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Note

Determination of the 5-lipoxygenase inhibitor (CGS 8515) in plasma by high-performance liquid chromatography using reductive electrochemical detection

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Methyl 2-[(3,4-dihydro-3,4-dioxo-1-naphthalenyl)amino]benzoate (CGS 8515, I, Fig. 1) is a potent 5-lipoxygenase inhibitor undergoing preclinical eval-

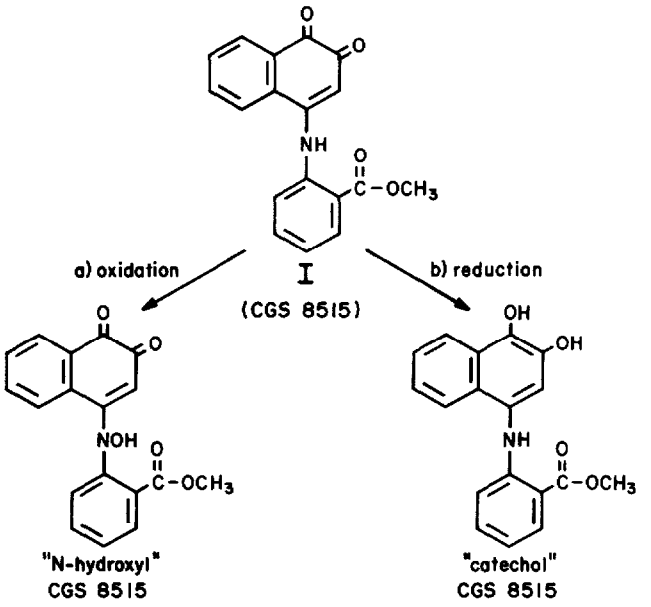

Fig. 1 Structure of CGS 8515 (I) and proposed (a) oxidation and (b) reduction products

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uation in animal models for asthma, psoriasis and rheumatoid arthritis [1]. The development of an analytical method to measure I in plasma is necessary to provide information to assess the relationship between plasma concentration and 5-lipoxygenase inhibition in these models. In anticipation of exceptionally low plasma concentrations, electrochemical detection was used to obtain greater sensitivity and selectivity.

Compound I contains both an oxidizable conjugated aromatic amine and a reducible naphthoquinone ring and is therefore amenable to detection by either oxidative or reductive electrochemical detection. Varney and Preston [2] and Radzik and Kissinger [3] have used oxidative electrochemical detection for the determination of aromatic amines and Fluck *et al.* [4] and others [5,6] have shown the feasibility of using reductive electrochemical detection for the measurement of naphthoquinones. Numerous analytical procedures have been reported using oxidative electrochemical detection [7], however, assays utilizing the reductive mode of detection are less common. The latter often require extensive modification in equipment and sample handling to eliminate background interference from dissolved oxygen [8].

In this report, we have compared a high-performance liquid chromatographic (HPLC) procedure using either oxidative or reductive electrochemical detection. Less interference from endogenous substances was obtained using reductive detection and an assay using this approach is described.

EXPERIMENTAL

Chemicals

HPLC-grade water, methanol and ethyl acetate were obtained from American Burdick and Jackson (Muskegon, MI, U.S.A.). HPLC-grade sodium phosphate and sodium acetate were from Fischer Scientific (Fair Lawn, NJ, U.S.A.). Compound I was supplied by Chemistry Research, Ciba-Geigy (Summit, NJ, U.S.A.).

Cyclic voltammetry

Cyclic voltammetry was performed using a CV-27 cyclic voltammograph from Bioanalytical Systems (West Lafayette, IN, U S.A.). A solution containing I at a concentration of 20 μg/ml in methanol–0.05 *M* sodium acetate buffer, pH 4.5 (60:40, v/v) was scanned at a rate of 40 mV/s. The voltage was set between +1.3 V and −0.3 V at a gain of 0.005.

Chromatography

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) 840 data and chromatography station, a WISP 710B automatic sample processor, a LDC/Milton Roy (Tampa, FL, U.S.A.) ConstaMetric pump and a Bioanalytical Systems LC-4B amperometric detector with a glassy carbon electrode and an Ag/AgCl reference electrode. Chromatography was performed at ambient tem-

perature (18–22°C) on a Waters μBondapak, 10 μm particle size, 300 mm × 4.6 mm I.D. octadecyl column. A Waters Guard-Pak precolumn module packed with octadecyl μBondapak was installed in-line prior to the analytical column. The mobile phase was prepared by mixing methanol with 0.15 *M* sodium acetate–hydrochloric acid buffer, pH 4.5 (60:40, v/v). The mobile phase was degassed and filtered through a 0.22-μm nylon membrane filter prior to use. The flow-rate was 1.3 ml/min. Several analogues of I were tested as internal standards, but the appearance of unknown metabolites of I having the same retention time required the use of the assay without an internal standard. Drug concentrations were calculated based on peak heights. Standard curves were evaluated by least-squares linear regression.

