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# DETERMINATION OF 2,3-DIHYDRO-6-[3-(2-HYDROXYMETHYL)-PHENYL-2-PROPENYL]-5-BENZOFURANOL IN PLASMA USING LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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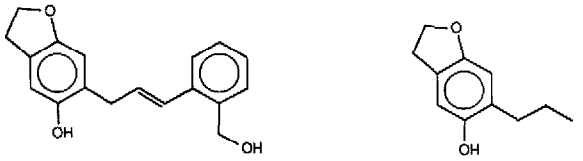
## SUMMARY

A reversed-phase column liquid chromatographic (LC) method with electrochemical detection (ED) is described for the quantification of 2,3-dihydro-6-[3-(2-hydroxymethyl)phenyl-2-propenyl]-5-benzofuranol (compound **1**), a new locally active dual inhibitor of leukotriene and prostaglandin synthesis, in plasma. After a single liquid–liquid extraction of the biological specimen, the extract was analyzed using a liquid chromatograph with an amperometric detector set at an oxidation potential of +0.55 V. The resulting chromatograms are free from endogenous interference and the limit of detection is 0.2 ng/ml. Several other analogous dihydrobenzofuranols were shown to be electrochemically active, permitting their determination using LC with ED. The described analytical method has been fully validated in the concentration range 0.5–20 ng/ml of plasma and utilized in the analysis of plasma samples from human clinical studies. The analytical methodology has also been adapted for analysis of compound **1** in human skin blister fluid after topical administration of **1**.

## INTRODUCTION

The 2,3-dihydro-5-benzofuranols were found to be potent 5-lipoxygenase (5-LO) inhibitors, in vitro [1]. 2,3-Dihydro-6-[3-(2-hydroxymethyl)phenyl-2-propenyl]-5-benzofuranol (compound **1**; Fig. 1), a member of this group, is a dual inhibitor of the synthesis of leukotrienes [concentration of the compound which gives 50% inhibition of the synthesis of leukotrienes or prostaglandins in mouse peritoneal macrophages ($IC_{50}$) = 0.6 $\mu M$] and prostaglandins ($IC_{50}$ = 2.2 $\mu M$) in mouse peritoneal macrophages [2]. In vivo, the compound is a potent topical antiinflammatory analgesic agent with a profile of activity extending beyond that of conventional cyclooxygenase inhibitors. The compound is not systemically active and is rapidly metabolized by the liver. It was chosen for clinical evaluation from 250 compounds synthesized [3].

In order to study the pharmacokinetics of **1** after both oral and topical administration of the drug to human and animal subjects, it was necessary to develop




(1) (2, IS)

Fig. 1. Chemical structures of **1** and internal standard (I.S., **2**).

an assay capable of determination of the drug in the range 0.5–5 ng per ml of plasma. This level of sensitivity could not be achieved using a simple liquid chromatographic (LC) method with ultraviolet (UV) detection [4], where the sensitivity limit was found to be 25 ng/ml, well above the concentrations of the drug in human plasma after topical drug administration. Therefore, the present study was undertaken to explore the feasibility of electrochemical detection (ED) for quantification of **1** and other analogous dihydrobenzofuranols. It was found that all of these compounds are electrochemically active and are oxidized at a very favorable, low oxidation potential (+0.5 to +0.8 V) permitting their sensitive and highly selective determination using LC–ED.

## EXPERIMENTAL

### *Materials*

HPLC-grade methanol, acetonitrile and water were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Monobasic sodium phosphate and ethylenediaminetetraacetic acid (EDTA) trisodium salt were reagent grade and were supplied by Sigma (St. Louis, MO, U.S.A.). Sodium hydroxide (Fisher Scientific, Pittsburgh, PA, U.S.A.) and dibasic sodium phosphate (Mallinckrodt, Paris, KY, U.S.A.) were also of reagent grade. Methylene chloride (HPLC grade), phosphoric acid (85%, LC grade), sodium hydroxide solution (10 *M*) and certified buffer solutions of pH 4 and 7 were also from Fisher. Compound **1** and 2,3-dihydro-6-propyl-5-benzofuranol (**2**, internal standard, I.S., Fig. 1) were obtained from Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.). Control human plasma was supplied by Sera-Tec Biologicals (North Brunswick, NJ, U.S.A.).

