Journal of Chromatography, 255 (1983) 491–496
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,153

ANALYSIS OF SK&F 82526 IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

SK&F 82526, a benzazepine with a catechol moiety, is a potent, specific renal vasodilator. The method described here for its determination in plasma uses an ethyl acetate extraction and an ether wash, followed by high-performance liquid chromatographic analysis with electrochemical detection. The detection limit is 160 p*M*. The method was proven accurate, precise and linear over three orders of magnitude (at plasma concentrations ranging from 50 pg/ml to 50 ng/ml). It has been successfully used for plasma determinations of SK&F 82526 in human volunteers following an oral dose of 25–100 mg.

INTRODUCTION

Endogenous catecholamines undergo rapid and extensive metabolism, keeping their plasma concentrations very low. Analysis of catecholamines in small volumes of plasma was made practical by the development of radioenzymatic assays using radiolabeled S-adenosyl methionine and catechol-O-methyl transferase[1]. However, these assays involve numerous manipulations of labile substances, the source of a variety of problems, as suggested by the number of method modifications appearing in the literature[2]. More recently plasma catecholamines have successfully been analyzed by high-performance liquid chromatography with electrochemical detection (HPLC–ElCD)[3,4]. These procedures are relatively simple and specific and achieve sensitivities in plasma comparable to the radioenzymatic assays.

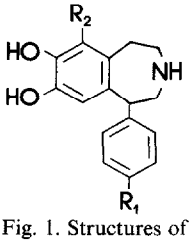

Fig. 1. Structures of SK&F 82526 (A) and SK&F 38393, internal standard, (B). R_1 = HO (A) or H (B); R_2 = Cl (A) or H (B).

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SK&F 82526 [6-chloro-2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-1*H*-3-benzazepine-7,8-diol] (Fig. 1A) is a specific renal vasodilator in rat, dog, monkey[5] and man[6]. Like other catecholamines, it rapidly decreases to very low levels in plasma after effective doses. The analytical procedure described for this drug in plasma employs organic solvent extraction for the initial sample preparation rather than adsorption onto alumina used in many other catecholamine assays. This, coupled with HPLC–ElCD, achieved subnanomolar sensitivity in the routine determination of SK&F 82526 in human plasma.

EXPERIMENTAL

Chromatographic apparatus

The HPLC–ElCD analyses were performed on a Perkin-Elmer 601 liquid chromatograph (Norwalk, CT, U.S.A.) and a thin-layer amperometric detector consisting of an LC-4A electronic controller, an LC-17 oxidative flow-cell (with a 2-μm PTFE gasket) and a TL-5 glassy carbon working electrode (Bioanalytical Systems, West Layette, IN, U.S.A.). The detector was set at the applied potential of +0.65 V *versus* calomel. The samples were injected by a WISP Model 710B autosampler (Waters Assoc., Milford, MA, U.S.A.).

Reagents

The citrate–acetate buffer[7] for preparation of mobile phase and reconstitution of samples was made (per liter) by dissolving the following in water: 11.0 g of sodium acetate trihydrate (Baker Analyzed reagent, J. T. Baker, Phillipsburg, NJ, U.S.A.); 10.5 g of citric acid monohydrate (Baker Analyzed reagent); 4.9 g of sodium hydroxide (electrolytic pellets, Fisher Scientific, Pittsburgh, PA, U.S.A.); 0.335 g of disodium ethylenedinitrilotetraacetic acid (A.C.S. reagent, Matheson, Coleman and Bell, Norwood, OH, U.S.A.); 48 ml of glacial acetic acid (Mallinckrodt, Paris, KY, U.S.A.). This buffer is approximately of 0.1 *M* ionic strength and pH 4.

The solvents used in sample preparation were ethyl acetate ("distilled in glass", Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and diethyl ether (anhydrous analytical reagent, Mallinckrodt). The acetonitrile used in the mobile phase was also from Burdick and Jackson Labs. ("distilled in glass", UV grade).

Materials

All samples were stored and extracted in polypropylene tubes (100 × 17 mm, No. 2059, Falcon Division, Becton, Dickinson and Co., Oxnard, CA, U.S.A.). Unopette shields (Becton–Dickinson and Co., Rutherford, NJ, U.S.A.) were used as limited-volume inserts in autosampler vials. Heparinized Vacutainers (Becton–Dickinson) were used for blood sample collections.

Chromatography conditions

Prior to the initial start-up of the HPLC–ElCD the liquid chromatograph pump and autosampler were passivated by pumping through them 500 ml of 6 *N* nitric acid and then 500 ml of 0.05 *M* phosphate buffer (pH 8) containing 2% $Na_2 \cdot$ EDTA. The mobile phase contained the citrate–acetate buffer and acetonitrile (92:8, v/v), degassed by filtering under reduced pressure through a 0.45-μm mem-

brane filter, type HA (Millipore, Bedford, MA, U.S.A.). Separations were performed on a 5-μm Partisil PXS ODS-3 5/25 column (Whatman, Clifton, NJ, U.S.A.). Each new column was conditioned by eluting with 100–200 ml of the mobile phase. The chromatography was performed at ambient temperature at a flow-rate of 1 ml/min. Injection volumes ranged from 15 to 200 μl, depending on drug concentration.

