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SIMULTANEOUS QUANTIFICATION OF CYCLOSERINE AND ITS PRODRUG ACETYLACETONYLCYCLOSERINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ULTRAVIOLET ABSORBANCE AND FLUORESCENCE AFTER POST-COLUMN DERIVATIZATION

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SUMMARY

D-Cycloserine is a broad-spectrum antibiotic used with other antibiotics to treat various forms of tuberculosis. Its prodrug sodium (*R*)-4-[(1-methyl-3-oxo-1-butenyl)amino]-3-isoxazolidinone hemihydrate, developed for better aqueous stability and solubility, is combined with another broad-spectrum antibiotic, fludalanine. An ion-pair, reversed-phase high-performance liquid chromatographic assay has been developed to simultaneously detect cycloserine and its prodrug in plasma and urine. The prodrug is detected directly by ultraviolet absorbance and cycloserine by fluorescence following post-column derivatization.

INTRODUCTION

D-Cycloserine (CS: D-4-amino-3-isoxazolidinone; Fig. 1, I), is a broad-spectrum antibiotic inhibiting the incorporation of alanine into the bacterial cell wall [1]. CS is clinically used in combination with other antibiotics to treat various forms of tuberculosis [2]. A prodrug of CS, sodium (*R*)-4-[(1-methyl-3-oxo-1-butenyl)amine]-3-isoxazolidinone hemihydrate, acetylacetonyl cycloserine (ACS; Fig. 1, II), was previously synthesized to enhance its aqueous stability and solubility in order to combine with another broad-spectrum antibiotic α-^{2}H-3-fluoro-D-alanine (Fig. 1, III), fludalanine [3]. This combination is synergistic in its antimicrobial effect and eliminates a self-reversal phenomenon of fludalanine's antimicrobial activity at therapeutic concentrations [4,5].

Previous quantitative methods for CS determination are mainly used for phar-

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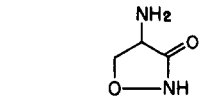

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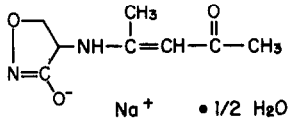

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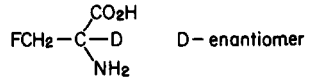

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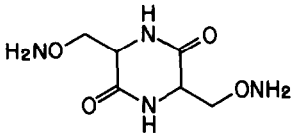

IV

Fig. 1. Structures for D-cycloserine (I), acetylacetonyl cycloserine (II), fludalanine (III) and dimer of D-cycloserine (IV).

maceutical preparations. These methods involve nitroprusside [6], ultraviolet spectrometry [7], and gas–liquid chromatography [8]. Microbiological [9] and photoelectrocolourimetric [10] methods are used for quantification of CS in biological fluids. Chemical manipulations of CS by these assays, however, may cause the degradation of CS [11–14]. Microbiological assays are inherently non-specific for any active degradate or metabolite. Currently, an assay has not been available to quantify the prodrug, ACS, in biological fluids, or to distinguish ACS from CS for quantification.

An ion-pair, reversed-phase high-performance liquid chromatograpic (HPLC) assay has been developed for the simultaneous determination of CS and ACS. ACS is detected by ultraviolet absorbance and CS by post-column derivatization with *o*-phthalaldehyde (OPA) and fluorescence. ACS and CS are quantified in plasma in the concentration ranges 0.3–7.5 μg/ml and 0.3–15.0 μg/ml, respectively. Both drugs are quantified in urine in the concentration range 2–100 μg/ml.