A phosphate buffer solution (0.1 *M*, pH 8.2) was prepared by mixing 466 ml of 0.2 *M* dibasic sodium phosphate and 34 ml of 0.2 *M* monobasic sodium phosphate, diluting (1:1) the mixture with water and adjusting the pH to 8.2 with 5 *M* sodium hydroxide.

### *Apparatus*

A Waters Assoc. 703 LC system equipped with a 730 data module, a 720 system controller, a WISP 710B automatic injector (limited-volume plastic inserts) and a 6000A chromatographic pump (Waters-Millipore, Milford, MA, U.S.A.) were used for all analyses. As a detector, the Bioanalytical Systems (West Lafayette, IN, U.S.A.) LC-17A electrochemical flow cell with dual glassy carbon working

electrode, RE-4 Ag/AgCl reference electrode and LC-4B amperometric controller were utilized. In order to improve sensitivity, the current from both carbon electrodes was combined and monitored. At the exploratory stages of this work, a variable-wavelength UV absorbance detector (Spectroflow 773, Kratos, Ramsey, NJ, U.S.A.) was placed in line between the LC system and electrochemical detector to allow for simultaneous monitoring of the UV and electrochemical signals. The detector output signal was interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, U.S.A.). A Chemcosorb 5-ODS-H $C_{18}$ column (15 cm×0.46 cm, 5 μm spherical particle size, DyChrom, Sunnyvale, CA, U.S.A.) and a Perkin-Elmer HS-3 $C_{18}$ column (8.3 cm×0.46 cm, 3 μm particle size, Perkin-Elmer, Norwalk, CT, U.S.A.) were used in series. As a guard column, a 8 cm×0.2 cm stainless-steel Whatman column, handpacked with ODS $C_{18}$ chemically bonded to 37–53 μm glass beads (Whatman, Clifton, NJ, U.S.A.), was utilized. For optimum chromatographic performance, the guard column had to be replaced after each 80–100 injections of plasma extracts. The LC columns were thermostatted using an Elilex Labs. (Menlo Park, CA, U.S.A.) column heater.

The UV spectra of **1** and its photolysis product in methanol (P, see below) were taken using either LC photodiode array UV detector (Polychrom 9060, Varian) or diode array spectrophotometer (Hewlett-Packard, Model 8451).

*Chromatographic conditions*

The aqueous part of the mobile phase was 0.125 *M* monobasic sodium phosphate in water containing 100 mg/l EDTA and adjusted to pH 3.0 with phosphoric acid. The organic portion of the mobile phase consisted of acetonitrile and the ratio of aqueous buffer/organic was 55:45. The analytical columns were maintained at 25°C at a flow-rate of 0.8 ml/min. The mobile phase was filtered through a 0.2-μm Nylon 66 filter (Rainin Instruments, Woburn, MA, U.S.A.). The working, glassy carbon electrodes were held at an oxidation potential of +0.55 V versus the Ag/AgCl reference electrode. The controller output was used at a sensitivity setting of 5 nA full scale and a filter setting of 0.1 Hz.

*Standard solutions*

A stock standard solution of compound **1** (1 mg/ml) was prepared in methanol. This solution was further diluted in the same solvent to give a series of working standards of **1**. The concentrations were 100, 10, 1 and 0.1 μg/ml.

The internal standard (**2**) was also prepared as a stock solution (10 μg/ml) in methanol. A working internal standard of 0.5 μg/ml was used for all plasma analysis.

Stock solutions were stored at −5°C and kept for a period of up to two weeks.

