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Note

High-performance liquid chromatographic assay of 2-[2-methoxy-4-(methylsulfinyl)phenyl]-1H-imidazo-[4,5-*c*]pyridine hydrochloride, a potent cardiotonic agent, and two metabolites in plasma

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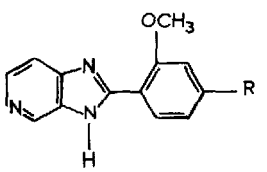
A number of 1H-imidazo-[4,5-*c*]pyridine derivatives have been studied for potential use as inotropic agents [1, 2]. Of these 2-[2-methoxy-4-(methylsulfinyl)phenyl]-1H-imidazo-[4,5-*c*]pyridine hydrochloride, (LY175326, I) (Fig. 1) is being actively investigated for its beneficial inotropic and vasodilator properties [3, 4]. Integral to the advancement of the drug has been the development of a suitable assay procedure capable of quantitating not only the parent drug but also the major plasma metabolites in the animal species used for pharmacological and toxicological studies. This paper deals with the development and use of a high-performance liquid chromatographic (HPLC) assay with fluorescence detection, applicable to both urinary and plasma fluids.



R	
$S(O)CH_3$	I (LY175326)
SO_2CH_3	II (LY163252)
SCH_3	III (LY137150)

Fig. 1. Chemical structures of I, II, and III.

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EXPERIMENTAL

Analytical-grade reagents and spectrophotometric-grade eluents were used. The internal standard, 1-(2-pyridyl)piperazine (n_D^{20} 1.5887), was purchased from Aldrich (Milwaukee, WI, U.S.A.) as was the ion-pairing agent 1-decanesulphonic acid, sodium salt. Both chemicals were used without further purification. Potential metabolites, 2-[2-methoxy-4-(methylsulfonyl)phenyl]-1H-imidazo-[4,5-*c*]pyridine hydrochloride (LY163252, II) and 2-[2-methoxy-4-(methylthiol)phenyl]-1H-imidazo-[4,5-*c*]pyridine hydrochloride (LY137150, III) as well as compound I were synthesized in the Lilly Research Labs. (Indianapolis, IN, U.S.A.).

HPLC determination

The HPLC apparatus was assembled with Waters Assoc. components consisting of a U6K injector connected to a WISP 710B. Pumps (6000A) were controlled by a 680 automated gradient controller. The detector was a Schoeffel FS 970 LC fluorometer equipped with a GM 770 monochromator. The excitation wavelength was 320 nm and the emission wavelength 360 nm. The column used was a 5-μm Partisil 5 ODS 3 RAC (Whatman; 10 cm × 9.4 mm I.D.) protected by a Brownlee RP-8 Spheri-10 guard cartridge (10 μm particle size, C_8 chain; 3 cm × 4.6 mm; Brownlee).

Satisfactory separation of I and potential metabolites (II, the sulfone derivative of I, and III, the sulfide derivative of I) was obtained using the following solvents and conditions at room temperature (22°C): solvent A: 1-decanesulphonic acid (Na^+), 1.1 g; glacial acetic acid, 10.5 ml; tetrahydrofuran (preservative-free), 50 ml; acetonitrile, 200 ml; made up to 1000 ml with purified water (Milli-Q Water System; Millipore); solvent B: 1-decanesulphonic acid (Na^+), 1.0 g dissolved in 1000 ml of methanol. The program used is given in Table I.

TABLE I

PROGRAM INDICATING TIME, FLOW-RATE, LINEARITY AND PERCENTAGES OF SOLVENTS USED IN THE HPLC ASSAY

Time (min)	Flow-rate (ml/min)	Percentage A	Percentage B	Curve
Initial	2.00	100	0	—
20.00	2.00	0	100	6 (linear)
25.00	1.50	0	100	6
30.00	2.00	100	0	6

Extraction procedure

To 1 ml of plasma were added 10 μl of 3.37 μl per 10 ml aqueous solution of internal standard and the soluton was thoroughly mixed. Proteins were precipitated by the addition of 1.0 ml of acetone followed by freezing in a solid carbon dioxide—acetone bath and thawing at room temperature. After thorough mixing, the suspension was spun in a centrifuge (Sorvall GLC-2) for 10 min at 2000 *g*. The supernatant was decanted into 1.0 ml of 0.2 *M* glycine—sodium hydroxide buffer, pH 10 and mixed. This solution was poured onto a 5-ml Clin-Elut column (Analytichem International). After 5 min the column

was eluted with 8 ml of dichloromethane–propan-2-ol solution (19:1). The elution with solvent was repeated once more and the pooled solvent evaporated to dryness in vacuo. The efficiency of extraction for I, the sulfone, and the sulfide compounds was greater than 90%. The residue was redissolved in 180 μl of a mixture of acetonitrile–methanol (1:1) and 15 μl were injected onto the HPLC column via the WISP.