Standard solutions

Dissolve 13.14 mg (10 mg base) of SK&F 82526 methanesulfonate in a 10-ml volumetric flask with water and store in plastic or silanized glassware (stable for at least two weeks at −20°C). Dilute 1:100 with water to make a 10-μg/ml solution; dilute this solution 1:100 with heparinized control human plasma to provide a 100-ng/ml plasma stock solution. Make appropriate dilutions of this stock with control plasma for standards 0.05–50 ng/ml (stable for at least two weeks at −20°C).

For the internal standard stock solution dissolve 11.4 mg (10 mg base) of SK&F 38393 HCl (1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride) (Fig. 1B) in a 10-ml volumetric flask with water and store in plastic or silanized glassware (stable for at least two weeks at −20°C). Make two 1:100 dilutions with water to provide an internal standard working stock solution of 100 ng/ml.

Extraction procedure

To each 2-ml plasma aliquot add 100 μl of internal standard working solution. Extract samples with 4 ml of ethyl acetate by shaking on a reciprocating shaker at 60 cycles/min for 10 min. Centrifuge. Transfer the ethyl acetate layer to a clean tube and evaporate to dryness under nitrogen in a warm (40°C) water bath. Reconstitute the residue in 300 μl of citrate–acetate buffer. Add 2 ml of ether and shake the samples for 5 min at 120 rpm. Centrifuge. Carefully aspirate and discard most of the ether layer; evaporate the remaining ether under nitrogen. Transfer the remaining buffer layer to plastic inserts in WISP vials for HPLC–ElCD analysis. The extracted samples are stable for at least 6 h at room temperature.

Quantitation

Divide SK&F 82526 peak height by internal standard peak height in each sample to obtain its peak height ratio. Plot peak height ratios *versus* concentration of the standards. Calculate the concentration of the unknowns from the slope (or linear response factor) of the standard curve.

RESULTS AND DISCUSSION

The hydrodynamic voltammogram for SK&F 82526, obtained with the HPLC–ElCD system described in Experimental, appears in Fig. 2. Maximal detector response occurred with potentials of +0.60 V and above. Thus the applied potential of +0.65 V assured optimal oxidation of SK&F 82526 while background current and oxidation of endogenous materials were maintained at relatively low levels. The glassy carbon electrode was selected for its durability and compatability with organic solvents. Noise characteristics and sensitivity of this electrode were found suitable for subnanomolar detection.

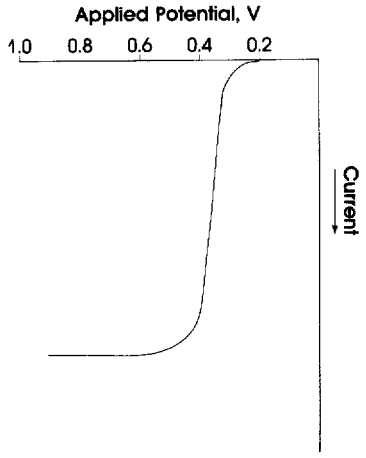

Fig. 2. Hydrodynamic voltammogram for SK&F 82526 in the described HPLC–ElCD system.

The wide range of SK&F 82526 concentrations in plasma necessitates a wide range of injection volumes. Injection of 10 to 200 μl SK&F 82526 in sample solvent (the mobile phase buffer) did not affect the response to amounts of drug injected, thereby column efficiency. The 5-μm ODS-3 column had a marked increase in efficiency over 10-μm C_{18} columns and a long life under these chromatographic conditions.

Unlike many other catecholamines adsorption of SK&F 82526 onto alumina from human plasma gave low inconsistent recoveries. However, extraction with 4 ml of ethyl acetate for 10 min without changing the pH of the plasma optimized drug recovery and minimized background. After reconstituting samples in buffer, the ether wash further reduced interference while not measurably extracting any drug from the acidic medium. Absolute drug recovery was determined by comparing extracted plasma standards prepared directly in the citrate–acetate buffer at concentrations corresponding to 100% recovery. The results showed *ca.* 90% recovery of drug by this extraction.

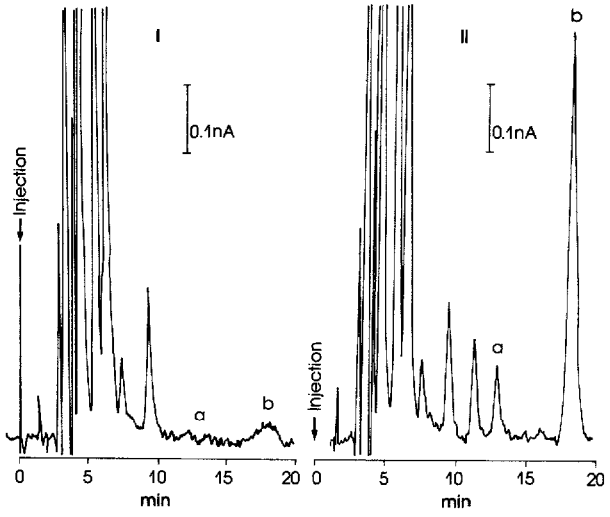

Fig. 3. Typical chromatograms of extracts of control human plasma (I) without and (II) with 1 ng/ml SK&F 82526 (a) and internal standard (b).