Hydrodynamic voltammograms were constructed using a mixture of working standard of **1** (1 μg/ml, 60 μl) and a working standard of **2** (0.5 μg/ml, 120 μl) with 2820 μl mobile phase. A 120-μl volume of this mixture, containing 2.5 ng of both **1** and **2**, was injected from the same LC vial at different oxidation potentials of the ED cell and the relative current was recorded.

*Plasma sample preparation*

The standard curve for **1** and its internal standard was constructed by spiking blank human plasma (1 ml) with known concentrations of **1** over the range 0.5–20 ng/ml, plus 5 ng/ml internal standard. This was done by the addition of standards (10 $\mu$l of working internal standard solution and 5, 10, 20 or 50 $\mu$l and 10 or 20 $\mu$l of 0.1 and 1 $\mu$g/ml working standard solutions, respectively) and 45, 40, 30 or 0 and 40 or 30 $\mu$l of methanol (total methanol content equal to 60 $\mu$l). The mixture of plasma and standards, placed in a 13-ml polypropylene centrifuge tube, was vortex-mixed (10 s), followed by the addition of phosphate buffer (50 $\mu$l, 0.1 *M*, pH 8.2), vortex-mixing (10 s) and extraction with 5 ml methylene chloride. The samples were shaken for 5 min at 240 strokes/min, centrifuged (3 min at 5000 *g*), the upper aqueous layer removed by vacuum suction, and the remaining organic layer evaporated to dryness under a stream of nitrogen at 45 °C. The residue was reconstituted in 250 $\mu$l of mobile phase by vortex-mixing (30 s), sonication (30 s), repeated vortex-mixing (30 s) and centrifugation (5 min at 5000 *g*). The solution was then transferred to the plastic autosampler vials using pipettes with plastic tips, and a 125-$\mu$l aliquot of each sample was injected directly on the LC columns for analysis.

Subjects' plasma samples were prepared for analysis in the same manner as plasma standards, substituting subjects' plasma (1 ml) for blank plasma and methanol for the working standard solutions.

*Precision, accuracy, linearity and recovery*

The precision of the method was determined by replicate analyses ($n=6$) of human plasma containing **1** at concentrations of 0.5, 1, 2, 5, 10 and 20 ng/ml. The accuracy of the assay was checked by preparing quality control samples at the start of the clinical study. As quality controls, plasma samples with known concentrations (2 and 15 ng/ml) of **1** were prepared and frozen at −20 °C. With each day's analysis, these quality control standards were assayed with the unknown samples over a period of two months, and the calculated concentrations were compared. Recovery from plasma in comparison with water was calculated by comparison of the peak-height ratios of spiked standards and internal standard from plasma with the respective ratios after extraction from water.

The linearity of each standard line was confirmed by plotting the drug concentration versus the ratio of drug to internal standard peak heights. The specificity of the assay was checked by running blank plasma and various patients' pre-dose plasma. No endogenous interference was encountered.

*Quantification*

A standard curve was prepared and assayed daily with the unknown samples. The concentration of **1** was determined from the standard calibration curve run on the same day. Calibration curves were constructed by plotting peak-height ratios of **1** to the internal standard versus concentration of **1** in plasma and fitting the data with linear regression.

## RESULTS AND DISCUSSION

The method described has been successfully applied to quantify **1** in human and dog plasma and in skin blister fluid of human subjects after topical administration of the drug. Since the anticipated concentrations of **1** were in the range 1–10 ng per ml of the biological fluid, the LC method with UV detection was inadequate and lacked the required sensitivity. Therefore, our effort concentrated on other, more sensitive detection methods which would allow direct measurements of **1** without its derivatization.

By analogy to *p*-methoxyphenol and other 1,0-alkyl-4-hydroxybenzene and 1,0-alkyl-4-hydroxy naphthalene derivatives [5], as well as to $\alpha$-tocopherol [6–8], dihydrobenzofuranols have the potential to undergo oxidation to quinoidal compounds. Based on this assumption, the electrochemical properties of **1** and **2** were examined and it was established that both compounds are electrochemically active, allowing their determination using LC with ED.

