Journal of Chromatography, 306 (1984) 249—256
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1951

SIMULTANEOUS ANALYSIS OF A NEW CARDIOTONIC AGENT, MDL 17,043, AND ITS MAJOR SULFOXIDE METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KENNETH Y. CHAN*, DAVID F. OHLWEILER, JAMES F. LANG and RICHARD A. OKERHOLM

Merrell Dow Research Institute, Merrell Dow Pharmaceuticals Inc., 2110 East Galbraith Road, Cincinnati, OH 45215 (U.S.A.)

(First received April 7th, 1983; revised manuscript received October 5th, 1983)

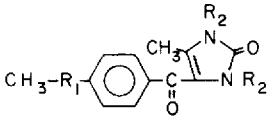
SUMMARY

MDL 17,043 or 1,3-dihydro-4-methyl-5-[4-(methylthio)benzoyl]-2H-imidazol-2-one, is a new cardiotonic agent being developed for the treatment of congestive heart failure. This communication describes a sensitive and selective analytical procedure for the simultaneous analysis of MDL 17,043 and its major oxidative metabolite in plasma. The method involves addition of internal standard and organic solvent extraction, followed by separation with high-performance liquid chromatography and detection by ultraviolet absorption. The assay has good precision and accuracy. Evidence for the positive identification of the sulfoxide metabolite is also presented.

INTRODUCTION

MDL 17,043, 1,3-dihydro-4-methyl-5-[4-(methylthio)benzoyl]-2H-imidazol-2-one (I) (Fig. 1) is a novel cardiotonic agent which is currently undergoing clinical evaluation in patients with congestive heart failure [1]. In vitro and in vivo studies in laboratory animals indicated that this compound possesses positive inotropic and vasodilatory activities [2, 3]. Its sulfur oxidation product, MDL 19,438 (II), has also been shown to have some qualitatively similar properties [4]. Biochemical studies have been carried out aiming to elucidate the mechanism of action of this new chemical entity on cardiac function. Results from these experiments suggested that the compound might exert its effect through the inhibition of cardiac high-affinity cyclic adenosine 5'-monophosphate (AMP) phosphodiesterase [5]. Previously an analytical method utilizing high-performance liquid chromatography (HPLC) in measuring plasma levels of I was reported [6]. In the course of preliminary

0378-4347/84/$03.00



MDL 17,043 (I) $R_1 = S$ $R_2 = H$

MDL 19,438 (II) (sulfoxide metabolite) $R_1 = S{=}O$ $R_2 = H$

MDL 18,763 (III) (internal standard) $R_1 = O$ $R_2 = C_2H_5$

Fig. 1. Chemical structures of MDL 17,043 (I), sulfoxide metabolite (II) and the internal standard (III).

investigation of plasma samples from volunteers receiving the drug, an oxidative metabolite was detected in relatively high quantities. In this present communication, we describe an analytical procedure for the simultaneous measurement of I and its major metabolite in plasma. Evidence for the positive identification of this metabolite in human plasma is also presented.

EXPERIMENTAL

Reagents and chemicals

Ethyl acetate, acetonitrile and chloroform (all glass-distilled) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Methanol (HPLC grade) and ammonium hydroxide (reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Glass-distilled water was used for the preparation of aqueous solutions. MDL 17,043 (I), the authentic sulfoxide metabolite (MDL 19,438) (II), and the internal standard (MDL 18,763) (III) (Fig. 1) were obtained from Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.).

Instrumentation

A component HPLC system (Waters Assoc., Milford, MA, U.S.A.) consisted of a Model 6000A pump, a WISP Model 710B auto-injector and a Model 440 UV absorption detector with a 313-nm filter. A pre-packed 25 cm × 4.6 mm I.D. Zorbax CN (6 μm particle size) HPLC column (DuPont, Wilmington, DE, U.S.A.) was operated with a methanol—water (45:55) mobile phase flowing at 1.0 ml/min. Detector output was recorded and chromatograms analyzed by a computer-automated laboratory system (CALS), (Computer Inquiry Systems, Englewood Cliffs, NJ, U.S.A.).

