CHROMBIO. 5207

Determination of a new anti-allergenic agent, 1-[4-[3-[4-[bis-(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxyphenyl]ethanone, and its active acidic metabolite in plasma by high-performance liquid chromatography

SUSAN V. GREENE[a],*, HERMAN A. KREBS[b], ROBERT E. LEMIEUX and LAWRENCE K. CHENG

Drug Metabolism Department, A.H. Robins Company, 1211 Sherwood Avenue, P.O. Box 26609, Richmond, VA 23220-6609 (U.S.A.)

SUMMARY

High-performance liquid chromatographic (HPLC) methods were developed for the analysis of two compounds in a series of new antiallergenic agents, 1-[4-[3-[4-[bis(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxyphenyl]ethanone and its active acidic metabolite in plasma. The methods utilize ultraviolet or fluorescence detection, liquid–liquid extraction or solid-phase extraction and reversed-phase HPLC. The drugs were quantitated in samples from bioavailability studies performed in dogs. Calibrations were in the ng/ml concentration range for both compounds in plasma.

INTRODUCTION

The compound 1-[4-[3-[4-[bis(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxyphenyl]ethanone has been isolated as the mandelic acid salt [1] (compound I, Fig. 1). It is a potent, long-acting, anti-allergenic agent that possesses activity at a number of sites involved in the immediate hypersensitivity response in animal models [2]. Preclinical studies have in-

---

[a]Present address: 9602 George's Bluff Road, Richmond, VA 23229, U.S.A.
[b]Present address: Pharmakinetics Laboratories, Inc., Baltimore, MD 21201, U.S.A.
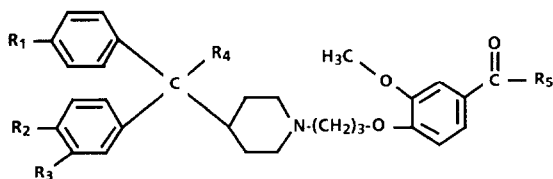
Compound	R_1	R_2	R_3	R_4	R_5
I	-F	-F	-H	-OH	-CH$_3$
II	-F	-F	-H	-OH	-OH
III	-H	-H	-CF$_3$	-OH	-CH$_3$
IV	-F	-F	-H	-H	-OH
V	-F	-F	-H	-OH	CH$_2$OH

Compound Name

I   1-[4-[3-[4-[bis(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxyphenyl]ethanone mandelic acid salt

II   4-[3-[4-[bis(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxybenzoic acid

III   1-[4-[3-[4-[Hydroxyphenyl[3-(trifluoromethyl)phenyl]methyl]-1-piperidinyl]propoxy]-3-methoxyphenyl]ethanone hemihydrate

IV   4-[3-[4-[bis(4-fluorophenyl)methyl]-1-piperidinyl]propoxy]-3-methoxy-benzoic acid hemihydrate

V   1-[4-[3-[4-[bis(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxyphenyl]-2-hydroxyethanone

Fig. 1. Structures of compounds I and II, their respective internal standards (III and IV) and a metabolite of compound I (V).

dicated that this compound inhibits the release of mediators from mast cells, and it also inhibits the effect of the mediators on the end organs [3,4]. Biotransformation studies in dogs using radioactively labeled drug showed that there was significant first-pass metabolism producing many metabolites, several of which are carboxylic acids. Among all the acidic metabolites of compound I isolated and identified, 4-[3-[4-[bis(4-fluorophenyl)hydroxymethyl]-1-piperidinyl]propoxy]-3-methoxybenzoic acid (compound II) constituted the greatest percentage formed. In addition, this acidic compound has pharmacological activity similar to the parent drug.

In order to support further development of this series of anti-allergenic agents, selective high-performance liquid chromatographic (HPLC) methods were developed for both compounds. Due to the widely differing chemical properties

of these compounds (compound I is slightly basic, compound II is acidic), the development of two separate extraction schemes (liquid–liquid extraction or solid-phase extraction) was necessitated.

The assay methods for compounds I and II and their applications to the analysis of plasma samples obtained from bioavailability studies in dogs are described herein.