The hydrodynamic voltammograms are shown in Fig. 2. Both **1** and **2** display a very similar oxidation profile with an oxidation potential at about +0.5 to +0.6 V in the mobile phase as a solvent.

The potential of +0.55 V was chosen experimentally taking into consideration both the required sensitivity and specificity of the assay. At this potential, 30–40 pg of **1** can be detected (amount of the injected standard, signal-to-noise ratio=3/1). This low oxidation potential was also very favorable to significantly reduce the baseline noise and prevent oxidation of the undesired endogenous and extractable impurities from plasma.

The tentative mechanism of electrochemical oxidation of dihydrobenzofuranols is represented in Fig. 3 and is based on the analogous mechanism advanced for 4-hydroxypropranolol [5], $\alpha$-tocopherol [6–8] and other preliminary electrochemical studies of compound **2** [9].

Compound **1** is a single isomer (*E*), but can exist in two geometrical isomeric forms (*E* and *Z*). The absorption spectrum of **1** in methanol is characterized by three absorption maxima at $\lambda$ 208 nm ($\epsilon_{max} = \sim 40\,000\ M^{-1}\,cm^{-1}$), 252 (14 800) and 306 (6400). The absorption in the long wavelength region extends to 330 nm, creating the possibility for photochemical interconversion or other photochemical transformations of the isomers when samples are prepared and handled in Pyrex glass tubes. The latter tubes are partially transparent to the radiation with wavelength above 300 nm. Therefore, the procedure for sample preparation, the design of the LC separation conditions and the choice of mobile phase had to take into account not only the compatibility of the system with ED but also the feasibility of separation of **1** and **2** from the potential products of the photochemical (see below) or in vivo transformation of the drug.

When standard solution (1 $\mu$g/ml) of **1** in methanol was placed in an LC autosampler Pyrex vial and exposed to room laboratory light, a new distinct and electrochemically active product (P) with a shorter retention time than **1** has been detected. After 24 h exposure, the area ratio of **1** to P was 75:25. Several photochemical reactions are possible for compounds with structure similar to **1**, among them $E \rightleftarrows Z$ photoisomerization, photodimerization and internal photo-

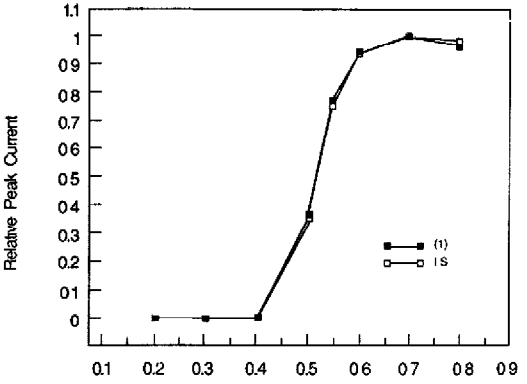
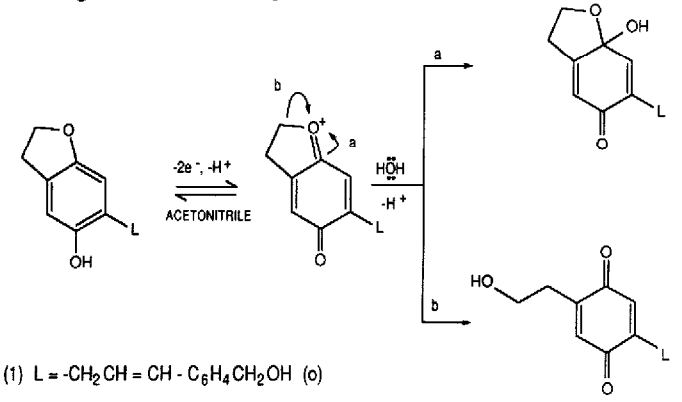



Fig. 2. Hydrodynamic voltammograms of **1** and **2** (I.S.). Peak current responses, relative to peak current at +0.7 V, were plotted as a function of applied potential. Each point represents a response of 2.5 ng of either **1** or **2** injected.