Electron impact mass spectrometry (EI—MS) was carried out using a Finnigan Model 3300 mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) operating at 70 eV. Sample was introduced by direct solid probe into the ionization chamber with gradual heating from ambient temperature to ca. 250° C for vaporization.

Plasma standard curve

A mixed stock solution of I and II was prepared by dissolving both compounds in methanol (300 μg/ml). Appropriate standard solutions were further diluted with methanol. Plasma standards were prepared by adding 0.1-ml aliquots of the standards to 1.9 ml of drug-free human plasma (NaEDTA anticoagulant) resulting in a range of concentrations as shown in Table I.

Internal standard solution was 6.0 μg/ml in methanol and 0.1 ml (600 ng) was added to each sample before extraction.

Validation study

To test the precision and accuracy of the assay, a validation study was carried out. For each run, a duplicate seven-point standard curve was processed. In three of the nine runs, plasma samples were analyzed that had added concentrations of I and II that were unknown to the analysts. In these particular samples, the concentrations of the compounds were not equal as they were in the standards. Rather, the relative concentrations were chosen to reflect the dynamic changes that might be expected during metabolism in vivo. Analyses were carried out by four analysts with three different groups of instruments so as to fully test variability.

Application of the method

To show the feasibility of the assay, oral solution doses of I in three graded single doses (1, 3 and 5 mg/kg) were given to three healthy volunteers. Periodic plasma samples taken from each subject were randomly coded and analyzed by this procedure. The analysts were unaware of the dose or sample time and the code was not broken until all of the samples were analyzed.

Extraction procedure

To each 2 ml of standard or unknown plasma sample were added 600 ng of internal standard in 0.1 ml of methanol. Then 3 ml of acetonitrile were added to each tube and vortexed to precipitate plasma protein. After centrifuging at ca. 900 *g* for 20 min, the supernatant was decanted into another 25-ml extraction tube which contained 9 ml of ethyl acetate. The compounds were extracted into the organic phase by shaking in an Eberbach reciprocating shaker for 20 min. After centrifuging for 5 min, 10 ml of upper organic layer were transferred to a 15-ml conical tube and placed in a heating block which was set at 50—55°C. The solvent was evaporated to dryness with the aid of a slow stream of nitrogen gas. For HPLC analysis, the extracted residue was redissolved in 100 μl of methanol, and 20 μl were injected into the HPLC column.

Calibration and calculations

The general form of the calibration equation was:

$$y^n = a + bx \tag{1}$$

where y is the peak height ratio (expressed as percent by CALS) of either I or II divided by the internal standard, a is the y-intercept, b is the slope of the curve and x is the concentration of compound. Using an iterative fitting

technique, the value of n was calculated to effectively make $a = 0$ (± 0.00001) when the slope and intercept in eq. 1 were calculated using linear regression.

Isolation and purification of plasma sulfoxide metabolite

A 60-ml volume of pooled plasma obtained from the volunteers in the feasibility study was divided into four equal portions. Plasma protein was precipitated by the addition of 15 ml of acetonitrile to each. After centrifugation, the supernatants were combined and extracted with 80 ml of ethyl acetate twice. The two ethyl acetate phases were combined and evaporated to dryness. The residue was redissolved in 0.1 ml of methanol. This extract residue solution was spotted on a thin-layer chromatographic (TLC) plate (Silica gel 60-F-254, 0.25 mm thickness, G. Merck, Darmstadt, F.R.G.) and developed in a mobile phase which consisted of chloroform—methanol—ammonia (80:20:1). The sulfoxide was located by viewing the plate under UV light at 254 nm and comparing with the R_F of the authentic compound. The band of the TLC plate corresponding to the metabolite was then scraped off and extracted with methanol. The material was further purified by repetitive injections of 15—20 μl of the TLC-isolated component into the HPLC system as described above. The peak corresponding to the sulfoxide was collected and the mobile phase evaporated. The purified metabolite was redissolved in approximately 10 μl of methanol and placed in the solid probe and the methanol solvent evaporated prior to MS analysis.