EXPERIMENTAL

Reagents and equipment

Compounds I–IV in Fig. 1 were synthesized by the A.H. Robins Company (Richmond, VA, U.S.A.). Hemihydrate compounds III and IV (Fig. 1) are the internal standards for compounds I and II, respectively. Reagents and sample preparation equipment necessary for performing the assays for compounds I and II are as follows: ammonium hydroxide, sodium phosphate (monobasic, monohydrate), methanol (HPLC grade), acetonitrile (HPLC grade), hydrochloric acid, phosphoric acid (85%) and C_{18} solid-phase extraction (SPE) columns, 3 ml capacity, were all purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.); hexane (UV grade) and methylene chloride were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); isopropanol (reagent grade) came from Fisher (Pittsburgh, PA, U.S.A.); methyl *tert.*-butyl ether, Omni Solv, was from E.M. Science (Cherry Hill, NJ, U.S.A.); the Vac Elut sample processing station (SPS)24, fitted with Teflon rather than stainless-steel delivery tips, was from Analytichem International (Harbor City, CA, U.S.A.).

Sample collection

Blood was drawn using a glass syringe and EDTA was used as the anticoagulant. The blood was centrifuged and the plasma transferred to storage tubes which were kept frozen until analyzed.

High-performance liquid chromatography

The assay methods for both compounds described herein employed the following equipment: M6000 pump and 710B WISP autosampler (Waters Assoc., Milford, MA, U.S.A.). Table I lists the instruments and operating conditions specific for each compound. All solutions were prepared using deionized, distilled water.

The output signal generated by the detector for each method was processed by a computer-automated laboratory system (Computer Inquiry Systems, Walwick, NJ, U.S.A.) and Hewlett-Packard Model 1000 computer (Palo Alto, CA, U.S.A.). The chromatographic tracings were recorded on a Kipp and Zonen 10-mV chart recorder, Model BD40 (Delft, The Netherlands).

TABLE I

HPLC CONDITIONS FOR METHODOLOGY OF EACH COMPOUND

	Compound I	Compound II
Column	Waters μBondapak C$_{18}$ 30 cm $\times$ 4.6 mm I.D. Temperature, ambient	Waters, Novapak C$_{18}$, 4 μm, end-capped, 15 cm $\times$ 3.9 mm I.D. Temperature, ambient
Detector	Schoeffel SF770 (Westwood, NJ, U.S.A.) UV monitor	Schoeffel 970 fluorometer
Detector settings	220 nm 0.02 a.u.f.s.	Excitation wavelength, 220 nm No filters in the emission except the PMT window Range, 0.5 Time constant, 2 s Sensitivity, 4.10 Reset module, 1.0 min
Mobile phase	Acetonitrile–0.05 M sodium phosphate (pH 3) (45:55)	Acetonitrile–0.05 M sodium phosphate (pH 3) (50:50)
Flow-rate	1.5 ml/min	0.7 ml/min
Run time	15 min	8 min

Standard solutions

Stock solutions, corresponding to 100 μg/ml of each of the two compounds and their respective internal standard compounds, were prepared separately in acetonitrile–water (1:9). The compound was first dissolved in acetonitrile and then water was added to volume. Using the stock standard 100 μg/ml solutions of compounds I and II, standard curves were prepared in drug-free human or dog plasma. The range of the standard curves was 2–500 ng/ml for compound I and 10–200 ng/ml for compound II. The 100 μg/ml internal standard solutions of compounds III and IV were diluted with deionized, distilled water to obtain final concentrations of 300 ng/ml and 1.0 μg/ml, respectively.

Sample preparation

Compound I. A 1.0-ml portion of either unknown or standard plasma samples was transferred into 15-ml centrifuge tubes. Diluted internal standard solution (0.5 ml), 0.5 ml of 5% ammonium hydroxide and 5.0 ml of methylene chloride–hexane (1:4) were then added to each tube. The tubes were shaken for 10 min in a reciprocating shaker and centrifuged at 550 g for 5 min. The organic phase was transferred to a clean tube and evaporated to dryness under nitrogen at 40°C. The residue was reconstituted in 0.2 ml of 10% acetonitrile in 0.05 M sodium phosphate (pH 3.0) and mixed thoroughly for 10 s. To this phase was added 0.5 ml of 5% isopropyl alcohol in hexane. After mixing for 10 s the tube was centrifuged for 5 min at 550 g. The upper layer was removed and

discarded; 150 μl of the acetonitrile–phosphate buffer layer was injected into the HPLC system.