(1) L = $-CH_2CH=CH-C_6H_4CH_2OH$ (o)

(2) L = $-(CH_2)_2CH_3$

Fig. 3. Tentative mechanism of electrochemical oxidation of some dihydrobenzofuranols.

condensation. Product P was found to be electrochemically active but only weakly absorbing at 254 nm. The UV spectrum of product P, taken with photodiode array UV detector, revealed that the spectrum of P is markedly different from **1**, and the intense maximum at 252 nm was absent in the product. Therefore, the photolysis product P can be easily overlooked if one follows the stability of **1** in methanol by LC with the UV detector set at 254 nm. Work is in progress to generate the product photochemically in sufficient quantities for its full spectral and structural characterization.

The selection of **2** as an internal standard was based not only on its chromatographic elution profile and electrochemical activity, but also on its photochemical stability and freedom from isomerism.

*Extraction and chromatography*

Several liquid–liquid and liquid–solid extraction methods using different combination of solvents (*tert.*-butyl ether; hexane–ethyl acetate–isopropanol, 90:7:3;

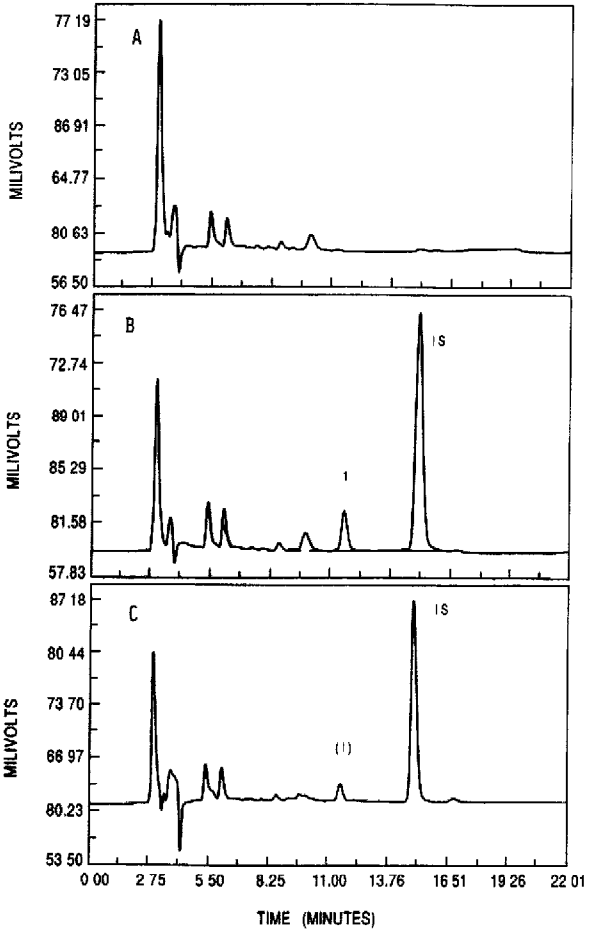



Fig. 4. Representative chromatograms of (A) plasma blank; (B) plasma blank spiked with compound **1** (1 ng/ml) and compound **2** (I.S., 5 ng/ml); (C) post-dose sample of a human subject spiked with I.S. (5 ng/ml). The estimated concentration of **1** is equal to 0.38 ng/ml.