RESULTS AND DISCUSSION

HPLC conditions

The DuPont Zorbax CN column chosen was ideal for this application. With a relatively non-polar C-8 column used previously [6], the sulfoxide metabolite, being more polar, was eluted almost unretained among several interfering endogenous plasma peaks in the solvent front. The CN column also eliminated the necessity of using a gradient system for the mobile phase. The HPLC conditions for the Zorbax CN column gave retention times for I, II and the internal standard that were about 6.8, 3.9 and 10.5 min, respectively. Figs. 2 and 3 show some typical chromatograms from extracted plasma samples.

Extraction efficiency

Using the procedure described above, the extraction efficiencies of I and II from plasma at 500 ng/ml were 97.4% and 69.6%, respectively.

Validation study

Composite results for the nine standardization runs are tabulated in Tables I and II. The standardization runs show good linearity and reproducibility. For I, the mean value for the correlation coefficient was 0.99984 ± 0.00011 S.D., the coefficient of variation (C.V.) was 0.01%. The slope of the equation (b) was 0.63818 ± 0.049 S.D. (C.V. 7.8%) and the mean power-fit (n) value was 1.0026 ± 0.01036 S.D. (C.V. 1.0%).

For the sulfoxide metabolite, the mean value for the correlation coefficient was 0.99952 ± 0.0043 S.D. (C.V. 0.4%). The slope (b) was 0.31136 ±

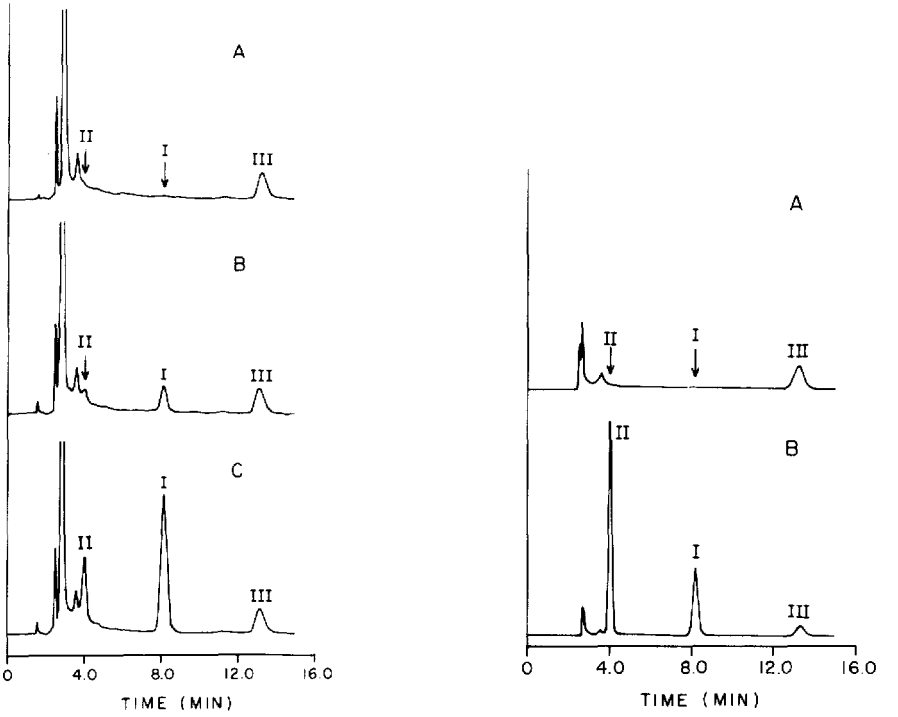


Fig. 2. Chromatograms of extracted plasma standards: (A) blank; (B) 125 ng/ml; (C) 750 ng/ml. Peaks: I = MDL 17,043; II = sulfoxide metabolite; III = internal standard.

Fig. 3. Chromatograms of extracted plasma samples from a patient receiving an oral 3 mg/kg dose of I: (A) pre-dose; (B) 2-h post-dose. Peaks as in Fig. 2.