Standard curves

Standard solutions of I were prepared by diluting a stock solution of I (1000 ng/ml) in methanol. A fresh stock solution was prepared every two weeks. For standard curves, six different standard solutions were prepared by appropriate dilutions of the stock solution such that 40 μl of each standard solution added to plasma (0.4 ml) gave final concentrations of 0, 5, 10, 25, 50 or 100 ng/ml.

Sample preparation

Sodium phosphate buffer, 0.2 *M*, pH 7.0 (0.4 ml), was added to 0.4 ml of plasma in a 75 mm × 12 mm glass tube. The sample was then extracted with 1.5 ml of ethyl acetate by vortex-mixing for 15 s, followed by centrifugation at 1000 *g* for 10 min. A 1-ml volume of the organic layer was transferred with an automatic pipetting device into another 75 mm × 12 mm glass tube. The organic extract was evaporated at room temperature (18–22°C) to dryness under a stream of nitrogen. The residue was immediately reconstituted in 60 μl of methanol and transferred to a WISP injection vial. A 30-μl aliquot was injected into the HPLC column.

Plasma samples

A pool of dog plasma containing 63 ng/ml I was prepared. Aliquots (0.4 ml) were placed in 75 mm × 12 mm glass tubes and stored at −20°C pending analysis. Plasma samples from rats, dogs and monkeys treated with I were obtained from the Department of Preclinical Drug Metabolism, Ciba-Geigy (Ardsley, NY, U.S.A.). The animals were treated orally with I (100–200 mg/kg) in a 3% (w/v) corn starch suspension. Samples were taken at 2 and 6 h after dosing. Samples were stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Compound I was found to undergo oxidation at potentials of +1.0 V or greater and reduction between −0.1 and −0.15 V by cyclic voltammetry. Radzik

and Kissinger [3] have shown that aniline has a half-wave potential of +0 82 V. Varney and Preston [2] have shown that other aromatic amines can undergo oxidation at potentials between +0.4 and +0.7 V. Therefore the potential for the oxidation of I at +1.0 V is in a similar range to the oxidation of aniline and probably represents oxidation of the nitrogen of I (Fig. 1). Fluck *et al.* [4] have demonstrated that naphthoquinones undergo reduction at potentials of −0.10 to −0.5 V. The reduction of I between −0.1 and −0.15 V is most likely is due to reduction of the naphthoquinone to the corresponding catechol of I (Fig. 1). To determine if the electrochemical properties could be used for detection of I under chromatographic conditions, an LC-4B amperometric detector was fitted in-line after a Waters μBondapak octadecyl column and the potential varied between +0.8 and +1.3 V and between −0.1 and −0.2 V. The background current and signal-to-noise ratio for chromatographed standards were optimal for oxidation at +0.1 V and for reduction at −0.15 V. A similar peak height was obtained when the same amount of I was injected on the column determined by either oxidation or reduction.

A variety of columns (C_{18}, phenyl, 3–10 μm particle size) and mobile phase compositions were investigated to select the best conditions for chromatography of I. After considerable experimentation, A Waters octadecyl μBondapak (10 μm particle size) column and a mobile phase consisting of methanol–0.05 *M* sodium acetate buffer, pH 4.5 (60:40, v/v) were selected. Using these conditions, compound I had a retention time of 8 5 min. The hydrolyzed ester and *o*-napthohydroquinone, which are potential metabolites, were also detectable using oxidative or reductive electrochemical detection. The retention time of the hydrolyzed ester was 4.2 min and the retention time of the *o*-naphthohydroquinone was 3.9 min. Neither compound interfered with the analysis of I.

The feasibility of developing an assay using the oxidative or the reductive mode of detection for I was evaluated by comparing an ethyl acetate extract of buffered plasma (pH 7.0) with an extract from buffered plasma supplemented with I (25 ng/ml). The chromatograms analyzed under the oxidative mode contained an interfering peak (Fig. 2). The interfering peak was not eliminated by using other liquid–liquid or liquid–solid extractions. When the ethyl acetate extracts of plasma were analyzed using the reductive mode of detection (−0.15 V), no interfering peaks were detected at this low potential (Fig. 2).