Standard curves

Curves were generated for known mixtures of I, II, and III in plasma (dog and rat). Concentrations of I ranged from 100 to 2000 ng/ml of plasma (r = 0.9973), and the sulfone (II) and sulfide (III) concentrations ranged from 10 to 500 ng/ml of plasma (r = 0.9965 and 0.9920, respectively). The standard curves for I, II, and III were linear within the limits quoted above. The coefficients of variation at the highest and lowest concentrations of drugs were 1.8 and 3.5, 2.8 and 6.4, 9.2 and 7.9 (n = 6) for I, II, and III, respectively.

Data were acquired and processed via an HP 1000 chromatography system using Lilly software allowing all data to be compared to standard curves giving both area and peak height values.

Plasma samples

As one example of the use of this assay, rats (Fischer 344, male, ca. 190 g, supplied by Charles River Breeding Labs.) were dosed orally with 20 mg/kg I. Animals were sacrificed at 0, 1, 2, 4, 6, 8 and 24 h (n = 3) and plasma was obtained from the blood by centrifugation (1800 g for 10 min). Of this plasma 1 ml was then assayed as described above.

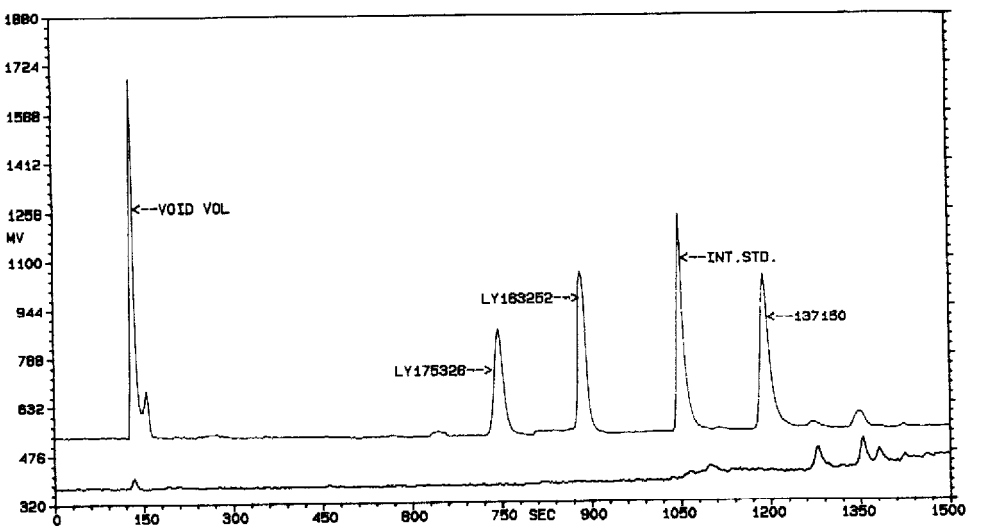

Fig. 2. Chromatogram of blank plasma (lower trace attenuation × 0.5), extracted and assayed by HPLC as in the Experimental section. The upper trace shows the relative retentions of I (LY175326); II (LY163252); the internal standard [1-(2-pyridyl)piperazine]; and III, (LY137510) in plasma (500, 100 and 50 ng of I, II, and III, respectively; attenuation × 0.2). The large peak in the void volume of the upper trace represents an impurity of III.

RESULTS

Fig. 2 illustrates both blank plasma and plasma (1 ml) to which was added internal standard, 500 ng I, 100 ng II, and 50 ng III. Blank and spiked plasmas were extracted and assayed as described under Experimental.

The realistic and useful limits of detection from plasma were 100 ng/ml for both I and II and 20 ng/ml for III. Fig. 3 shows an extract of rat plasma 1 h after oral dosing with I at 20 mg/kg. For the purpose of clarity, a scan of the standard compounds, extracted from plasma, is included.

Table II lists the concentrations of I, II, and III in rat plasma over a 24-h period after an oral dose of 20 mg/kg. From Table II the steadily declining