EXPERIMENTAL

Chemicals

Isopropanol (HPLC grade) used in the mobile phase and in sample extractions was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Glacial

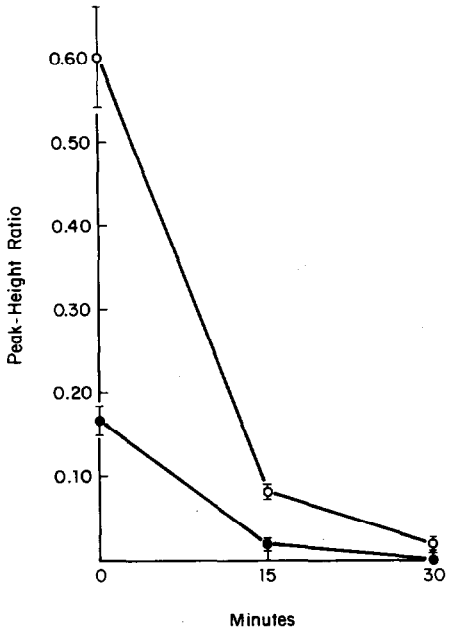

Fig. 2. Stability of ACS in human urine at 37°C over time at 5 μg/ml (●) and 15 μg/ml (○). Each point is the mean of five replicates and the bars indicate the standard deviation from the mean.

acetic acid, potassium hydroxide, boric acid and sodium carbonate were obtained from Fisher Scientific (Springfield, NJ, U.S.A.). Fluoraldehyde (*o*-phthalaldehyde and 2-mercaptoethanol reagent solution), post-column derivatizing reagent, was obtained from Pierce (Rockford, IL, U.S.A.). 6-Aminocaproic acid (6-ACA), internal standard for cycloserine plasma, and 5-methoxyindole-3-acetic acid (5-MIAA), internal standard for ACS in plasma and urine, were purchased from Aldrich (Milwaukee, WI, U.S.A.). α-Aminobutylhistidine, internal standard for cycloserine urine, was purchased from Chemalog Chemical Dynamics (South Plainfield, NJ, U.S.A.). Water was purified on a Milli-Q water purification system from Millipore (Bedford, MA, U.S.A.). CS and ACS were supplied by Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.) [3].

Apparatus

The HPLC system for plasma and urine matrices consists of two M6000A solvent delivery systems, an M-730 data module computing integrator, a Model 730 system control, a Model 710B autosampler (WISP), a Model 441 absorbance detector, all from Waters Assoc. (Milford, MA, U.S.A.). The analytical columns consist of a Co:Pell ODS (30–38 μm) guard column from Whatman (Clifton, NJ, U.S.A.) and an ODS-Hypersil column (240 mm×5 mm I.D.; 10 μm) from Shandon Southern Instruments (Sewickley, PA, U.S.A.). The columns were kept at ambient temperature. CS and its prodrug are detected simultaneously in a plasma or urine matrix by a post-column reactor fluorometer and an ultraviolet detector, respectively. The post-column, packed-bed reactor is an empty stainless-steel column (25 cm×4.5 mm I.D.) packed dry with glass beads (50 μm) from Whatman. The reactor is housed in a column heater from Eldex Labs. (Menlo Park,

TABLE I

STABILITY OF D-CYCLOSERINE IN URINE CONTAINING 100 mg/ml SODIUM CARBONATE AT 2°C

Time (h)	Percentage CS remaining	
	20 μg/ml	80 μg/ml
0.5	107.1	100.0
1.0	105.0	98.4
2.0	107.9	97.5
3.4	112.9	100.0

CA, U.S.A.) set at 40°C. The fluorometer from Perkin-Elmer (Norwalk, CT, U.S.A.), Model 650-10S, with a xenon power supply is set at an excitation wavelength of 340 nm (slit 5 mm) and an emission of 455 nm (slit 5 mm). The ultraviolet detector from Waters Assoc., Model 441, is set at 313 nm.

HPLC conditions

Chromatography of CS and its prodrug involved ion-pair separation on a stationary reversed-phase. The mobile phase consists of 75 ml isopropanol (65 ml

TABLE II

STABILITY OF ACS AND CS IN PLASMA AND TREATED PLASMA AT AMBIENT TEMPERATURE

Components were mixed in the same standard at 7.5 μg/ml. Values represent percentages of zero-hour value.