TABLE I

I (MDL 17,043) STANDARDIZATION RESULTS FOR NINE RUNS

MDL 17,043 plasma concentration (ng/ml)	*n*	Mean percent peak height ratio	S.D.	C.V. (%)
0	18	0	—	—
62.5	18	38.3	1.80	4.7
125	18	78.3	3.53	4.5
375	18	232.9	11.39	4.9
750	16	464.1	18.61	4.0
1500	18	924.9	35.50	3.8
3000	17	1840.0	62.95	3.4

0.01507 S.D. (C.V. 1.5%) and the mean power fit (n) value was 1.01536 ± 0.15965 S.D. (C.V. 15.7%).

The accuracy of the assay was demonstrated by analyzing 58 unknowns in a randomly coded fashion. These results are shown in Tables III and IV. The mean overall accountability for I was 98.8% while that for the sulfoxide was 98.6%. At the extreme of added concentration differences, the presence of I at

TABLE II

II (MDL 19,438) STANDARDIZATION RESULTS FOR NINE RUNS

MDL 19,438 plasma concentration (ng/ml)	*n*	Mean percent peak height ratio	S.D.	C.V. (%)
0	18	0	—	—
62.5	18	18.4	3.92	21.3
125	18	35.8	3.73	10.4
375	18	107.8	8.52	7.9
750	16	215.9	10.75	5.0
1500	18	430.5	22.82	5.3
3000	17	839.4	35.70	4.3

TABLE III

ANALYSIS OF PLASMA SAMPLES CONTAINING UNKNOWN ADDED AMOUNTS OF I

Concentration added (ng/ml)	*n*	Mean concentration found (ng/ml)	S.D.	C.V. (%)	Recovery (%)
0	8	0	0	—	—
29.5	8	30.7	1.87	6.1	104.1
73.7	8	73.4	3.02	4.1	99.6
147	6	144.7	2.52	1.7	98.4
295	8	293.5	13.9	4.7	99.5
737	10	734.7	22.4	3.0	99.7
1473	10	1431.0	49.0	3.4	97.1

TABLE IV

ANALYSIS OF PLASMA SAMPLES CONTAINING UNKNOWN ADDED AMOUNTS OF II

Concentration added (ng/ml)	*n*	Mean concentration found (ng/ml)	S.D.	C.V. (%)	Recovery (%)
0	10	0	0	—	—
48.3	10	46.4	8.1	17.5	96.1
121	8	115.6	4.4	3.8	95.5
242	8	244.4	11.8	4.8	101.0
483	6	472.0	21.2	4.5	97.7
1208	8	1241.0	79.8	6.4	102.7
2415	8	2404.0	75.8	3.2	99.5

1473 ng/ml to plasma in the absence of any II did not show measurable II. Likewise, II added at 2415 ng/ml to plasma did not show measurable I when none was added. These results show that the procedure does not give oxidative or reductive changes that are likely to give artifically high or low values for either compound.

Identification of plasma sulfoxide metabolite

The solid probe EI mass spectra of the purified plasma metabolite and the authentic sulfoxide are shown in Fig. 4. Both mass spectra show an abundant

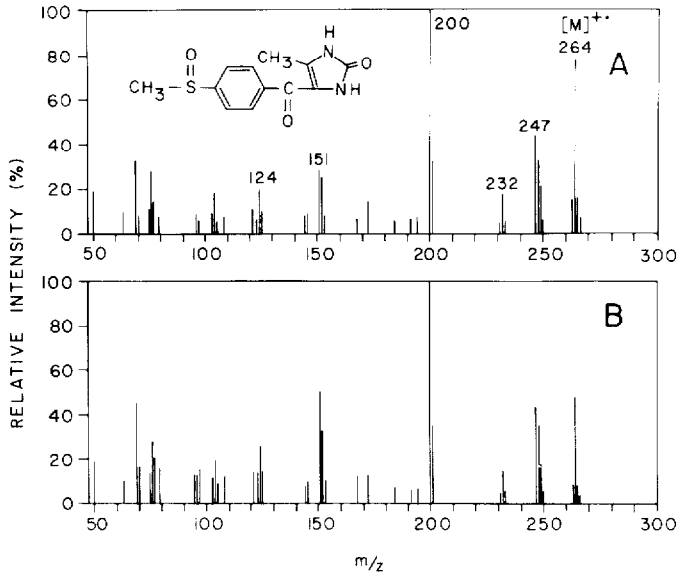


Fig. 4. Solid probe EI mass spectra: (A) authentic sulfoxide; (B) purified plasma metabolite.

molecular ion at *m/z* 264 as well as other similar characteristic fragments. Hence, from the evidence of identical TLC R_F values, HPLC retention times and solid probe mass spectra, it was concluded that the sulfoxide chemical structure was correctly assigned to the plasma metabolite.