hexane–ethyl acetate, 95:5; *n*-pentane–*tert.*-butyl ether, 3:2; methylene chloride), different solid-phase extraction cartridges ($C_2$, $C_8$, $C_{18}$, Si, CN, $NH_2$) and extraction from plasma at various pH values (4.8, 7.2, 8.2) were examined. Poor recoveries and/or presence in the extracts of interfering endogenous impurities prevented the use of most of these methods in the assay. However, very clean chromatograms were obtained when liquid–liquid extraction was performed with methylene chloride and after adjusting the pH of plasma to 8.2. Fig. 4 illustrates the chromatograms of **1** and **2** after extraction from human plasma. The absolute recovery from plasma, calculated by comparison of the peak areas of the extracted standards with the peak areas of the standards injected directly on-column, following liquid–liquid extraction, evaporation and transfer into autosampler vials and using the extraction method described in detail in Experimental, was found to be 70–80% at 1.5 ng/ml of plasma. The mean recoveries of **1** from plasma in

comparison with the recovery from water was found to be 98 ± 10% in the concentration range 1–20 ng/ml.

Different LC columns and various mobile phases were also examined to separate **1** and **2**, not only from endogenous plasma interferences but also from the potential product P. The combination of the mobile phase and two different columns in series (see Experimental) was finally selected as meeting all of the requirements listed above and allowing separation of the sample components in a reasonably short analysis time (22 min) required for multi-sample assay of specimen from clinical trials. Baseline separation of all the components was achieved, and the retention times were 10.1, 11.5 and 15.1 min for P, **1** and **2**, respectively.

The stability of **1** and **2** in the plasma extracts after reconstitution in the mobile phase was also studied. Twenty plasma extracts containing 5 ng of both **1** and **2** were prepared, placed directly on the LC autosampler carousel at room temperature and analyzed in four sets of five over a period of 48 h. The concentrations of **1** and **2** were found to be the same, within experimental error, for all of the analyzed samples, indicating that the applied procedure and the stability of **1** and **2** are adequate for overnight, multi-sample analyses of clinical specimens.

Special precautions were also taken during sample preparation and extraction to avoid an unnecessary exposure of samples to room laboratory light. Standard solutions of **1** in methanol were prepared in amber volumetric flasks. All Pyrex glassware was replaced by polypropylene labware (extraction tubes, micropipettes, autosampler vials, etc.). Under these conditions, photochemical degradation of **1** was not observed. The use of plastic labware was also beneficial to improve the recovery of **1** at the levels below 10 ng/ml of plasma. It was found that in mobile phase, **1** strongly adsorbed to the silica surface of microvials made of Pyrex glass, leading to very poor reproducibility and recoveries. When these microvials were replaced by plastic autosampler inserts, very consistent results and high recoveries were noted.

*Linearity, precision and accuracy*

The assay for **1** was linear in the range 0.5–20 ng/ml of plasma (mean correlation coefficient of fourteen standard curves run over a period of three months was 0.9912). Since the anticipated concentrations of **1** from human studies were in this range, no attempt was made to extend this range above 20 ng/ml. The within-day precision of the assay was less than 10% for all concentrations within the standard curve range (Table I). Inter-day precision, as measured by the concentration of quality control standards over a period of two months, was 8.0 and 11.4% at 2 and 15 ng/ml, respectively (Table II). The accuracy of the assay expressed as (mean observed concentration/expected concentration) multiplied by 100 was 97–104% (Table II).

*Sensitivity*

The lowest quantifiable concentration of **1** in plasma was 0.5 ng/ml (~2 pmol/ml). The limit of detection, with signal-to-noise ratio of 3:1, was at least 0.2 ng/ml as illustrated in Fig. 4C for a plasma sample from a human subject after topical administration of **1** on the human skin. The estimated concentration of

TABLE I

INTRA-DAY PRECISION DATA FOR THE ANALYSIS OF **1** IN PLASMA

| Concentration (ng/ml) | Coefficient of variation ($n=6$) (%) |
|---|---|
| 0.5 | 4.3 |
| 1.0 | 9.5 |
| 2.0 | 6.3 |
| 5.0 | 6.1 |
| 10.0 | 5.5 |
| 20.0 | 4.8 |

TABLE II

INTER-DAY VARIABILITY FOR THE ASSAY OF QUALITY CONTROL PLASMA SAMPLES SPIKED WITH **1**

| Nominal concentration (ng/ml) | Determined concentration (mean ± S.D.)* (ng/ml) | Coefficient of variation (%) |
|---|---|---|
| 2 | 2.08 ± 0.17 | 8.0 |
| 15 | 14.50 ± 1.66 | 11.4 |

*Mean of ten analyses performed over the period of two months.

