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Capillary gas chromatographic–electron-capture assay for the aldose reductase inhibitor imirestat in lens and plasma[a]

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ABSTRACT

A sensitive and selective gas chromatographic–electron-capture assay was developed for the determination of the aldose reductase inhibitor imirestat in lens and plasma. The method involves solid-phase extraction of drug and internal standard from the plasma specimen or lens sample homogenate using "Baker"-10 SPE™ extraction columns followed by derivatization with pentafluorobenzyl bromide and further purification. Derivatives of drug and internal standard were separated on a fused-silica capillary column and analyzed using a ^{63}Ni electron-capture detector. The limit of detection was 2.5 ng per lens or ml of plasma. The method was used to evaluate the pharmacokinetics of imirestat in human subjects and to quantitate imirestat in animal lens tissue following topical ocular administration.

INTRODUCTION

The enzyme aldose reductase has been implicated in the pathological conditions associated with diabetes mellitus [1–3]. Aldose reductase inhibitors have been found to prevent some of the pathological changes associated with experimental diabetes [4–6]. Imirestat [2,7-difluorospiro-(9H-fluorene-9,4′-imidazolidine)-2′,5′dione; AL∅1576; HOE 843] is a potent aldose reductase inhibitor that has been shown to prevent the development of cataracts, altered nerve function, and sorbitol accumulation in peripheral nerves and lens of diabetic animals [7–9]. Pharmacokinetic studies of imirestat in animals and humans require sensitive and reproducible analytical methods for the quantitation of the drug in biological fluids and tissues. This report presents the selective gas chromatographic (GC)–electron-capture assay method used to evaluate the pharmacokinetics of imirestat in human subjects [10]. This assay has greater selectivity and sensitivity than the bioassay for imirestat reported previously [11] and has been applied to other animal models and biologic matrices.

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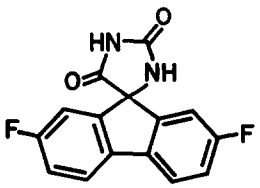
EXPERIMENTAL

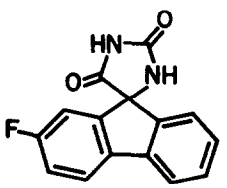
Reagents and standards

Imirestat (I, Fig. 1) for use as a standard and spiro(2-fluoro-9H-fluorene-9,4′-imidazolidine)-2′,5′dione; AL⌀1567; II, Fig. 1) the internal standard, were synthesized at Alcon Labs. HPLC grade methanol, methylene chloride, heptane, hexane, and ethyl acetate were purchased from J. T. Baker, (Phillipsburg, NJ, U.S.A.), as were analytical reagent grade acetic acid, ammonium hydroxide, sodium hydroxide, sulfosalicylic acid, and anhydrous dibasic sodium phosphate. Tetrabutylammonium hydrogensulfate was purchased from Sigma (St. Louis, MO, U.S.A.). Pentafluorobenzyl bromide was purchased from Pierce (Rockford, IL, U.S.A.). Water, high purity, was obtained from Burdick and Jackson Labs. (Richmond, CA, U.S.A.).

The stock solution of imirestat (10 μg per 25 μl of methanol) was serially diluted with methanol to prepare working standard solutions at fourteen different concentrations ranging from 1.0 to 200 ng per 25 μl. Plasma standards were prepared by spiking the appropriate volume of drug-free plasma (0.5–1.0 ml) with a 25-μl aliquot of each working standard solution at appropriate concentrations and a fixed amount of internal standard (25 ng per 25 μl of methanol). For lens standards, prior to the placement of the lens in the homogenization tube, a 25-μl aliquot of each working standard solution at appropriate concentrations and a fixed amount of internal standard (25 ng) was added to the tube and evaporated to dryness with nitrogen. On each day of analysis, either a full plasma or lens standard curve or appropriate quality control standards which bracketed the expected sample concentrations were analyzed concurrently with the samples.



I



II

Fig. 1. Structures of imirestat (I) and internal standard (II).

Extraction of plasma samples

Depending upon the volume of sample chosen for extraction (normally 0.5 ml), the appropriate octadecyl (C_{18}) extraction columns (1 or 3 ml capacity, J. T. Baker) were placed into the luer fittings of the "Baker"-10 SPE™ extraction system vacuum manifold cover. Columns were conditioned by aspiration of two column volumes of HPLC grade methanol, followed immediately by two column volumes of high-purity water through the column under a vacuum of 380–500 mmHg. Vacuum was turned off before the solution was totally aspirated through the column to prevent drying of the sorbent.