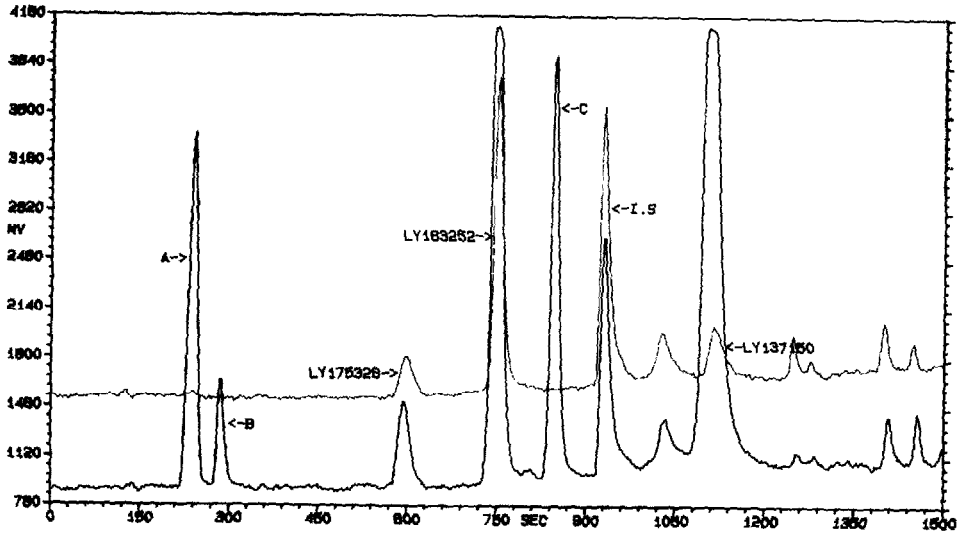

Fig. 3. Chromatogram of an extract of rat plasma assayed 1 h after the oral administration of 20 mg/kg I (lower trace). The upper trace represents the standards I (LY175326) (1000 ng), II (LY163252) (100 ng), and III (LY137510) (100 ng) in a plasma extract, assayed by HPLC as in the Experimental section. Attenuation × 0.5).

TABLE II

PLASMA LEVELS OF I, II, AND III IN FISCHER 344 RATS DOSED ORALLY WITH I AT 20 mg/kg

Time (h)	Plasma level (mean ± S.D., n = 3, ng/ml)		
	I	II	III
1	2614 ± 81	310 ± 12	494 ± 16
2	1544 ± 720	291 ± 25	424 ± 106
4	673 ± 90	298 ± 50	326 ± 40
6	334 ± 47	307 ± 15	233 ± 17
8	206 ± 32	283 ± 25	73 ± 28
24	106 ± 10	N.D.*	N.D.

*N.D. = Not detected.

plasma levels of I are apparent ($t_{½\alpha}$, 1—8 h, 1.9 h). Interestingly, levels of II seem to remain relatively constant over the 1—8 h period. Over this same time period, levels of III also declined from the highest recorded value at 1 h.

DISCUSSION

The HPLC assay with fluorescence detection reported here has thus far proven satisfactory for monitoring plasma levels of the cardiotonic drug I as well as two plasma metabolites, II, the sulfone, and III, the sulfide. The accuracy, precision, and reproducibility of the assay has proven to be acceptable. One disadvantage of the assay is the complexity of the eluent solvents. However, in order to gain a satisfactory separation of unchanged I, two plasma metabolites and the internal standard, such a system was necessary, being superior to other tertiary solvent systems and the use of other ion-pairing agents such as heptanesulphonic acid. While investigating other solvent systems, other columns were also tried. Indeed, very successful results were obtained using a Zorbax TMS column (Dupont) and a simple methanol—acetonitrile gradient utilizing fluorescence detection. However, this particular column proved to be unique in its resolving properties and results could not be repeated on either old or new Zorbax TMS columns. (New columns were obtained from at least two suppliers). The unchanged drug, I, has been successfully detected by HPLC utilizing variable wavelength detection (320 nm) on a Whatman ODS-3 column with an ion-pair elution system [5], but assays of biological media have proven to be two insensitive using this mode of detection.

The fluorescent intensities of I, II, and III vary considerably. Thus, in Fig. 3, the peak corresponding to III is by far the largest on the chromatogram and yet perusal of Table II shows the concentration to be approximately five times less than I. Also visible in the chromatogram shown in Fig. 3 are some, thus far, unidentified metabolites.

The entire assay system has been less well characterized for the quantitation of urinary metabolites but initial studies have been extremely favorable. Of importance has been the use of the Clin-Elut columns as against solvent extraction (e.g. ethyl acetate or dichloromethane) which have given rise to extraneous peaks in the HPLC chromatograms.

The plasma metabolite II has been demonstrated to exhibit cardiotonic activity [2] and while the data are not shown, the assay described herein for I may also be usefully applied to plasma after dosing animals with II.

In summary, an HPLC method utilizing fluorescence detection has been elaborated to investigate plasma levels of I, a potent cardiotonic drug, as well as two plasma metabolites II, the sulfone analogue of I and III, the sulfide analogue. This assay has been used to investigate the time course of these three compounds in the plasma of rats dosed orally with I at 20 mg/kg. This assay is also being used to evaluate the plasma from rodents and dogs treated over a 90-day period with I. Additionally, the assay is also applicable to the examination of these compounds in urine after the administration of I.

REFERENCES

1 D.W. Robertson, E.E. Beedle, J.H. Krushinski, G.D. Pollock, H. Wilson and J.S. Hayes, 188th National Meeting of the American Chemical Society, Philadelphia, PA, U.S.A. August 26–31, 1984, Abstract, MEDI 10.
2 D W. Robertson, E.E. Beedle, J.H. Krushinski, G.D. Pollock, H Wilson, V.L. Wyss and J.S. Hayes, J. Med. Chem., 28 (1985) in press.
3 J.S. Hayes, G.D. Pollock, H Wilson, N. Bowling and D.W. Robertson, Pharmacologist, 26 (1984) 165.
4 J.S. Hayes, G D. Pollock, H. Wilson, N. Bowling and D.W. Robertson, J. Pharm. Exp. Ther., (1985) in press.
5 E. Jensen, personal communication.