Time h	Control plasma		Plasma with sodium borate buffer		Plasma with sodium carbonate	
	ACS	CS	ACS	CS	ACS	CS
1	93.4	98.3	98.2	95.3	96.2	94.6
2	100.2	108.1	102.5	109	99.6	95.2
5	83.3	93.0	90.4	100.5	89.6	88.6
7	72.7	106.9	92.5	98.4	91.4	105.0
24	126.2	76.5	104.9	89.9	107.2	76.2

TABLE III

LINEAR REGRESSION ANALYSIS OF CS AND ACS IN PLASMA AND URINE

Matrix	Compound	Slope	Intercept	r^2	Range (μg/ml)
Plasma	ACS	0.018	0.159	0.996	0.3–7.5
	CS	0.008	0.065	0.993	0.3–15.0
Urine	ACS	0.018	0.040	0.997	2.0–100.0
	CS	−0.009	0.013	0.999	2.0–100.0

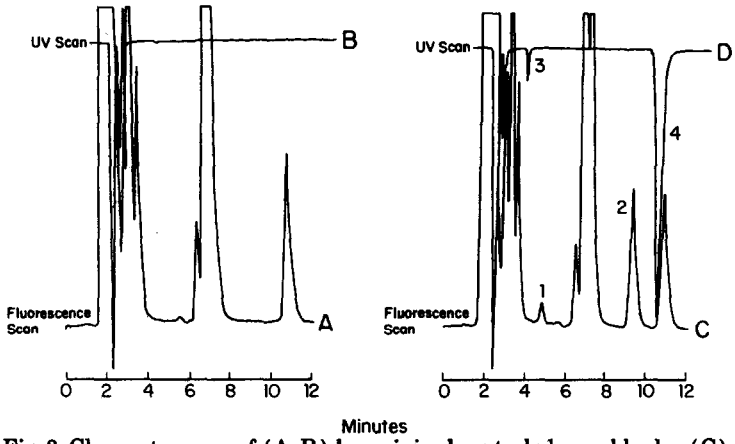

Fig. 3. Chromatograms of (A, B) heparinized control plasma blanks, (C) control plasma containing 1.4 μg/ml CS and 6-ACA (internal standard) and (D) control plasma containing 1.6 μg/ml ACS and 5-MIAA (internal standard). (A and C) Fluorescence detection; (B and D) ultraviolet detection. Peaks: 1 = CS; 2 = 6-ACA; 3 = ACS; 4 = 5-MIAA.

for the urine assay), 800 ml water, 5 ml glacial acetic acid and 0.5 decanesulfonate titrated with 1 *M* potassium hydroxide to pH 4.4. The mobile phase flows at a rate of 2.3 ml/min through the analytical columns and ultraviolet detector to a low-dead volume tee. The eluent mixes with the fluoraldehyde reagent at 1.2 ml/min and continues to react in the reactor (dead volume 1.5 ml). The reactants then flow through the fluorometer.

Sample preparation

Plasma. A frozen sample (at −70°C) is thawed to a liquid, mixed by vortex, and a 1-ml aliquot is transferred to a 13-ml tube. Each aliquot is then mixed with 0.25 ml of 0.2 *M* sodium borate buffer, pH 9.75 (12.4 g boric acid with 100 ml of 1 *M* sodium hydroxide, diluted to 250 ml with water), 30 μl of 6-ACA (240 μg/ml) and 50 μl of 5-MIAA (6.5 mg/ml). Each aliquot is then transferred to a Centriflo ultrafilter from Amicon (Danvers, MA, U.S.A.) and centrifuged for 15 min at 723 *g*. An injection volume of 20–25 μl of the ultrafiltrate is analyzed for CS and its prodrug.