Compound II. The C_{18} solid-phase extraction columns employed in the analysis of plasma samples containing compound II were first conditioned by washing under low pressure (30–38 cmHg) using one column volume of each solution, applied in the following order: (1) methanol; (2) water; (3) 0.05 M hydrochloric acid.

A 0.5-ml portion of either unknown or standard plasma samples was transferred to a 10-ml disposable centrifuge tube. A 0.5-ml aliquot of 0.5 M hydrochloric acid and 100 μl of internal standard were added to the samples and the samples were mixed for 15 s. With the Vac Elut vacuum off, the acidified plasma was poured on the top of the solid-phase extraction column. Then the vacuum pump was turned on and the acidified plasma was pulled to waste. The column was washed with 3 ml of each of the following solutions in the following order: (1) methanol–water (10:90); (2) methyl *tert.*-butyl ether. Each wash was discarded.

The compounds of interest were eluted with two successive 0.5-ml washings of acetonitrile–0.5% hydrochloric acid in methanol (1:4). The total eluate was pooled and evaporated to dryness at 50°C under nitrogen. The samples were reconstituted in 200 μl of the appropriate mobile phase, and 40 μl were injected into the HPLC system.

Precision and accuracy

To test the precision of these two methods, six individual standard curves for each of compounds I and II were run on consecutive days. The slope, intercept and correlation coefficients of the daily standard curves were calculated.

The accuracy of the method was determined by assaying thirty randomized samples spiked with various concentrations of either compound I or II. The concentration of the given compound in the samples was unknown to the analyst at the time of analysis.

RESULTS AND DISCUSSION

Representative chromatograms generated from each of the two methods are shown in Figs. 2 and 3. There was a 3-min delay in data acquisition for both compounds I and II. This data acquisition delay suppressed acquisition of the solvent peak and prevented the software from normalizing the chromatogram using the solvent peak as the largest peak.

Included for each method in Figs. 2 and 3 are drug-free and spiked standard curve points as well as dog plasma samples obtained from bioavailability studies. The chromatograms for the drug-free dog and human plasma show no interference peaks at the retention times of the compounds of interest and their respective internal standards. There was another metabolite of compound I
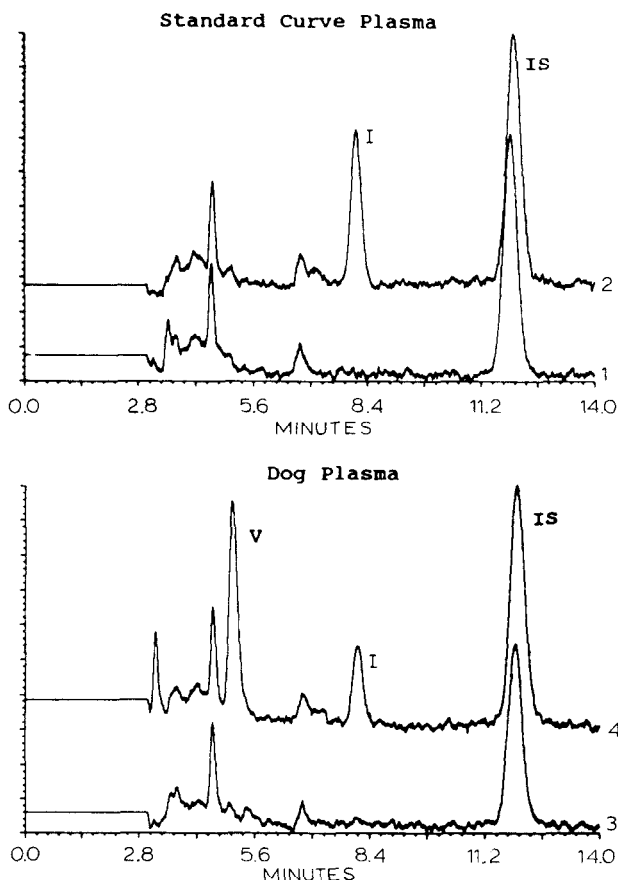
Fig. 2. Representative chromatograms for compound I. Standard curves for compound I were prepared in drug-free dog plasma. All samples shown contain 150 ng/ml internal standard (IS). (1) Drug-free dog plasma; (2) drug-free dog plasma spiked with 50 ng/ml compound I; (3) 0-h dog plasma before an oral dose of 10 mg/kg compound I; (4) 5-h dog plasma after an oral dose of 10 mg/kg. The sample contains 25.6 ng/ml compound I.

co-extracted which elutes at 5.5 min (Fig. 2, chromatogram 4). Using mass spectrometry, nuclear magnetic resonance spectrometry and chemical reduction, the compound was identified as compound V (Fig. 1).