A 200-μl volume of 0.1% acetic acid was added to each column followed by the appropriate plasma aliquot. If a standard sample was analyzed, 25 μl of working standard solution plus 25 μl of internal standard were added. If an unknown sample was analyzed, only the internal standard was added. On top of each standard and sample aliquot, an additional 200 μl of 0.1% acetic acid were added forcefully to mix. The order of addition of the standard and internal standard solution to the plasma, either prior to or after its placement on the C_{18} column, has no effect on the extraction procedure. The vacuum was turned on and the components of interest were retained by the extraction column. Each column was washed with 4–5 ml of 0.1% acetic acid to remove unwanted polar plasma constituents. Imirestat and the internal standard were eluted from the extraction column by the addition of two 250-μl aliquots of HPLC grade methanol. The total eluates were evaporated in a stream of nitrogen. The sample was then ready for the derivatization procedure.

Homogenization of lens samples

A 25-μl volume of the internal standard was added to a 1.5-ml Eppendorf microcentrifuge tube and evaporated to dryness prior to placing the lens in the tube for homogenization. If a standard sample was analyzed, 25 μl of the appropriate working standard solution were added. The preweighed lens was initially solubilized by homogenization in 0.25 ml of 0.1% sodium hydroxide. The addition of 0.1% sodium hydroxide resolubilized the internal standard and standard which was adsorbed on the walls of the microcentrifuge tube. Precipitation of the protein was completed by the addition of two 0.25-ml aliquots of 1% sulfosalicylic acid (homogenizing after each addition). The micro-pestle was rinsed three times with 0.25 ml of 0.5 *M* phosphate buffer, pH 4, and the rinses collected in the same tube. The tube was vortexed and the precipitated protein was separated by centrifugation at approximately 14 000 *g* for 10 min. The supernatant, which had a final pH of 5.5, was removed for extraction on the Baker columns as described in the plasma extraction procedure.

Derivatization procedure

Following evaporation of the methanol eluates, the sample was reconstituted in 0.25 ml of 0.8 *M* ammonium hydroxide. To this, 0.25 ml of 0.4 *M* tetrabutyl-

ammonium hydrogensulfate, 0.5 ml of methylene chloride, and 5 μl of pentafluorobenzyl bromide were added. The sample was vortex-mixed and allowed to sit for 15 min at room temperature. At the completion of the reaction time, the sample was vortex-mixed and centrifuged for 5 min at approximately 14 000 *g*. The organic phase was transferred and evaporated to dryness with nitrogen. The derivatized sample was reconstituted in 500 μl of heptane and vortex-mixed.

Reduction of electron capture-sensitive background

The entire heptane solution was added to an individual 1 ml "Baker" SPE cyano (CN) extraction column preconditioned by aspiration of two column volumes of HPLC grade methanol, followed immediately by one column volume of heptane under a vacuum of 380–500 mmHg. The tube was rinsed with another 500 μl of heptane with the rinsings also added to the column. The vacuum was turned on and the compounds of interest were retained by the extraction column. Each column was washed twice with hexane to remove derivatization impurities. The derivatives of imirestat and the internal standard were eluted from the extraction column by the addition of three 200-μl aliquots of ethyl acetate–hexane (50:50). The eluates were evaporated to dryness with nitrogen. The samples were reconstituted in 200 μl of heptane for GC analysis.

Gas chromatography

Injections of 1–2 μl were analyzed on a Hewlett Packard Model 5890A gas chromatograph (Hewlett Packard, Palo Alto, CA, U.S.A.) equipped with a ^{63}Ni electron-capture detector, HP Model 7673A automatic injector and split–splitless capillary inlet system operating in the split mode. The column was a 30 m × 0.25 mm I.D. fused-silica capillary column with a chemically bonded SPB-1 stationary phase at a film thickness of 0.25 μm (Supelco, Bellefonte, PA, U.S.A.). The make-up gas was ultra-high-purity nitrogen (99.999%) at a flow-rate of about 70 ml/min. Ultra-high-purity hydrogen (99.999%) was the carrier gas at a linear velocity of 51 cm/s (1.5 ml/min) giving a split ratio of 1:15 at an oven temperature of 240-245°C. The injection port was heated to 275°C and the detector temperature was 300°C. The chromatographic peaks were integrated by a Hewlett Packard 3392A networking integrator with an attenuation setting of 2^3 and an HP5890A range setting of 0. Typical retention times for imirestat and internal standard were 12.5 and 14.0 min, respectively.