Urine. A 1-ml aliquot from a thawed sample is mixed with 30 μl of 6-ACA, 50 μl of α-aminobutylhistidine (3 mg/ml) and 1 ml of a sodium carbonate solution (200 mg/ml water). Only five to six samples should be prepared at a time at this step (because of CS instability). The aliquots are then mixed by vortex with 2 ml of isopropanol and centrifuged at 723 *g* for 5 min. The top organic layer is analyzed by HPLC with an injection volume of 25 μl.

Protocol of clinical study (study design)

A clinical study was conducted to determine the safety, tolerance and pharmacodynamics of this pentizidone prodrug of CS. Normal male volunteers were randomly assigned to receive orally the prodrug (360 mg every 8 h) or placebo for five days (informed consent was obtained from the subjects). Following a four-day washout, each of the volunteers was crossed over to receive the alternate

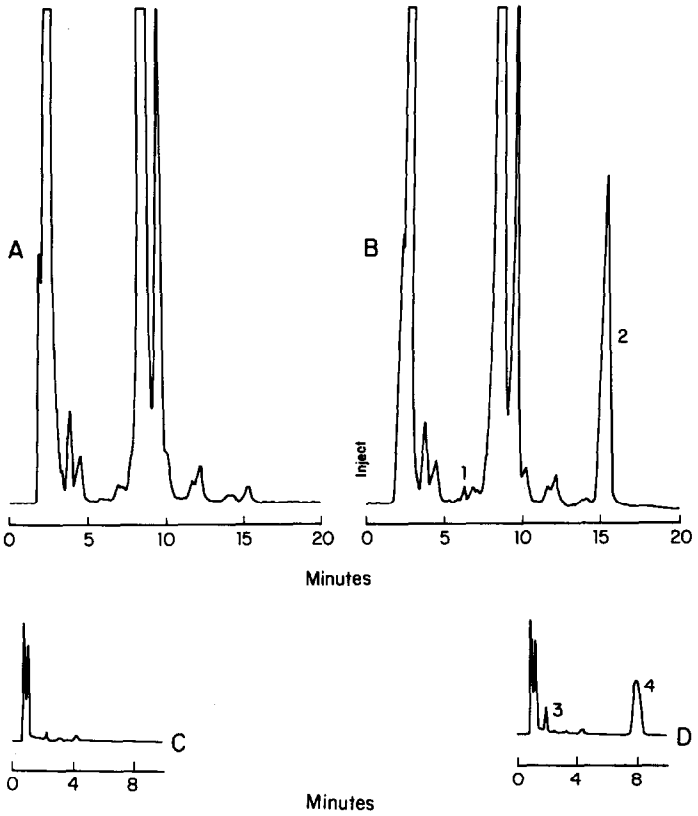

Fig. 4. Chromatograms of (A) a control urine blank, (B) control urine containing 5 μg/ml CS and α-aminobutylhistidine (internal standard), (C) a control urine blank and (D) control urine containing 10 μg/ml ACS and 5-MIAA (internal standard). (A and B) Fluorescence detection; (C and D) ultraviolet detection. Peaks: 1 = CS; 2 = α-aminobutylhistidine; 3 = ACS; 4 = 5-MIAA.

treatment. All medications were given orally as matching capsules under double-blind conditions with a 250-ml glass of water. Other medications or alcohol were not permitted seven days prior to the initiation of the study and throughout the duration of the study. Treatments were initiated while volunteers fasted and a normal diet was resumed 3 to 4 h after dosing.

Blood samples were collected from the forearm (anticubital fossa) of subjects into Vacutainers containing heparin. The samples were immediately centrifuged and plasma aliquots were frozen at −70°C. Each urine collection was transferred into a sterile flask, volume was measured and, thereafter, an aliquot was buffered with sodium carbonate to pH 9–10 (for CS stability) and frozen at −70°C.