The stability of I was determined at various stages of the procedure. Compound I was stable in methanol solutions stored at −4.0°C for at least two weeks. The stability of I during the evaporation step was evaluated by adding I directly to tubes containing 1.0, 2.0 and 3.0 ml of ethyl acetate. The samples were then evaporated with nitrogen, and compound I was found to undergo slight decomposition when 2.0 or 3.0 ml of ethyl acetate were used. Greater decomposition was observed at 3.0 ml than at 2.0 ml. Different solvents were also examined. Greater decomposition was observed when methanol was used and less decomposition was observed when hexane was used. These observations suggest that the

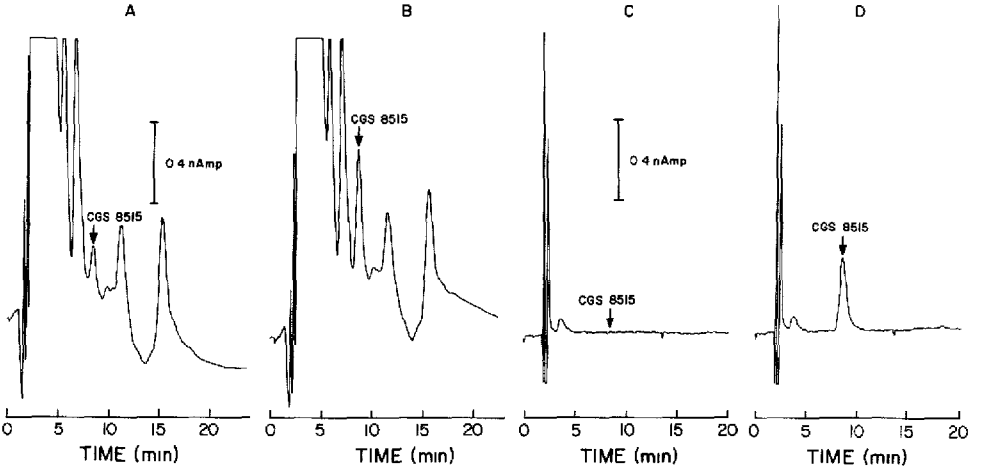

Fig. 2 Chromatograms of plasma extracts of (A) drug-free plasma analysed with oxidative mode (+1 0 V), (B) plasma supplemented with 25 ng/ml I and analyzed with oxidative mode, (C) drug-free plasma analyzed with reductive mode (−0 15 V) and (D) plasma supplemented with 25 ng/ml I and analyzed with reductive mode.

organic solvent *per se* was not responsible for the decomposition, but rather the time required for evaporation of the solvent. Decreasing the time of evaporation by using less volume or a more volatile solvent such as hexane resulted in no decomposition; increasing the time required for evaporation by increasing the volume or using a less volatile solvent such as methanol increased the amount of decomposition. The stability of I in the dried residue after evaporation was also evaluated. The immediate reconstitution of the residue in methanol was critical for minimizing decomposition. Compound I was, however, stable for at least 24 h after reconstitution in methanol. The method selected for extraction of I from plasma consisted of buffering 0.4 ml of plasma with 0.4 ml of sodium phosphate buffer (0.2 *M*, pH 7.0), followed by extraction with 1.5 ml of ethyl acetate. After centrifugation, 1 ml of the organic phase was evaporated at room temperature (18–22°C) and immediately reconstituted in 60 μl of methanol. Despite the limitations of the extraction procedure, the absolute recovery of I from plasma was high and reproducible. At 5.0 ng/ml, 90 ± 7.7% (S.D.) of drug was extracted, while the recovery at 50 ng/ml was 87 ± 2.6% (S.D.).

Standard curves over the range 5.0–100 ng/ml were determined on five separate occasions. The standard curves were linear within the range tested and the mean (± S.D.) correlation coefficient was 0.998 ± 0.0023. The detection limit of the assay was estimated as 1.0 ng/ml at a signal-to-noise ratio of 3 using 0.4 ml of plasma. The within-day imprecision of the assay was determined by the replicate analysis of six plasma samples containing either 10 or 100 ng/ml. The coefficients of variation (C.V.) were 9.2 and 2.7% for the 10 and 100 ng/ml samples, respec-

tively. The inter-assay precision is shown in Table I which also shows that above 50 ng/ml the analytical recovery was quantitative and below that the level the analytical recovery was better than 90%.

The stability of I was determined by supplementing a pool of plasma at 63 ng/ml and storing at −20°C. On five separate occasions over four months the samples were found to contain 63 ± 4.0 ng/ml. This suggests that compound I was stable over this period.