TABLE III

PLASMA CONCENTRATIONS OF SOME HUMAN SUBJECTS ENROLLED IN THE STUDY ON THE EFFECT OF TOPICAL **1** ON CHRONIC PLAQUE PSORIASIS

| Subject | Concentration (ng/ml) | | |
|---|---|---|---|
| | Day 0 | Day 22* | Day 29* |
| A | 0 | 1.94 | 0.56 |
| B | 0 | 2.10 | 0.57 |
| C | 0 | 0.92 | 1.25 |

*Plasma samples were collected on a given day, 12 h after last topical application of **1**.

**1** in this plasma sample was 0.38 ng/ml, outside the reliable range of quantification of the drug (0.5–20 ng/ml). However, Fig. 4C nicely illustrates the sensitivity of the method below 0.5 ng/ml.

*Analysis of samples from human clinical studies*

The assay was utilized for analysis of plasma and skin blister fluid samples from human subjects after topical administration of **1**. For blister fluid assay, a similar method as for plasma was employed, but 1 ml of plasma was replaced by a mixture of 10 μl of blister fluid (or its appropriately water-diluted aliquot) and 0.99 ml of control, blank plasma. Similarly to blank plasma, no interfering peaks were present in the chromatograms of the blister fluid mixtures with plasma even

when 100 $\mu$l of blister fluid blank was mixed with 900 $\mu$l of control plasma and extracted according to the described procedure.

The representative concentrations of **1** in human plasma of several subjects enrolled in a study on the effect of topical **1** on chronic plaque psoriasis are given in Table III.

The assay was also utilized for determination of **1** in dog plasma after oral administration of the drug.

## CONCLUSIONS

The results demonstrate the utility of ED for the quantitative determination of dihydrobenzofuranols in biologicl fluids. The specificity of the detector when coupled with a carefully designed liquid–liquid extraction method provided a remarkably clean chromatogram from biological media. The sensitivity of the LC–ED assay is almost two orders of magnitude better than that of the analogous assay with UV detection.

The described methodology was successfully applied for the analysis of plasma samples from human clinical studies after topical administration of the drug. The same assay procedure, with the modification described in the Results section, was also found to be fully adequate for assaying **1** in skin blister fluid of human subjects and determination of **1** in dog plasma.

## REFERENCES

1 R.J. Bonney, P. Davie, H. Dougherty, R.W. Egan, P.H. Gale, M. Change, M. Hammond, N. Jensen, J. McDonald, K. Thompson, R. Zambias, E.E. Opas, R. Meurer, S. Pacholak and J.L. Humes, Biochem. Pharmacol., 36 (1987) 3885.
2 R.J. Bonney, American Oil Chemists Society 78th Annual Meeting, New Orleans, LA, May 17–21, 1987.
3 M.N. Change, M.L. Hammond, R.J. Booney, H.W. Dougherty and J.L. Humes, 193rd American Chemical Society National Meeting, Denver, CO, April 5–10, 1987, Abstract No. MEDI-83.
4 B.M. Sweeney and H.B. Hucker, unpublished results.
5 Cyclic Voltammetry Notes, Bioanalytical Systems, West Lafayette, IN, 1980.
6 M.F. Marcus and M.D. Hawley, Biochim. Biophys. Acta, 201 (1970) 1.
7 M.F. Marcus and M.D. Hawley, J. Org. Chem., 35 (1970) 2185.
8 M.F. Marcus and M.D. Hawley, Biochim. Biophys. Acta, 222 (1970) 163.
9 J. Walsh, G. Miwa and D. Ellison, unpublished results.