Determination of CS-045 (U-87326) in Rat Serum Using LCEC

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Pharmacokinetic evaluations of CS-045, a novel thiazolidinedione hypoglycemic, needed an assay with better selectivity, precision and greater sensitivity than that afforded by the LCUV assay method currently in use. By using electrochemical detection the sensitivity and selectivity of the assay was increased by approximately a factor of 10, as was the assay accuracy and precision.

CS-045 (chemically known as (±)-5-(4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl)-2, 4-thiazolidinedione, U-87326) was developed by Sankyo Co., Ltd., Tokyo, Japan, as a potential oral antidiabetic drug (***F1***). This drug is part of the novel thiazolidinedione class of compounds and has been shown to reduce insulin resistance, or potentiate insulin action, in genetically diabetic and/or obese animals (1). The mode of action differs from the most commonly used oral hypoglycemics (sulfonylureas), which potentiate insulin action and secretion in peripheral tissues and has potential undesired side effects. When administered orally to KK mice, ob/ob mice and Zucker fatty rats, CS-045 showed potent hypoglycemic and hypolipidemic effects (2,3). Clinical studies have revealed that an oral administration of CS-045 improved various parameters of glycemic control (2). An earlier method for determination of the concentration of CS-045 in serum was developed by the Pharmaceutical Development Division of Takeda Chemical Industries, Ltd., Japan, which utilized an acetonitrile precipitation of serum proteins followed by an LCUV analysis (4). The chromatography obtained with blank rat serum prepared by this method had a number of endogenous peaks which eluted close enough to CS-045 to limit the sensitivity of the assay. We have modified this LCUV method by using solid phase extraction (SPE) on a phenyl SPE column to improve selectivity and therefore, sensitivity. To further improve the assay selectivity and sensitivity, a method using electrochemical detection (LCEC) was developed which improved the method sensitivity to a low limit of quantitation at 0.01 µg/mL. The redox chemistry of this compound is similar to that observed for tocopherols (***F2***) (5). This report describes the LCEC method development, as well as the comparison with the modified LCUV method.

Experimental

Materials

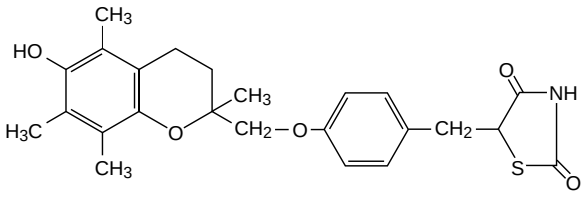
CS-045 (lot # M683-R1102) was obtained from Takeda Chemical Industries (Tokyo, Japan). The internal standards, U-91325 (lot # 22057-EJJ-137B) and U-80036E (lot # 22057-EJJ-54A) were obtained from Pharmacia & Upjohn, Inc. (Kalamazoo, MI, USA). Acetonitrile (high purity solvent grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). Glacial acetic acid and ammonium hydroxide were of analytical grade and obtained from Mallinckrodt (Paris, KY, USA). The ethylenediaminetetraacetic acid, disodium salt dihydrate was obtained from the Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Purified water was generated by a Milli-Q reagent water system (Millipore Corporation, Bedford, MA, USA).

Cyclic Voltammetry

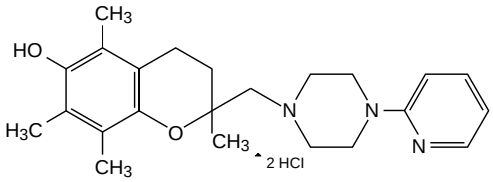
Cyclic voltammetric (CV) measurements were performed on a Bioanalytical System's Model BAS100A Electrochemical Analyzer (West Lafayette, IN) at room temperature (21-23 °C) using a glassy carbon working electrode, Ag/AgCl reference electrode and

F1

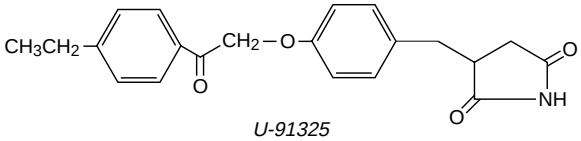
Structures of CS-045 (U-87326) and U-80036E and U-91325, the internal standards (IS).