RESULTS AND DISCUSSION

The present assay for CS and ACS involved the simultaneous detection of both components in plasma or urine without lengthy isolation procedures before HPLC analysis. Both compounds are unstable in acidic media (14–16] and are not

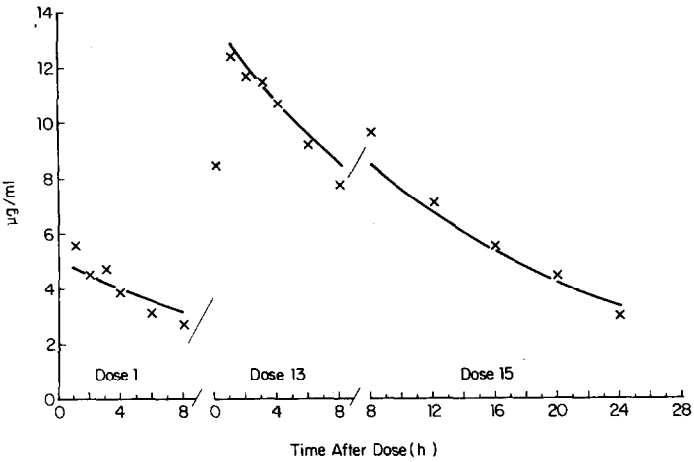

Fig. 5. Representative plasma concentration profile of CS in one subject receiving multiple doses of ACS (360 mg every 8 h) for five days.

appreciably soluble in organic solvents that might be used for sample extraction [17]. CS will hydrolyze to β-aminoxy-*D*-alanine and will subsequently in high concentrations form a dimer (Fig. 1, IV) [14]. The prodrug will hydrolyze to CS and acetylacetone [3].

The HPLC system was designed to utilize the inherent structural characteristics of both compounds for detection. In a plasma or urine matrix ACS is quantified by an ultraviolet detector (313-nm filter, λ_{max} 313 nm, molar absorptivity 20 900) [3]; CS is derivatized with *o*-phthalaldehyde and 2-mercaptoethanol, post-column, and quantified as an isoindole by a fluorometer. ACS does not derivatize as an enamine adduct of acetyl-acetone; CS does not absorb ultraviolet light above 226 nm [18].

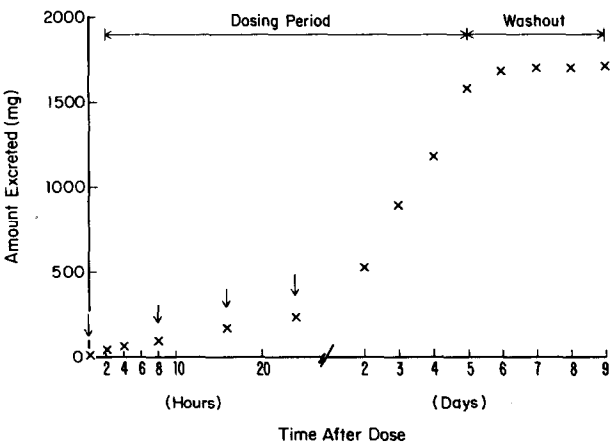

Fig. 6. Accumulative urinary levels of CS in one subject receiving multiple doses of ACS.

Aliquots of plasma samples containing ACS and CS are buffered to pH 9.0 for stability, mixed with internal standards 5-MIAA and 6-ACA (or α-aminobutylhistidine), deproteinated by ultrafiltration and analyzed by ion-pair, reversed-phase chromatography. The ion-pair chromatography is necessary to retain CS but not ACS on reversed-phase. The specificity of the chromatography and the selectivity of the detection system allow for minimal sample preparation of both unstable components.

Urine samples containing CS and ACS are mixed with the internal standards above, saturated with sodium carbonate and extracted into isopropanol. The extract is analyzed with the same chromatography without any further manipulations for both components. A less successful approach involved separating ACS from CS with C_{18} Sep-Pak (from Waters Assoc.) and then isolating CS from the eluent on a weak cation-exchange resin (phosphonic acid, proton form). This procedure was long and variable.

