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Note

Determination of lodoxamide in plasma using ion-pairing and reversed-phase high-performance liquid chromatography

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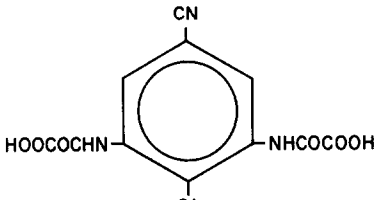
Lodoxamide (I), N,N′-(2-chloro-5-cyano-*m*-phenylene)dioxamic acid is currently under investigation as an orally and inhalation active antiasthmatic agent [1, 2]. An inhibitor of IgE-mediated allergic reactions, I is chemically different from disodium cromoglycate, a currently marketed antiasthmatic drug. Lodoxamide is 2500 times more active in rats than disodium cromoglycate when assayed by the passive cutaneous analphylaxis test [2].



Lodoxamide
I

In continuing efforts to apply high-performance liquid chromatography (HPLC) to pharmaceutical analysis [3], a HPLC procedure for the separation and quantitation of I in human plasma is reported. The separation is effected utilizing ion-pair formation with tris(hydroxymethyl)aminomethane on an octadecylsilane column. The chromatographic separation takes approximately 20 min. The overall analysis time is about 90 min, which includes extraction of the drug from plasma followed by HPLC separation and quantitation.

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EXPERIMENTAL

Materials

A powdered sample of lodoxamide tromethamine (Upjohn Company, Kalamazoo, MI, U.S.A.) was used in the preparation of standard solutions. Tris(hydroxymethyl)aminomethane (Sigma, St. Louis, MO, U.S.A.) was used as the ion-pair reagent in the mobile phase. All other chemicals and solvents were the highest grade of commercially available materials.

HPLC conditions

The HPLC analyses were performed on a Waters Assoc. (Milford, MA, U.S.A.) Model ALC202 equipped with an M-6000 pump, a U6K injector, and a 254-nm fixed-wavelength UV detector. The column was a 10-μm μBondapak C_{18} column (300 mm × 4 mm I.D.) (Waters Assoc.). The mobile phase used was absolute methanol—0.05 *M* aqueous tris(hydroxymethyl)aminomethane (10:90) and was degassed before use. The pH of the mobile phase was adjusted to pH 6 with concentrated phosphoric acid and the flow-rate was set at 1.0 ml/min (82.74 bar).

Standard solutions for calibration curve

A stock solution of lodoxamide tromethamine (74 μg/ml) was prepared by dissolving a weighed amount of the powder in distilled water. The resulting solution of the salt was equivalent to 40 μg/ml of lodoxamide free acid. In addition, an internal standard stock solution (115 μg/ml) of *p*-nitrocinnamic acid in absolute methanol—distilled water (50:50) was prepared.

Plasma calibration procedure

Into individual 15-ml centrifuge tubes were placed 1.0-ml quantities of drug-free human plasma. Accurately measured volumes of 10, 5, and 2.5 μl of lodoxamine tromethamine stock solution were added such that the final concentration of lodoxamide free acid was 400, 200, and 100 ng/ml, respectively. Internal standard stock solution (10 μl) and concentrated hydrochloric acid (100 μl) were added to each tube, followed by mixing on a vortex mixer (30 sec) and heating on a steam bath (1 min). After cooling to room temperature, ethyl acetate (5 ml) was added, and the mixture vortexed (45 sec) followed by centrifugation at 860 *g* (10 min). The organic phase (4 ml) was removed with the aid of a volumetric pipette, and transferred to a clean 15-ml centrifuge tube. Upon evaporation of the ethyl acetate to dryness on a water bath with the aid of a nitrogen stream, the residue was dissolved with shaking in 100 μl of distilled water—absolute methanol (50:50) containing 0.05 *M* tris(hydroxymethyl)aminomethane (pH 6). If the resulting solution was cloudy, the sample was centrifuged at 1239 *g* (5 min), the clear supernatant transferred to a clean tube with the aid of a disposable pipette, and a 25-μl aliquot was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

The HPLC analysis of I in plasma necessitated the examination of two

separate problems: (a) the development of HPLC operating parameters that would separate I from plasma components without interference, and (b) the detection and quantitation of I at expected plasma levels (100—400 ng/ml) following a 2—4 mg total oral dose.

Initially, the chromatographic process was investigated to determine if a non-extraction sample preparation method similar to the one recently reported by this laboratory for *p*-aminosalicylic acid in plasma would be feasible for the analysis of I [4]. In the method, an initial protein denaturation step is performed using equal volumes of plasma and an organic solvent such as acetonitrile or methanol. After centrifugation, an aliquot of the supernatant fluid is injected into the liquid chromatograph. Retention times and other chromatographic parameters were determined for I and/or I-tetrabutylammonium ion-pair on phenyl and octadecylsilane columns. Analysis of I using a phenyl column with mobile phases of methanol—water in the pH 2—4 range or acetonitrile—water containing 0.01 *M* tetrabutylammonium ion at pH 7 was unsuccessful either due to lack of suitable resolution of I from endogenous plasma components or unacceptable band broadening due to the presence of several ionic species of I. Mobile phases containing methanol—water and/or acetonitrile—water in differing ratios and pH values (4—7 range) with and without tetrabutylammonium ion were used to evaluate the retention of I and/or I—tetrabutylammonium ion-pair on the octadecylsilane column. Acetonitrile—water (30:70) containing 0.01 *M* tetrabutylammonium ion at pH 6 was the most suitable mobile phase on the octadecyl column since it provided adequate chromatographic resolution of I from endogenous plasma components and was compatible with the use of acetonitrile in the sample pretreatment step as plasma protein precipitant. However, I still exhibited excessive tailing.