CS-045 (U-87326)



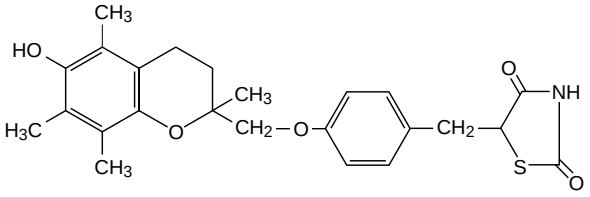
U-80036E



U-91325

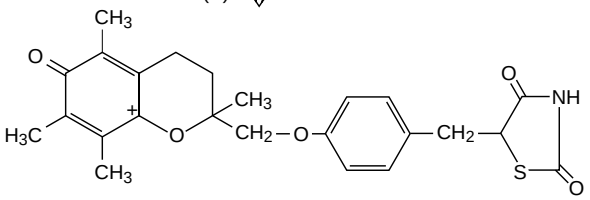
F2

Redox Chemistry of CS-045.

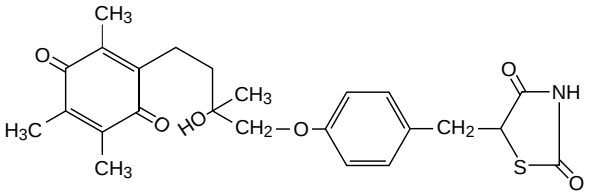


CS-045 (U-87326)

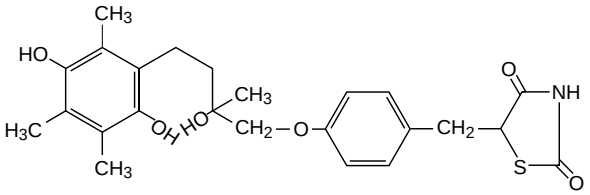
(A) ⇓ *-2e-, -H+*



⇓ *H2O*



(C) ⇑⇓ *+2e-, +2H+* (B)



platinum auxiliary electrode. Sample solutions were sparged with high purity helium (99.995%, AGA Specialty Gas, Maumee, OH) for two minutes prior to CV evaluation to minimize interferences from adventitious dioxygen during the reductive sweep segments. Default scan conditions were set as follows: Sweep segment 1, 1 to -400 mV; Sweep segment 2, -400 to 1500 mV; Sweep segment 3, 1500 to -400 mV; Scan rate, 50 mV/s; and Sample interval, 2 mV. The cyclic voltammetry evaluations were conducted at a drug concentration of approximately 20 mM in 25 mM sodium acetate buffer adjusted to pH 5.0 with glacial acetic acid. Samples were accurately weighed and dissolved in the solvent immediately prior to CV evaluation.

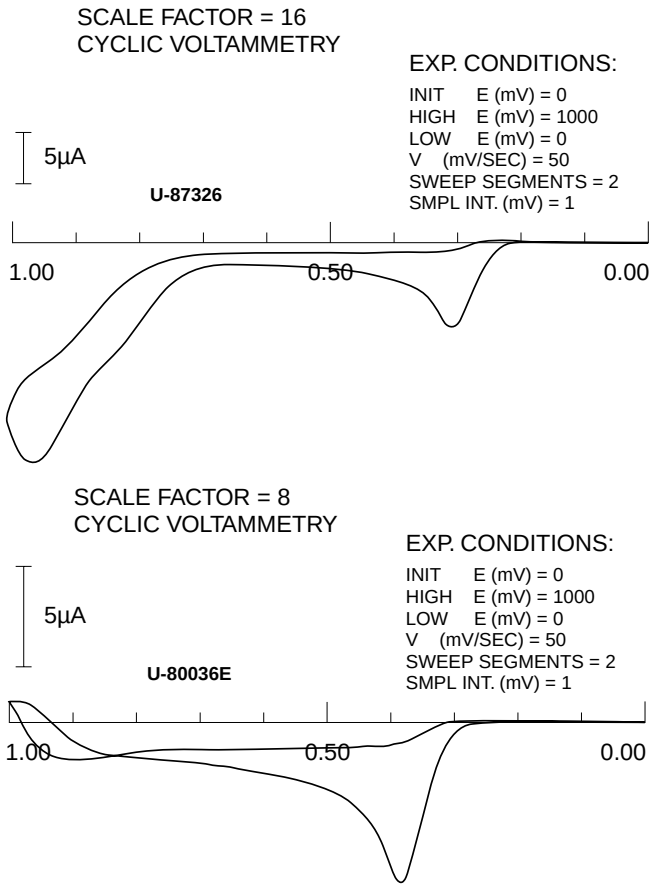
Observed peak potential maxima were determined using the instrument's internal peak finding software. Representative voltammograms were plotted on a Hewlett Packard ColorPro X-Y Plotter. It should be noted that the observed peak potential (E_{pa}) is not a direct measure of the redox potential (E_o), but they are related. Compounds exhibiting diffusion controlled electrode behavior, an unstable oxidation product with respect to chemical reaction, and those with no observable peaks on the reverse scan, share the following relationship:

$$Epa = E^o - 0.9\,(RT/nF) + (RT/3nF)\ln(2kCRT/3nF\upsilon)$$

where *C* is the initial concentration, υ is the potential scan rate, and *k* is the rate constant for a bimolecular chemical reaction presumed to occur for the initial oxidation product (6). True redox potentials have not been calculated for the current evaluation, but the lowest observed oxidation potentials are presented to allow relative comparison of oxidative susceptibility of a series of closely related

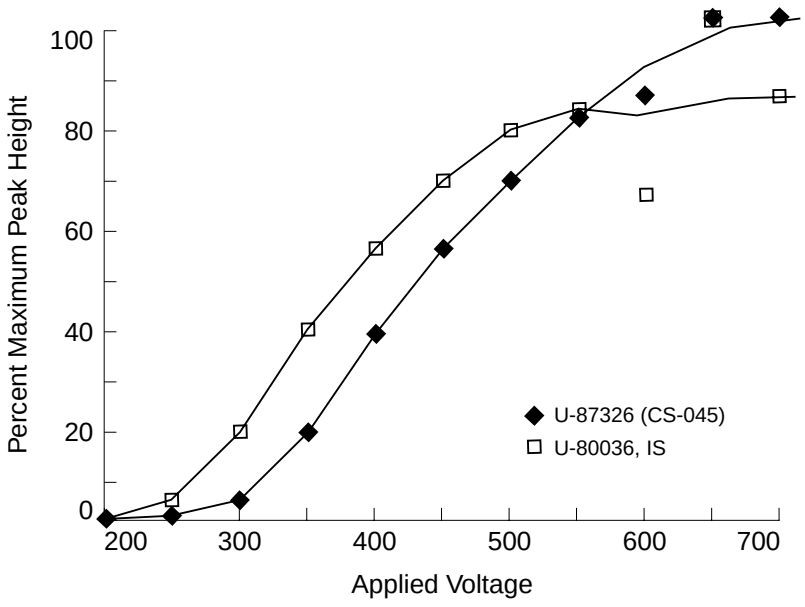
F3

Cyclic Voltammetry of CS-045 (U-87326) and U-80036E.



F4

Hydrodynamic Voltammograms of CS-045 (U-87326) and U-80036E (IS).



analogs to facilitate LC method development.

Hydrodynamic Voltammogram

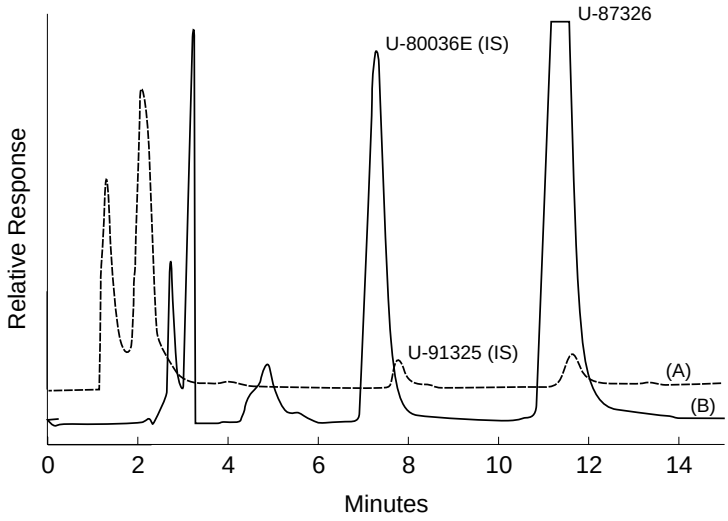
A 0.5 µg/mL solution of CS-045 and a 0.8 µg/mL of U-80036E was prepared in acetonitrile:water (2:1 v/v). A 100 µL sample was injected by an ISS 100 Autosampler (Perkin Elmer, Norwalk, CT) onto an Inertsil PH reverse phase column (250 x 4.6 mm ID, 5 µm particle size) (MetaChem Technologies Inc., Redondo Beach, CA). An SP8800 HPLC pump (Spectra Physics, Fremont, CA) delivered the mobile phase of acetonitrile:water (56:44 v/v) containing 4 mL/L glacial acetic acid and 0.1 mM ethylenediaminetetraacetic acid, disodium salt dihydrate (pH was adjusted to 5.50 ± 0.05 with ammonium hydroxide) at 1 mL/min. The eluate was monitored by amperometric detection using a BAS LC-4C (with a CC-5) amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN) set to applied voltages in 50 mV intervals from +100 to +700 mV. Peak height was determined by in-house chromatography system software on a Harris Nighthawk Computer System (7).