Application of the method

Results of the feasibility study are shown in Fig. 5. I was found to be readily absorbed and showed appreciable plasma levels. Its sulfoxide metabolite levels were found to be even higher. The assay was judged to provide adequate sensitivity as the therapeutic dose was estimated to be about 3 mg/kg. The method has been applied successfully in studying single oral and intravenous doses in normal subjects as well as in patients with congestive heart failure.

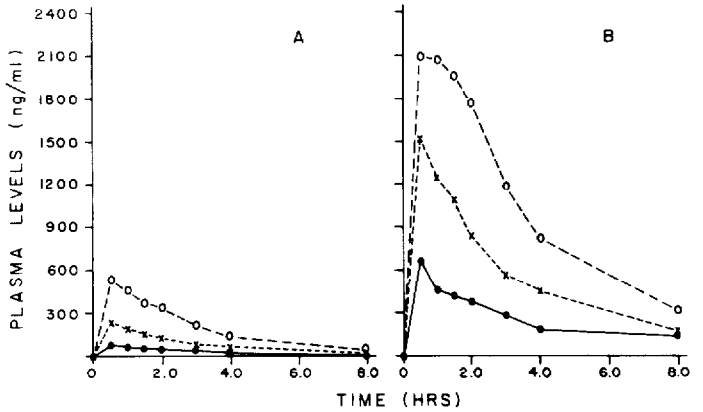


Fig. 5. Mean plasma concentrations of I (A) and the sulfoxide metabolite (B) after single oral doses of (•) 1 mg/kg; (×) 3 mg/kg; and (○) 5 mg/kg (n = 3).

TABLE V

CONCOMITANT MEDICATIONS TAKEN BY HEART PATIENTS THAT SHOW NO INTERFERENCE WITH THE HPLC ASSAY

Chlorthalidone	Isosorbide dinitrate
Clonidine • HCl	Methyldopa
Digitalis	Nitroglycerin
Digoxin	Phenazopyridine
Furosemide	Synthroid
Gantanol	Tolinase
Hydralazine	Zomepirac sodium
Hydrochlorothiazide	

Analysis of plasma from laboratory animals such as dogs and rats also showed the same levels of precision and accuracy.

Stability and interferences

Comparison of freshly prepared plasma standards with those prepared and kept frozen at −20° C showed no significant variations through a period of six months.

Control plasma samples were obtained from heart patients before they received therapy with I. Even though these patients, as a group, were receiving the concomitant medications listed in Table V, there were no substances present in the control samples that showed any potential interference with the analysis of I, II or the internal standard.

CONCLUSION

In conclusion, the validity of the HPLC method to simultaneously measure plasma concentrations of I and its sulfoxide metabolite was demonstrated. The sulfoxide metabolite present in human plasma was also detected in plasma and urine of other species such as rat and dog. Hence, oxidation of the sulfur atom in the molecule appears to be a common and important metabolic pathway of this compound.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Dr. Churby Clowers and Mr. William T. Magner for their excellent technical assistance. They also thank Mrs. Christine E. Wainscott for the preparation of the manuscript.

REFERENCES

1 B.F. Uretsky, T. Generalovich, P.S. Reddy, R.B. Spangenberg and W.P. Follensbee, Clin. Res., 30 (1982) 762A.
2 R.C. Dage, L.E. Roebel, C.P. Hsieh, D.L. Weiner and J.K. Woodward, J. Cardiovasc. Pharmacol., 4 (1982) 500.
3 L.E. Roebel, R.C. Dage, H.C. Cheng and J.K. Woodward, J. Cardiovasc. Pharmacol., 4 (1982) 721.
4 R.C. Dage, L.E. Roebel and H.C. Cheng, personal communication.
5 T. Kariya, L.J. Wille and R.C. Dage, J. Cardiovasc. Pharmacol., 4 (1982) 509.
6 K.Y. Chan, J.F. Lang and R.A. Okerholm, J. Chromatogr., 272 (1983) 396.