The precision data generated by the set of six standard curves for each of compounds I and II are shown in Table II. The curves are all linear within the concentration range prepared for each drug with correlation coefficients of at least 0.999. The intercept values of the regression lines were always negligible. The coefficient of variation of the average peak-height ratio in each set of the six standard curves was generally less than 12% for compound II and less than 10% for compound I.
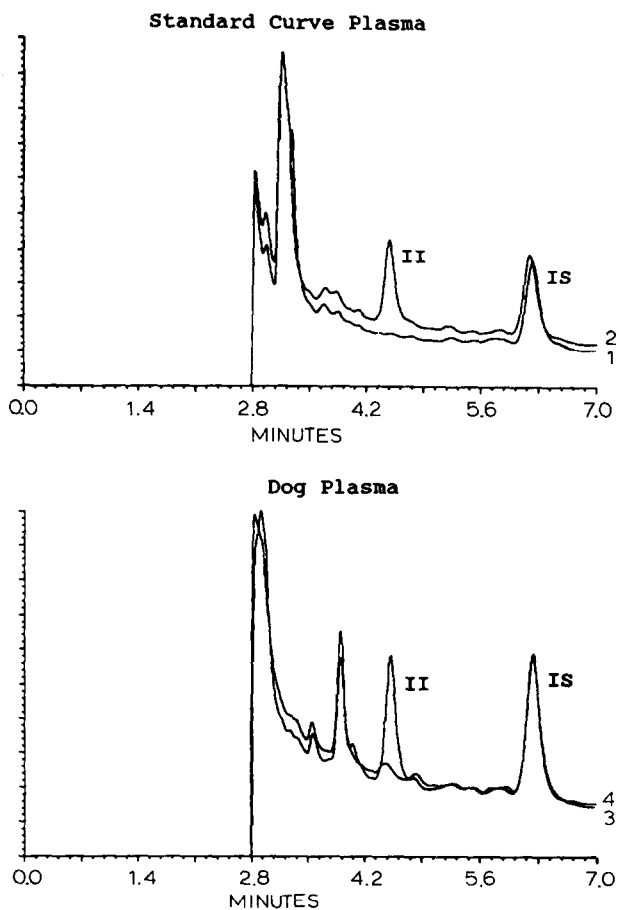
Fig. 3. Representative chromatograms for compound II. Standard curves for compound II (metabolite of compound I) were prepared in drug-free human plasma. The dog plasma samples shown (chromatograms 3 and 4) are the same plasma samples as those shown in Fig. 4 (chromatograms 3 and 4). All samples shown contain 200 ng/ml internal standard (IS). (1) Drug-free human plasma; (2) drug-free human plasma spiked with 100 ng/ml compound II; (3) 0-h dog plasma before an oral dose of 10 mg/kg compound I; (4) 5-h dog plasma sample after an oral dose of 10 mg/kg compound I. The sample contains 102.9 ng/ml compound II.

TABLE II

PRECISION DATA FOR THE TWO METHODOLOGIES

Compound	Linear range (ng/ml)	n	Slope (mean $\pm$ S.D.)	Correlation coefficient (mean $\pm$ S.D.)
I	0–500	6	0.0088 $\pm$ 0.0003	0.9995 $\pm$ 0.0003
II	0–200	6	0.006 $\pm$ 0.001	0.999 $\pm$ 0.001