LCEC

To determine the concentration of CS-045, 0.1 mL of rat serum was vortex-mixed with 0.3 mL acetonitrile which contained 0.83 ng U-80036E/mL (***F1***) as the internal standard (IS) in a 1.5 mL polypropylene conical centrifuge tube. The tube was centrifuged at 14000 rpm for 2 minutes to precipitate the proteins. The organic acetonitrile supernatant was removed and transferred to a clean, labeled autosampler vial containing 200 µL of distilled water. The autosampler vial was capped and vortex-mixed. The mixture was then analyzed by LC on an Inertsil PH reverse phase column (250 x 4.6 mm ID, 5 µm particle size) (MetaChem Technologies Inc., Redondo Beach, CA) with a mobile phase of acetonitrile:water (56:44 v/v) containing 4 mL/L glacial acetic acid and 0.1 mM ethylenediaminetetraacetic acid, disodium salt dihydrate (pH was adjusted to 5.50 ± 0.05 with ammonium hydroxide). An SP8800 HPLC pump (Spectra Physics, Fremont, CA) delivered the mobile phase at 1 mL/min. One hundred µL of sample was injected by an ISS 100 Autosampler (Perkin Elmer, Norwalk, CT). The eluate was monitored by amperometric detection at an applied voltage of +500 mV using a BAS LC-4C (with a

F5

Comparison of Chromatograms of CS-045 and IS Fortified Rat Serum Samples Assayed by the Two Methods: A - LCUV; B - LCEC.



T1

Calibration Curve Data for CS-045, LCEC Method.

Theoretical Concentration µg/mL	Peak Height Concentration µg/mL		
0.01	0.0102	0.0105	0.0107
0.05	0.0446	0.0553	0.0472
0.10	0.0953	0.1057	0.1095
0.50	0.389	0.5082	0.4387
1.0	1.025	0.9387	0.9843
2.0	2.015	2.0282	2.0230
Original linear	0.0045	0.1837	0.1258
Linear coefficient of variation	2.2	1.3	1.4
Original correlation coefficient	0.9987	0.9996	0.9995
Relative weight response coefficient of variation	4	2.2	8.6

CC-5) amperometric detector (Bioanalytical Systems, Inc., West Lafayette, IN). Quantitation was accomplished by linear regression (force through the origin with no weighting) based on a peak height ratio of the drug to the IS using in-house chromatography system software on a Harris Nighthawk Computer System (7).

LCUV

Each serum sample, 0.1 mL, was briefly mixed with 0.3 mL of water and 0.025 mL of U-91325 (IS, 10 µg/mL). This mixture was loaded onto a phenyl solid phase extraction column (SPE, 100 mg/mL, Varian, Harbor City, CA) that had been prewashed with 1 mL of acetonitrile followed by 1 mL of water at 10 in. Hg. The column was dried at 20 in. Hg for 5 min. The drug and IS were eluted from the SPE column with 0.6 mL acetonitrile into a 10 x 75 mm disposable glass culture tube. The eluate was taken to dryness and redissolved in 0.3 mL acetonitrile:water (1:1 v/v) with vigorous vortex-mixing for the purpose of LC analysis. The LC conditions were the same as described above for the LCEC method, except a UV wavelength of 280 nm was used for detection. Quantitation was accomplished by linear regression based on peak area ratio of drug to the IS using in-house chromatography system software on a Harris NightHawk Computer System (7).

The assay method was evaluated for precision, accuracy and suitability in three analytical runs following the guidelines given by Shah et al. (8).

