the robustness test

Parameter	Lower level (−)	Nominal level (0)	Higher level (+)
ACN (%)	3.5	4.0	4.5
pH	2.6	2.8	3.0
SOS (mM)	18	20	22
Temperature (°C)	43	45	47

3.4. Robustness

In this study, the influence of four chromatographic parameters on the separation was investigated. These parameters were examined using a central composite design.

The parameters examined were the volume of ACN in mobile phase A, the pH of the buffer, and the SOS molarity and the temperature of the column. Each of these parameters was investigated at three levels: a lower, a nominal and a higher level. The chromatographic parameter settings in the experimental design are shown in Table 2. For robustness a 4 days old 0.5 mg/ml D-cycloserine sample in mobile phase A kept at room temperature was used to avoid complexity of the degradation profile (Fig. 3d). This resulting solution was frozen in small vials for injection to keep the same degradation profile during the performance of the experimental design. For each experiment one vial was taken and brought to room temperature before injection.

Investigation of the four parameters ($k = 4$) at three levels ($n = 3$) requires $2^k + 2k + n = 27$ experiments. The parameters were examined by calculating the resolution for four peak pairs as response factors. Fig. 4(a–d) show the regression coefficient plots for the resolution between peak pairs 1–2, 2–3, 3–4 and 1–3, respectively (for the peak numbering see Fig. 3d).

In Fig. 4 the regression coefficients, which correspond to the effect of a factor, are given by a bar and the 95% confidence limits by an error line. A regression coefficient smaller than the confidence limits means that the variation in the response, caused by changing the variable, is smaller than the experimental error. A positive effect means that an increase of the factor value increases the response. A negative effect means that an increase of the factor value causes a decrease of the response studied. The regression coefficient plots of Fig. 4(a) and (b) show that the SOS concentration and the pH in mobile phase A have a slightly positive effect on the respective resolutions. The remaining factors and all interactions are not significant within the range studied. There is no effect of the examined parameters on the resolution between the system suitability test (SST) peak and the main peak, as can be seen from Fig. 4(d). Therefore, it can be concluded that the method is robust within the ranges tested.

3.5. Quantitative aspects

3.5.1. Sensitivity

The limits of detection and quantification, corresponding to a signal-to-noise ratio of 3 and 10, respectively, obtained for D-cycloserine amounted to 0.003% and 0.01%, respectively. The percentage was calculated with respect to the nominal concentration of the sample (0.5 mg/ml = 100%).

3.5.2. Linearity and repeatability

The linearity for the related substances test was investigated by evaluating the response of D-cycloserine solutions at 0.01%, 0.02%, 0.05%, 0.1%, 0.5%, 1.0%, 2%, 5% and 10% of a fresh 0.5 mg/ml (100%) solution of D-cycloserine. The method can also be used for the assay of D-cycloserine, but the concentration has to be decreased because overloading was noticed with a 0.5 mg/ml solution. So, the linearity was tested by evaluating the response from 25% to 125% of a fresh 0.1 mg/ml solution (100%) of D-cycloserine. Each solution was injected three times. The results are shown in Table 3.