Concurrent with these preliminary HPLC studies, it was determined that a non-extraction sample preparation method would not be sensitive enough to allow detection of I at the expected plasma levels due to lack of sufficient molar absorptivity of the drug at the concentration available in the injection volume. It was decided to utilize a sample preparation procedure involving solvent extraction followed by a sample concentration step in order to improve the UV detectability of I.

Further HPLC investigations revealed that ion-pairing of I with tris(hydroxymethyl)aminomethane on an octadecylsilane column was a more successful approach to the chromatographic separation of I. The effect of mobile phase composition and pH on the retention time of the ion-pair is shown in Table I. The ion-pairing mobile phase increased the capacity factor (k') for I so that the drug would be adequately resolved from any endogenous plasma components extracted in the assay procedure. Retention time of the ion-pair increased with increasing water content and decreasing pH (4—8 range).

The ion-pair was most propitiously separated using absolute methanol—distilled water (10:90) at pH 6. Fig. 1 shows a typical chromatogram of the separation of lodoxamide and *p*-nitrocinnamic acid (internal standard) in a spiked human plasma sample using tris(hydroxymethyl)aminomethane at pH 6. Under the chromatographic conditions chosen, endogenous plasma constituents do not interfere with the assay.

A flow-rate of 1.0 ml/min (82.74 bar) allowed the separation to be obtained

TABLE I

EFFECT OF MOBILE PHASE COMPOSITION AND pH ON RETENTION TIME OF LODOXAMIDE USING OCTADECYLSILANE COLUMN

Mobile phase*		pH				
		8	7	6	5	4
A		278**	478	684	798	825
		(40)***	(53)	(60)	(71)	(80)
	k'	0.54	1.8	2.8	3.4	3.6
B		192	366	400	441	488
		(30)	(39)	(36)	(36)	(50)
	k'	0.67	1.0	1.2	1.45	1.7
C		188	228	252	261	296
		(21)	(27)	(27)	(29)	(33)
	k'	0.04	0.30	0.40	0.50	0.60

*Solvent composition: A, absolute methanol—distilled water (10:90) containing 0.05 *M* tris(hydroxymethyl)aminomethane; B, absolute methanol—distilled water (20:80) containing 0.05 *M* tris(hydroxymethyl)aminomethane; C, absolute methanol—distilled water (30:70) containing 0.05 *M* tris(hydroxymethyl)aminomethane.

**Retention time expressed as seconds measured as elapsed time between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using a 254-nm UV detector.

***Base peak width expressed as seconds.

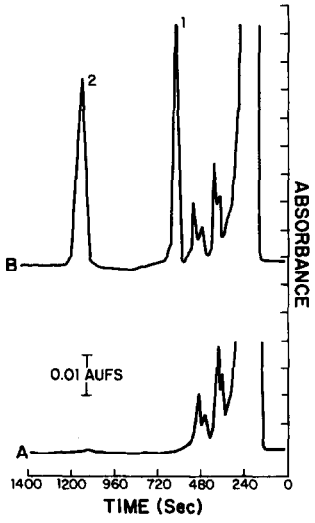

Fig. 1. Typical chromatogram of lodoxamide (1) and *p*-nitrocinnamic acid (2) (internal standard) in a human plasma sample spiked with 200 ng/ml of drug (B). For comparison, a chromatogram of blank plasma (A) is shown. Conditions: column, octadecylsilane (300 mm × 4 mm I.D.); eluent, absolute methanol—distilled water (10:90) containing 0.05 *M* tris(hydroxymethyl)aminomethane adjusted to pH 6 with concentrated phosphoric acid; flow-rate, 1 ml/min; UV detector set at 254 nm.

in approximately 20 min. The void volume of the column was 2.6 ml. A fixed-wavelength detector (254 nm) was suitable for the assay since I showed a broad UV absorption spectrum with a maximum at 249.5 nm. Thus, maximum

detector sensitivity was available for the low concentration levels present in the extracted plasma samples.

A calibration curve for lodoxamide in the anticipated therapeutic concentration range (100—400 ng/ml of plasma) based on a total oral dose of 2—4 mg was performed. The digital determinations of peak heights of drug and internal standard on the chromatograms were determined with an electronic integrator (Spectra-Physics Autolab Minigrator, San Jose, CA, U.S.A.). The ratio of lodoxamide peak height to the height of the internal standard (D/IS) was calculated for each chromatogram. Regression analysis of these data at the various concentrations of drug gave slope, 0.0702; intercept, 0.0037; and correlation coefficient, 0.9955 (n = 11). The minimum detectable quantity of lodoxamide that can be measured using this procedure is 20 ng/ml based upon extraction of drug from plasma. The percent recovery of lodoxamide using the procedure described herein was 98.90 ± 1.60% (mean ± S.D., n = 4).

TABLE II

ANALYSIS OF LODOXAMIDE IN SPIKED PLASMA SAMPLES

Initial concn. (ng/ml)	Conc. found* (ng/ml)	Relative standard deviation (%)	Relative error (%)
150	150.07 ± 4.79	3.19	0.05
300	306.23 ± 3.12	1.02	2.08

*Mean ± S.D. based on triplicate determinations of each sample.

Human plasma samples containing spiked quantities of lodoxamide in the therapeutic concentration range were chromatographed concurrently with the calibration solutions and the ratios of drug peak heights to internal standard peak heights were calculated. The slope and intercept data from regression analysis for lodoxamide calibration solutions were used to calculate the concentration in the spiked samples: D/IS = (slope × concentration) + intercept. The data in Table II demonstrate the quantitative results obtained from these spiked plasma samples. The utility of HPLC in the assay of lodoxamide plasma levels using ion-pairing with tris(hydroxymethyl)aminomethane is clearly demonstrated with relative error < 5%.

ACKNOWLEDGEMENT

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