TABLE III

DETERMINATION OF UNKNOWN AMOUNTS OF COMPOUND ADDED TO HUMAN PLASMA

Concentration added (ng/ml)	n	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Found (%)
Compound I				
0	3	BQL[a]	—	—
2.2	3	2.1 ± 0.3	12.8	95.5
20.0	6	19.8 ± 0.9	4.4	99.0
60.0	6	59.6 ± 2.0	3.4	99.3
120.0	3	127.9 ± 3.5	2.7	106.6
240.0	6	255.5 ± 5.8	2.3	106.5
480.0	3	485.5 ± 8.4	1.7	101.1
Compound II				
0	5	BQL[b]	—	—
11.0	5	10.6 ± 1.0	8.9	96.6
35.0	5	35.0 ± 3.3	9.4	100.1
85.0	5	84.8 ± 4.3	5.0	99.7
125.0	5	122.6 ± 4.4	3.6	98.1
195.0	5	188.9 ± 4.2	2.2	96.9

[a]BQL = Below quantifiable limit; i.e., concentration is less than 2 ng/ml.
[b]BQL = Below quantifiable limit; i.e., concentration is less than 10 ng/ml.
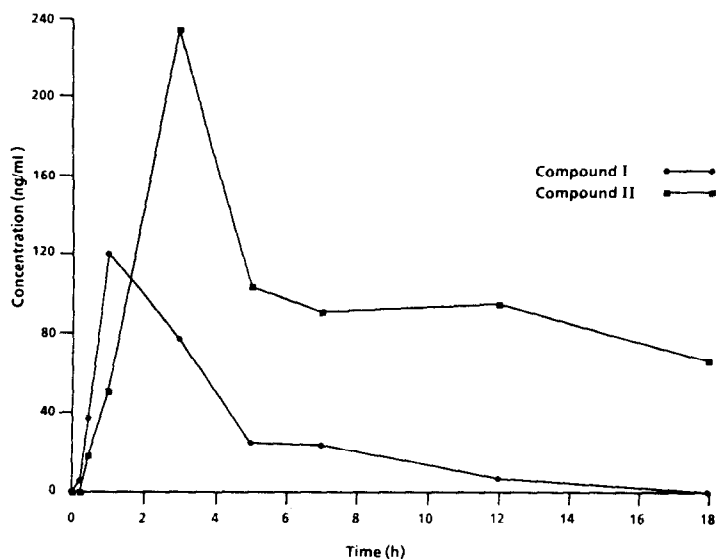

Fig. 4. Dog plasma concentration–time curves of unchanged drug and metabolite (compound II) after a single oral dose of 10 mg/kg compound I.

Table III shows the accuracy of each method determined using its set of thirty spiked plasma samples. The percentage of the compound found was generally within 7% of the theoretical concentration added. The recovery of both compounds I and II was at least 80%.

Automation of the solid-phase extraction sample preparation for compound II was demonstrated on the MilliLab Workstation, Waters Assoc. The preliminary precision data generated under the automated conditions were as good or better as those generated by manually preparing the samples.

These two methods were employed in the analysis of plasma samples obtained from bioavailability studies in beagle dogs. Fig. 4 shows typical drug concentration–time profiles for compound I and its metabolite, compound II. The range of the standard curves for compounds I and II was found to be adequate for up to 10 mg/kg doses in dogs. Human plasma collected after 12.5- to 50.0-mg doses of compound I showed that only a few samples had detectable drug levels because of the extensive first-pass metabolism. However, appreciable concentrations of compound II in those same samples was detected ranging from 15 to 250 ng/ml in plasma.

CONCLUSIONS

Sensitive and selective HPLC methods were developed for the determination of anti-allergy compounds I and II in dog and human plasma. The concentration–response curves were linear from 0 to 500 ng/ml and 0 to 200 ng/ml, respectively. The methods have a high degree of precision and accuracy. They were successfully utilized in the analysis of plasma samples obtained from bioavailability studies. In the method where solid-phase extraction can be used, automation has been demonstrated which will increase the sample throughput without any loss in precision and accuracy.

ACKNOWLEDGEMENTS

REFERENCES

1   D.A. Walsh and J.M. Yanni, 193rd National ACS Meeting, Denver, April 1987, Abstract No. MEDI-85.
2   D.A. Walsh and J.M. Yanni, Drugs Future, 13 (1988) 605–609.
3   J.M. Yanni and M.H. Foxwell, Int. Arch. Allergy Appl. Immunol., 85 (1988) 428–433.
4   J.M. Yanni, R.E. Halliwell and C.H. Tracy, J. Vet. Pharmacol. Ther., 11 (1988) 221–225.