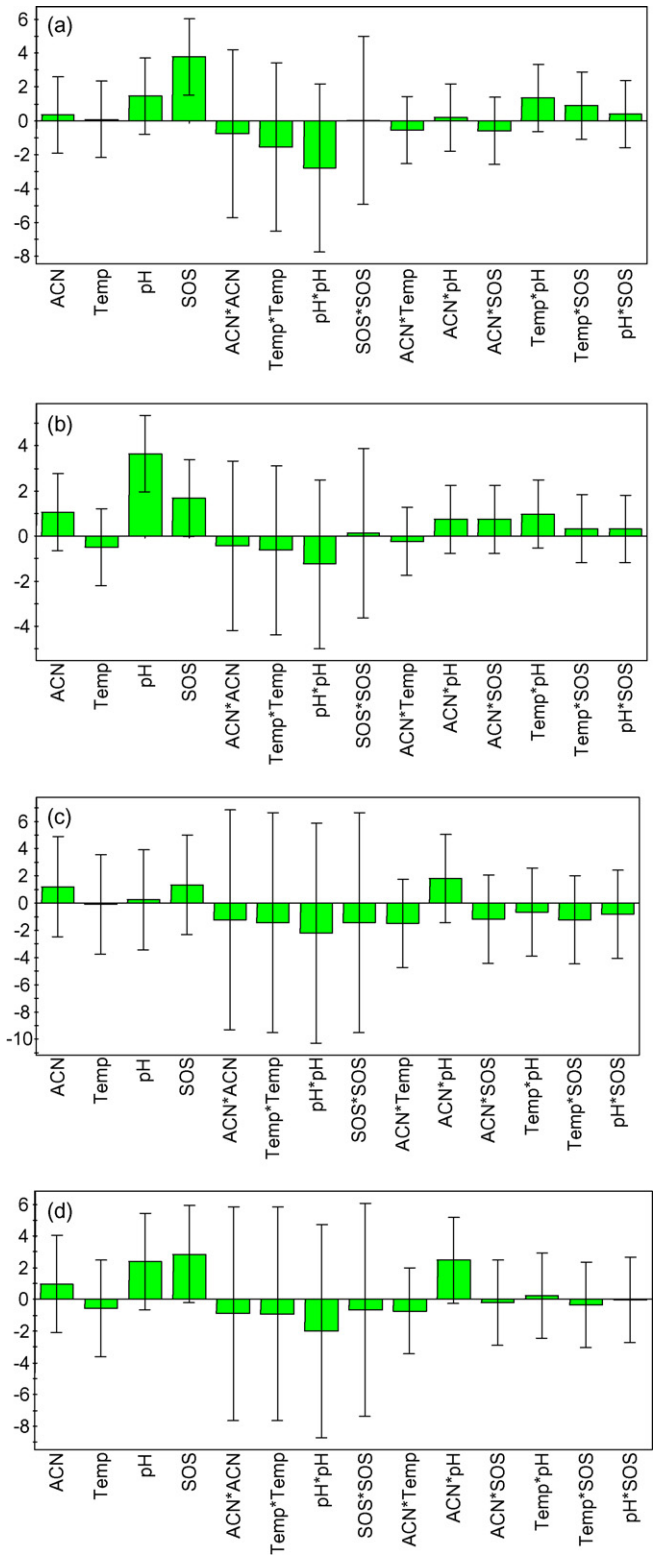

Fig. 4. Regression coefficient plots for the effects of the parameters on the resolution between peak pairs (a) 1–2, (b) 2–3, (c) 3–4 and (d) 1–3.

Table 3
Linearity and repeatability data

	Repeatability RSD (%) ($n = 6$)	Linearity			
		Range x (%) ($n = 3$)	R^2	y	$S_{y,x}$
Related substances (0.5 mg/ml as 100%)	0.18	0.01–10	0.999	$7.47x + 0.02$	0.09
Assay (0.1 mg/ml as 100%)	0.23	25–125	0.999	$1.38x - 0.25$	0.61

RSD: relative standard deviation; range: percentage range studied; n: number of injections per concentration; R^2: coefficient of determination; y: peak area; x: concentration (%); $S_{y,x}$: standard error of estimate.

Table 4
Analysis of a commercial D-cycloserine drug substance and a drug product

Ret. time	Related substances (%)	
	Drug substance	Drug product (capsules)
3.42	–	0.04
3.89	–	0.03
5.29	–	0.16
5.85	0.06	0.06
16.26	0.04	0.09
17.64	–	0.03
18.34	–	0.03
25.26	0.06	0.02
Total impurities	0.17	0.45
Assay (%)	100.4	99.3
RSD	0.60	0.31

Values for the relative standard deviation (RSD) at the 100% level for the related substances and the assay, respectively, are shown in Table 3. These results demonstrate the good linearity and repeatability of the method.

3.5.3. Quantitative analysis

Using the final method, determination of related substances and assay were carried out on a commercial D-cycloserine drug substance (raw material) and a drug product (capsules). Results are shown in Table 4. It was found that the content of individual impurities is below 0.1% for the drug substance and below 0.2% for the drug product. The percentage content was 100.4% and 99.3% for the drug substance and drug product, respectively. The assay value for the drug substance was calculated by taking into account the loss on drying.

4. Conclusion

A gradient LC method was developed for the analysis of D-cycloserine and its impurities. The developed method is able to separate the main compound D-cycloserine from its degradation impurities. The method is robust, linear, repeatable, sensitive, selective and easy to perform.

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