Quantitation

The peak-height ratios of the drug to internal standard were obtained by the aid of a data automation system. To construct the standard curve, the peak-height ratios of plasma or lens standards were analyzed by linear regression using a SAS program with respect to their amounts. The amount of imirestat in unknown plasma or lens samples was determined by inverse prediction from the constructed plasma or lens standard curve. The amount found was then normal-

ized for the plasma volume or for the lens weight. The SAS linear regression analysis generates the slope, intercept, the standard error of both, and the correlation coefficient. The intercept can be assumed to be zero if no interferences are detected in standard sample blanks analyzed during prestudy validation. The standard curve may be truncated to the appropriate range which will effectively

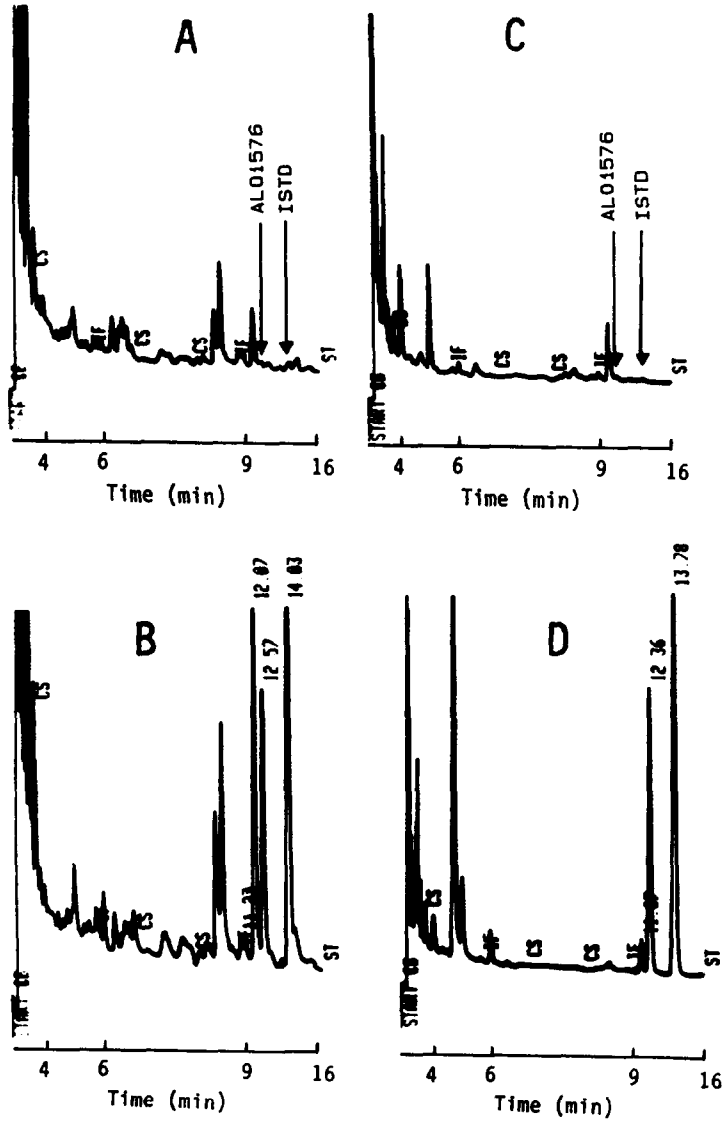


Fig. 2. Typical chromatograms of imirestat in biologic matrices at identical sensitivity settings. (A) Extracted human plasma blank; (B) extracted imirestat sample in human plasma from a dosed subject; (C) extracted rabbit lens blank; (D) extracted imirestat sample in rabbit lens from a dosed animal.

bracket the concentration of the samples to be analyzed. The accuracy of the calculations, especially at concentrations below 60 ng/ml of plasma or 30 ng per lens will be significantly improved by this procedure.

RESULTS AND DISCUSSION

Typical chromatograms of extracted human plasma and rabbit lens samples before and after dosing with imirestat are shown in Fig. 2. No interfering peaks were observed in either the drug-free rabbit lens or human plasma samples in the region of the drug or internal standard. The assay procedure involved solid-phase extraction of imirestat and the internal standard from plasma or lens homogenate and purification of the pentafluorobenzyl derivatives of both compounds by the same technique. The assay has been easily adapted for the analysis of imirestat in rat, dog and human lens, as well as, cynomolgus monkey, rat, dog, and rabbit plasma.