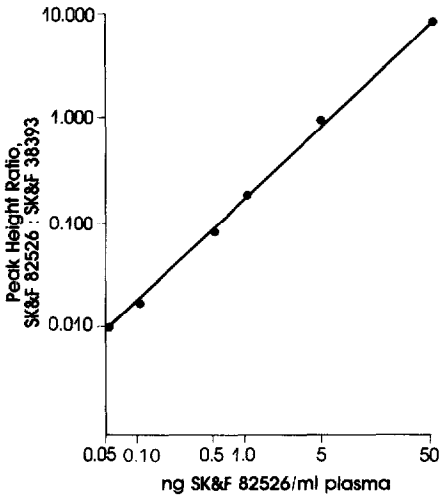

Fig. 4. Response curve of SK&F 82526 in human plasma from Day 1 of method validation.

Fig. 3 shows typical chromatograms of extracts from contral human plasma with and without SK&F 82526 and internal standard added. The retention time was 12.7 min for SK&F 82526 and 18.0 min for the internal standard. There were no interfering peaks. SK&F 82526 analysis of plasma samples from human subjects given therapeutic doses of hydrochlorothiazide or furosemide and 100 mg of SK&F 82526-J concomitantly showed no interfering peaks when compared to the diuretics administered alone.

To validate the accuracy and precision of this method SK&F 82526 plasma standards at 10 ng/ml and 500 pg/ml were analyzed in triplicate on three different days. Analysis of each day's standard curve by linear regression ($r = 0.9999$) showed the analysis to be linear over three orders of magnitude, 50 pg/ml to 50 ng/ml (Fig. 4).

TABLE I

VALIDATION OF SK&F 82526 ANALYSIS BY HPLC–EICD IN HUMAN PLASMA FOR PRECISION AND ACCURACY

Day	*Measured concentrations in standard replicates*	
	10 ng/ml	*500 pg/ml*
1	9.0	460
1	9.3	530
1	8.8	450
2	10.8	480
2	11.3	540
2	11.0	400
3	11.4	500
3	10.7	500
3	11.3	–
Mean ± S.D.	10.4 ± 1.1 (104% ± 10.2%)	485 ± 47 (96% ± 9.7%)

The percent of measured *versus* actual drug concentrations averaged for the 3 days (mean ± S.D.) was 96 ± 10% at 500 pg/ml and 104 ± 10% at 10 ng/ml (Table I).

The lower limit of sensitivity for this method, 160 p*M* (50 pg/ml), is comparable to detection limits reported for epinephrine (100 p*M*) by either radioenzymatic or HPLC–ElCD assays[2].

This assay was applied in a study of human volunteers receiving a single oral dose of 25, 50 and 100 mg (base) of SK&F 82526 methanesulfonate. Blood samples were collected in heparinized vacutainers, and plasmas were frozen at −20°C. Plasma level curves for three of these subjects are shown in Fig. 5. The drug was readily detectable through 2 h even after the lowest dose.

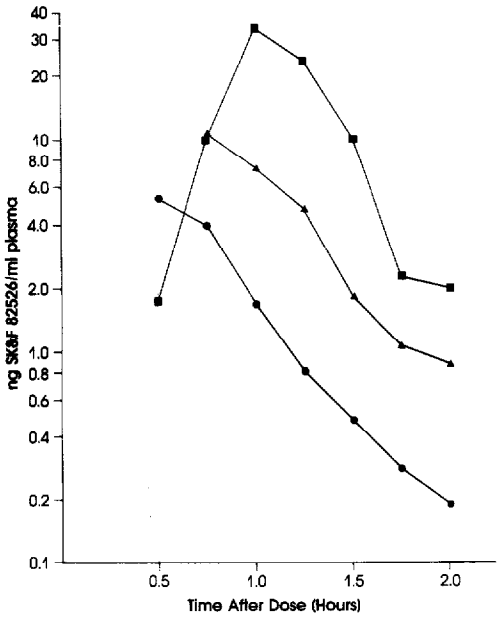

Fig. 5. Plasma levels of SK&F 82526 in human volunteers following a single oral dose of 25 mg (●), 50 mg (▲) and 100 mg (■).

The method described for SK&F 82526 determination in human plasma is accurate, precise and linear over a wide concentration range. Sample preparation is simple enough for routine analysis. The lower limit of sensitivity is adequate for following the absorption and distribution of SK&F 82526 in plasma of human subjects given a 25-mg oral dose.

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