Results and Discussion

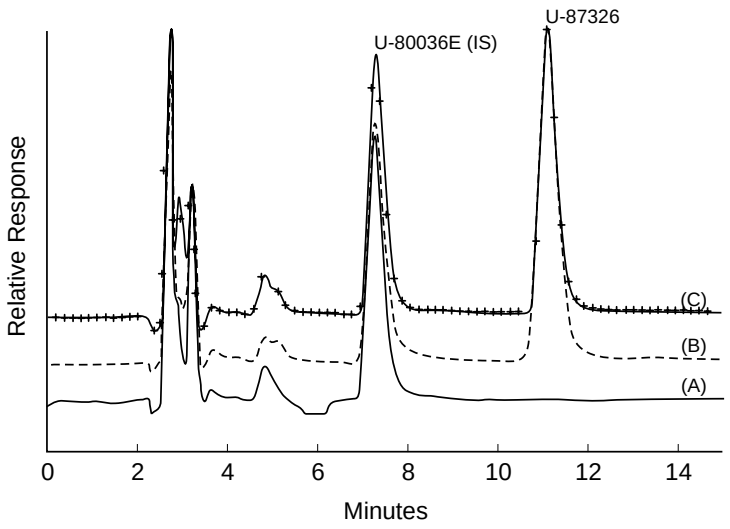
The initial assay of samples from rats administered with CS-045 was accomplished using the LCUV method. Serum concentrations less than 0.1 µg/mL could not be detected using this method. Although the application of solid-phase extraction to this LCUV method improved the selectivity, it did not yet meet the requested sensitivity. To further improve the selectivity and sensitivity, a method employing EC detection was used instead of UV detection.

The structural analogy of the chroman ring of CS-045 and of α-tocopherol provided the basis for attempting EC detection of CS-045. The oxidation of α-tocopherol to an intermediate carbonium ion and the subsequent addition of a hydroxyl in the presence of water could be carried out on the chroman of CS-045 (***F2***) to break the ring. The redox properties of CS-045 were initially established from cyclic voltammetry of the compound and the internal standard (IS) U-80036E (***F2***). The CV diagrams for CS-045 and IS are shown in ***F3***. Maximum response for both compounds appeared to peak around 500 mV. This was confirmed by preparing a hydrodynamic voltammogram for both CS-045 and IS as shown in ***F4***.

T1 presents the calibration curve data obtained for the valida-

F6

Chromatograms of a Predose Rat Serum Sample (A), a Fortified Rat Serum Standard (B, 1000 ng/mL), and a 0.5 hr Rat Serum Sample from a Toxicity Study (C, DSR Protocol 93-237)



T2

Concentration-Time Data of CS-045 in rats administered the drug at different dose levels in a Tween 80 (TW) or Polyethylene Glycol (PG) formulation. CS-045 concentrations are in mg/mL serum. (Protocol 93-237).

Time (hr)	Rat #32 160 mg/kg (TW)	Rat #31 160 mg/kg (TW)	Rat #20 160 mg/kg (PG)	Rat #7 160 mg/kg (PG)
0.5	1.02	0.41	2.04	0.22
2	3.25	1.29	0.42	0.05
4	0.3	0.76	0.52	0.03
23	0.6	1.08	0.0	0.0

tion of the LCEC method. A linear relationship (Y *IS = aX, force through the origin with no weighting) was found between the peak height ratio and concentration in the range of 0.010 to 2 µg/mL for CS-045. Detector response for concentrations above 2 µg/mL was not linear. The low limit of quantitation (LLOQ) was 0.01 µg/mL for CS-045. The extraction recovery mean ranged from 80% to 101.3% for CS-045. The IS had an extraction recovery that ranged from 87% to 106%. The inter-assay precision, as expressed by the coefficient of variation of the back calculated concentrations of the calibration standards among the three analytical runs, ranged from 5.7% to 14.6%.

F5 (previous page) compares the chromatograms of a rat serum sample fortified with CS-045 and assayed using the two LC methods. The chromatogram of the UV detected sample (A) was fortified with 10 µg/mL. The chromatogram of the EC detected sample (B) was fortified with 1 µg/mL. EC detection improved the sensitivity by approximately a factor of 10. With these considerations, and the desire for more selectivity and sensitivity in the assay of serum samples for future studies, the LCUV method was dropped in favor of the LCEC method.

F6 shows typical chromatograms of the extract of a rat serum blank with IS, a fortified serum standard containing 1 µg/mL of U-873326 and a serum sample from a rat dosed with CS-045. There is no interference at the retention times of CS-045 or U-80036E, IS.

Rat serum samples from two subsequent studies were successfully analyzed using this LCEC method. The serum concentrations of U-87326 for a toxicokinetic study are shown in ***T2***.

Conclusion

The assay was greatly improved by use of electrochemical detection, which had much higher selectivity and sensitivity when compared to the UV detection. The LCEC method is simple and rapid. The assay precision, as well as the increased selectivity and sensitivity, has proved to be suitable for pharmacokinetic/toxicokinetic evaluation.

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