Table I shows the inter-assay statistics for imirestat standard curves prepared with human plasma. The inter-assay statistics for imirestat standard curves in rabbit lens are presented in Table II. The data in Table I show good inter-assay precision for imirestat in human plasma over a seven-month period with coefficients of variation (C.V.) ranging from 11.2% (n = 16) at 2.5 ng to 3.8% (n = 11) at 200 ng. At imirestat amounts below 30 ng in human plasma (60 ng/ml) a standard curve truncated to a 0–30 ng range will improve the C.V. to 4.5% at 2.5 ng and 1.4% at 30 ng. Good inter-assay precision for imirestat in rabbit lens is evident from the data in Table II with the C.V. ranging from 17.5% (n = 4) at 2.5

TABLE I

INTER-ASSAY PRECISION FOR IMIRESTAT IN HUMAN PLASMA

Number of runs	Amount added (ng)	Amount found (mean ± S.D.) (ng)	Coefficient of variation (%)	Mean percentage difference[a]
16	2.5	2.96 ± 0.33	11.24	+18.40
20	5	5.57 ± 0.51	9.10	+11.40
19	10	11.08 ± 0.83	7.46	+10.80
21	20	21.22 ± 1.02	4.82	+ 6.10
19	40	40.36 ± 1.60	3.96	+ 0.90
18	60	57.39 ± 2.20	3.83	− 4.35
20	80	80.63 ± 2.76	3.42	+ 0.79
18	100	99.08 ± 3.38	3.41	− 0.92
11	160	157.75 ± 4.99	3.16	− 1.41
11	200	200.33 ± 7.55	3.77	− 0.17

[a] Mean percentage difference = $\frac{\text{mean amount found} - \text{amount added}}{\text{amount added}} \times 100$

TABLE II

INTER-ASSAY PRECISION FOR IMIRESTAT IN RABBIT LENS

Number of runs	Amount added (ng per lens)	Amount found (mean ± S.D.) (ng per lens)	Coefficient of variation (%)	Mean percentage difference[a]
4	2.5	1.96 ± 0.34	17.48	−21.60
5	5	5.15 ± 0.35	6.71	+ 3.00
5	10	10.12 ± 0.60	5.90	+ 1.20
5	20	21.75 ± 0.72	3.31	+ 8.75
5	30	30.23 ± 1.47	4.87	+ 0.77
5	40	38.99 ± 1.21	3.09	− 2.59
4	50	52.16 ± 2.28	4.37	+ 4.32
5	60	58.53 ± 1.35	2.31	− 2.45

[a] Mean percentage difference = $\frac{\text{mean amount found} - \text{amount added}}{\text{amount added}} \times 100$

ng to 2.3% (n = 5) at 60 ng. The accuracy in human plasma, as evaluated by mean percentage difference, ranged from +18.4% at 2.5 ng to −0.17% at 200 ng, while in rabbit lens the mean percentage difference ranged from −21.6% at 2.5 ng to −2.5% at 60 ng. With a 0–30 ng standard curve range in human plasma the accuracy will improve to 14.0% at 2.5 ng and 1.2% at 30 ng.

Linear regression parameters covering each standard curve were determined for 21 separate sets of standards analyzed in human plasma. The relationship between peak-height ratio and amount appeared linear over a 1–200 ng range with a mean correlation coefficient of 0.999 ± 0.001 (C.V. = 0.067%). Slope values for imirestat in human plasma were highly reproducible with a mean slope of 0.049 ± 0.004 (C.V. = 7.5%). For five separate sets of standards analyzed in rabbit lens good linearity between peak-height ratio and amount over a 2.5–60 ng range was evident with a mean correlation coefficient of 0.995 ± 0.002 (C.V. = 0.24%). Highly reproducible slope values were determined for imirestat in rabbit lens with a mean slope of 0.040 ± 0.002 (C.V. = 4.3%) and mean intercept of 0.064 ± 0.010 (C.V. = 15.6%). The minimum quantifiable level was determined to be 2.5 ng in either rabbit lens or human plasma based on the C.V. values of 17.5 and 11.2%, respectively. Extraction efficiency was determined to be quantitative at all levels by comparison of the non-extracted *versus* extracted peak height of imirestat.

Several human plasma samples were refrozen and placed in storage at −20°C for five months. After thawing and reanalysis, the results for more than 50% of the reassayed samples did not differ by more than 10% from the original concentration. The representative data in Table III show a slight increase in concentration for approximately 47% of the samples rather than the loss which would be expected if there was degradation of imirestat. For samples with concentrations

TABLE III

STABILITY OF IMIRESTAT IN PLASMA FROZEN FOR FIVE MONTHS

Sample No.	Original assay concentration (μg/ml)	Reassay concentration (μg/ml)	Percentage difference[a]
1	0.408	0.370	− 9.3
2	0.418	0.435	+ 4.1
3	0.494	0.485	− 1.8
4	0.395	0.402	+ 1.8
5	0.455	0.498	+ 9.5
6	0.369	0.390	+ 5.7
7	0.657	0.605	− 7.9
8	0.533	0.475	−10.9

[a] Percentage difference $= \frac{\text{reassay} - \text{original assay}}{\text{original assay}} \times 100$

higher than 200 ng/ml, smaller plasma aliquots were diluted or taken to provide concentrations within the validated range.