The instability of ACS and CS in urine was extensively studied to determine the viability of a urine assay. The stability of ACS in urine at 37°C was tested since these conditions may exist for a sample between collections in the bladder of a human subject. It was found that ACS in urine (5 and 15 μg/ml) incubated at 37°C degraded within 30 min (Fig. 2). In urine (e.g. pH 6.7) at ambient temperature, ACS (12.5 μg/ml) hydrolyzed with a half-life of 2.4 h. In urine treated with a sodium borate buffer at pH 9.0 or sodium carbonate at pH 10, ACS did not appreciably hydrolyze over 7 h. Thus, urine samples containing ACS could be collected and prepared with minimal drug loss, but the significance of the assay appeared negligible since ACS could not survive in gastric acid fluids after oral administration or in urine held in the bladder. CS in untreated urine degraded 15–17.5% at ambient temperature (20 and 80 μg/ml) over 3 h; in urine saturated with sodium carbonate for isopropanol extraction (100 mg/ml), it degraded 18.6–23.1% (43.0–129.0 μg/ml) over 3 h. However, there was little loss of CS in treated urine cooled in wet ice (Table I) or in an isopropanol extract.

The stability of both ACS and CS in plasma (combination) was investigated in control human plasma (pH 8.2), and plasma treated with sodium borate buffer (pH 9.0) or with sodium carbonate (pH 10) at ambient temperature. The data in Table II show, for all conditions, a slight loss for ACS and gain for CS. The degradation for ACS, however, appeared less pronounced in the treated plasmas, especially with the sodium borate buffer. Therefore, plasma samples were treated with a sodium borate buffer before preparation for analysis.

Ultrafiltration of plasma standards containing ACS and CS gives linear coefficients of determination (Table III) from a minimum concentration of 0.3 μg/ml for both components. Similarly, extraction of urine standards (Table III) gives a linear plot from a minimum concentration of 2.0 μg/ml. Assay specificities for plasma and urine matrices are depicted in Figs. 3 and 4. In the CS assay, fludalanine elutes before CS and the dimer of CS elutes later. In the ACS assay, fludalanine and the CS dimer do not absorb at 313 nm.

Recoveries of ACS and CS from plasma by ultrafiltration (compared with direct aqueous standards) are 81.2 ± 8.9 and 90.1 ± 10.6%, respectively; from urine

extraction, 86.4 ± 5.1 and 60.4 ± 9.1%, respectively. Intra-day reproducibility of standard replicates ($n = 6$) for the CS plasma assay varied from 6.1% at 0.3 μg/ml to 2.8% at 1.5 μg/ml; ACS reproducibility varied from 5.9% at 0.3 μg/ml to 2.3% at 1.5 μg/ml. For CS urine, reproducibility varied from 12.3 to 4.6% at 2.0 to 100.0 μg/ml; for ACS, 7.3 to 2.1%.

Inter-day variability of standard replicates for CS plasma kept at −70°C was 11.0 and 4.9% at 2.0 and 9.0 μg/ml, respectively; variability of ACS in the same standards was 11.6 and 6.5% at 1.5 and 6.0 μg/ml. For CS urine replicates (containing sodium carbonate) kept at −70°C (twenty days), variability was 5.2 and 3.2% at 20 and 80 μg/ml, respectively. For ACS urine kept at −70°C (twenty days), 6.1% at 5.0 μg/ml. ACS standards (4.0 μg/ml) kept at −20°C did degrade over twenty days with first-order kinetics (half-life 11.5 days).

The plasma concentration profile of CS for a typical subject (No. 8) is given in Fig. 5. The plasma drug concentration peaked at or before the first sample and henceforth decreased monoexponentially. Essentially, no ACS was detected in the plasma collections. For this subject, 57% of the dose was excreted in the urine as CS (Fig. 6). It suggested an important role of the kidney for the drug's elimination.

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