The procedure was used to evaluate plasma samples from rats, dogs and monkeys treated orally with I. Chromatograms of plasma from untreated rats, dogs or monkeys did not show any interfering peaks (Fig. 3). Plasma from rats treated orally with I showed parent drug and two apparent metabolites with retention times of 5.8 and 13.7 min. Plasma samples from dogs treated with I showed parent drug and one apparent metabolite having a retention time of 5.2 min. Plasma samples from monkeys treated with I contained parent drug and four apparent metabolites with retention times of 4.2, 5.7, 7.4 and 13.0 min. These metabolites did not interfere with the determination of I but did interfere with the determination of several of the analogues of I tried as an internal standard for the assay. Fortunately, the reproducibility of the assay without the use of an internal standard was found to be within acceptable limits, and consequently the examination of other analogues of I suitable as an internal standard was not further investigated.

In summary, compound I was detectable by electrochemical detection in either the oxidative or reductive mode. However, it was only feasible to develop an assay using the reductive mode. At the low potential required for the reduction of the naphthoquinone ring, dissolved oxygen or interference from endogenous substances was not a significant problem For drugs containing easily reducible moieties such as a naphthoquinone, quinone or hydroquinone ring [8,9], reductive electrochemical detection may be a simple and sensitive method for their analysis.

TABLE I

INTER-ASSAY PRECISION OF COMPOUND I

Concentration added (ng/ml)	*n*	Concentration found (mean ± S D) (ng/ml)	Analytical recovery (%)	C V (%)
5 0	4	4.6±0.97	92±19	21
10.0	4	9.1±1 4	91±14	11
25 0	5	23 ±1 5	92±6 0	6 4
50.0	5	51 ±3 4	102±6 8	6 7
100 0	5	100 ±1.5	100 ±1 5	1 5

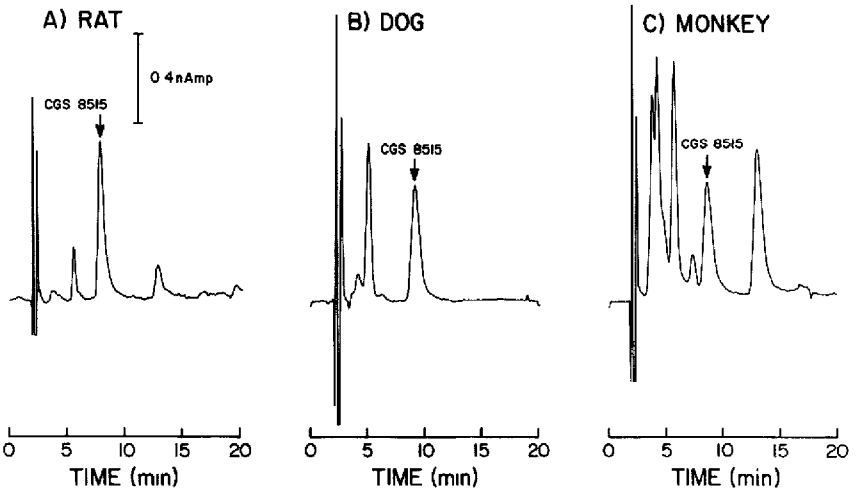

Fig. 3 Chromatograms of plasma extracts after the oral administration of I from (A) rat (100 mg/kg, 2 h after dosing), (B) dog (250 mg/kg, 2 h after dosing) and (C) cyanomolgus monkey (127 mg/kg; 6 h after dosing).

REFERENCES

1 E C Ku, A Raychaudhuri, G Ghai, E. F Kimble, W. H Lee, C. Colombo, R Dotson, T D. Oglesby and J W F Wasley, *Biochim. Biophys. Acta*, 959 (1988) 332
2 M S. Varney and M. R. Preston, *J Chromatogr.*, 348 (1985) 265.
3 D M Radzik and P T Kissinger, *Anal. Biochem.*, 140 (1984) 74.
4 D. S. Fluck, S M Rappaport, D. A. Eastmond and M. T. Smith, *Arch. Biochem Biophys* , 235 (1984) 351
5 S M. Rappaport, Z Jin and X Xu, *J. Chromatogr* , 240 (1982) 145
6 Z Zin and S M. Rappaport, *Anal. Chem* , 55 (1983) 1778
7 P T. Kissinger, K Bratin, W. P King and J R Rice, *ACS Symp. Ser.*, 136 (1981) 57.
8 K. Bratin and P. T. Kissinger, *J Liq. Chromatogr.*, 4 (1981) 321
9 K. Brunt, C. H P Bruins and D A Doornbos, *Anal. Chim. Acta*, 125 (1981) 85