The stability of the pentafluorobenzyl derivatives of imirestat and the internal standard was also evaluated following storage at room temperature for thirteen months. The data in Table IV supply evidence that the imirestat derivative is stable for over a year at room temperature. The standard curve slope shows only a slight increase of 5–7% rather than a decrease which would be expected if there was degradation of imirestat.

In conclusion, the above data indicate that the GC–electron-capture method described is precise, sensitive, and selective for the analysis of imirestat in human

TABLE IV

STABILITY OF PENTAFLUOROBENZYL DERIVATIVE OF IMIRESTAT

Original assay date	9-1-87	6-6-88	9-8-88
Stability reinjection	10-6-88	10-5-88	10-10-88
Number of months	13	4	1
Amount range (ng)	1–60	1–60	1–60
Original slope	0.0481	0.0462	0.0402
R^2	0.997	0.999	0.997
S.E. × 10^{-3}	0.920	0.299	0.824
Reinjection slope	0.0515	0.0492	0.0422
R^2	0.995	0.999	0.999
S.E. × 10^{-3}	1.200	0.276	0.368
Percentage difference	+7.07	+6.49	+4.98

plasma and rabbit lens. The method has been used successfully to evaluate the pharmacokinetics of imirestat in human subjects and to quantitate imirestat in animal lens following topical ocular administration.

Application

Imirestat concentrations have been determined with this assay in lens and plasma samples from various studies over a period of three years. In one such study a 0.025% imirestat ophthalmic suspension was administered to New Zealand rabbits as part of a topical multiple dosing protocol. A 30-μl volume of the formulation, corresponding to 7.5 μg, was instilled into the right eye of each rabbit twice daily at 8-h intervals with the contralateral eye serving as the control.

Three animals were humanely sacrificed with 50 mg/kg pentobarbital at each of the following times: 8 h after the morning dose on days 3, 7, 14, 21, and 28. The eyes were rinsed with saline, and the lenses were surgically removed and stored deep frozen (-20°C) until analyzed by the method described. The lens concentration–time profile is shown in Fig. 3. The mean maximum imirestat lens concentration reached after 28 days dosing was 227 ng/g of lens.

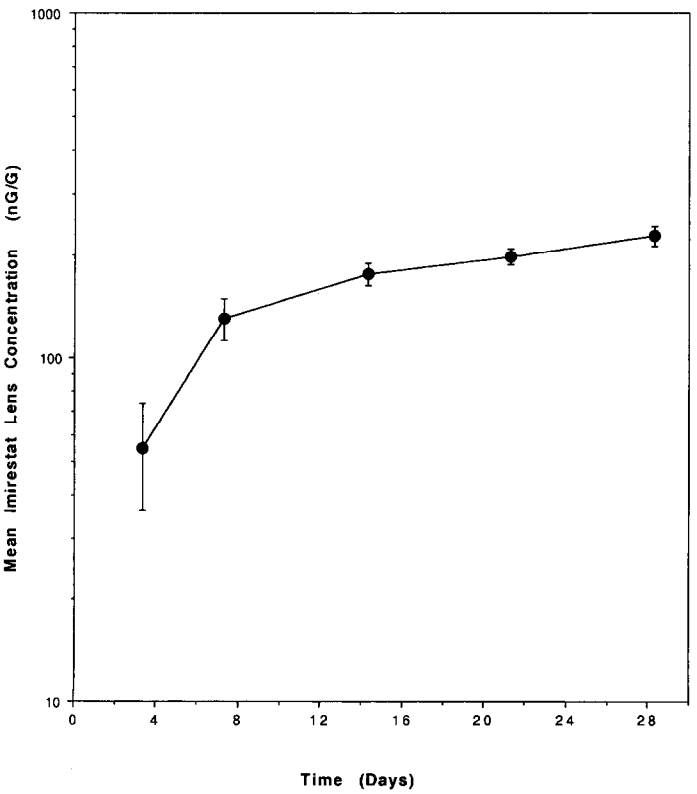


Fig. 3. Mean imirestat concentrations in the rabbit lens following daily administration of 0.025% imirestat ophthalmic suspension.

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