Journal of Chromatography, 305 (1984) 153—161
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1896

DETERMINATION OF THE IMIDAZO QUINAZOLINE DERIVATIVE Ro 13-6438 IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 6th, 1983; revised manuscript received August 10th, 1983)

SUMMARY

A high-performance liquid chromatographic assay has been developed for the imidazo quinazoline derivative Ro 13-6438 [D-(—)-6-chloro-1,5-dihydro-3-methylimidazo(2,1-*b*)-quinazolin-2(3H)-one], which is under clinical investigation as a cardioactive drug.

The drug is extracted from biological fluids into 1-chlorobutane—1-hexanol (90:10) and back-extracted into perchloric acid. This extract is chromatographed directly, using a reversed-phase high-performance liquid chromatographic system with ultraviolet detection at 254 nm. The detection limit in plasma is about 1 ng ml^{-1}, using a 1-ml sample. The assay is rapid, accurate and sufficiently sensitive for the study of the single-dose kinetics of Ro 13-6438 in man following a 7.5-mg intravenous dose.

No instability of the unchanged substance was observed in plasma during storage for one day at room temperature and for five months at —20°C.

INTRODUCTION

Ro 13-6438 (I) is an imidazo quinazoline derivative [D-(—)-6-chloro-1,5-dihydro-3-methylimidazo(2,1-*b*)quinazolin-2(3H)-one, Fig. 1] with positive inotropic properties and is under clinical investigation as a cardiotonic drug.

For pharmacokinetic studies of I a suitable method had to be developed to determine the unchanged drug in biological fluids. High-performance liquid chromatography (HPLC) has proved to be a convenient procedure for this purpose, and, because of its higher selectivity, fluorescence detection is used whenever possible [1—3]. Unfortunately, I has no native fluorescence as extensive experiments with a large number of solvents and different test conditions showed. Therefore, the ultraviolet (UV) absorbance of the substance had to be used for quantitative determination. The UV absorbance of I is pH-

0378-4347/84/$03.00

dependent. In aqueous solution at pH 1 two absorption maxima were found, one at 275 nm and a second at 222 nm, the latter having an extinction coefficient about twice that at 275 nm. If the pH was raised, the maximum at 275 nm disappeared; at a pH of 6.5—7.0 and above, a maximum at 250—255 nm could be observed with an extinction coefficient nearly as high as that at 222 nm and a pH of 1.

During the development of the HPLC system, several possibilities were tried. In a preliminary method for pharmacokinetic studies in dogs, normal-phase chromatography was used. Due to endogenous interferences which could not be eliminated and inadequate sensitivity, this approach was not suitable for human plasma. Reversed-phase systems with acidic or neutral to slightly alkaline mobile phases and detection at 222 nm and 254 nm, respectively, were compared. Less base-line noise was encountered at 254 nm; therefore this approach was used to develop an assay for I in biological fluids.

Direct injection of the biological material was not possible [4, 5] due to endogenous interferences. For reversed-phase HPLC, the extraction of a drug from plasma or urine into an organic solvent followed by back-extraction into an aqueous phase, which could then be directly chromatographed, has proved to be an elegant procedure [6, 7]. This approach was applied to I, thus avoiding evaporation and redissolving of the extract.

EXPERIMENTAL

Materials

The following solvents and reagents were used without special purification: *n*-hexane p.a., 1-hexanol for synthesis, methanol p.a., perchloric acid p.a. about 70% (E. Merck, Darmstadt, F.R.G.); 1-chlorobutane HPLC grade (Fisons, Loughborough, U.K.); acetonitrile HPLC grade S (Rathburn, Walkerburn, U.K.).

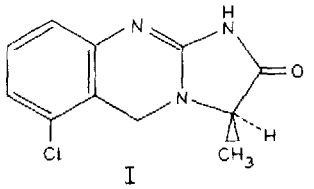
A 0.05 *M* (0.5%) perchloric acid solution was prepared by making up 4.3 ml of perchloric acid (70%) with deionized water to 1000 ml. For extraction, a mixture of one part 1-hexanol and nine parts 1-chlorobutane was used. Buffer solution, pH 6.8 (0.05 *M*), was prepared by dissolving 13.8 g of $NaH_2PO_4 \cdot H_2O$ (p.a. Merck) in about 1950 ml of water (bidistilled), adding about 7 ml of 8 *M* sodium hydroxide, adjusting the pH to 6.8, and making the volume up to 2000 ml with water.

I and the internal standards II and III (for structures see Fig. 1) were first synthesized by Dr. M. Chodnekar and Dr. F. Kienzle of Roche, Basle.

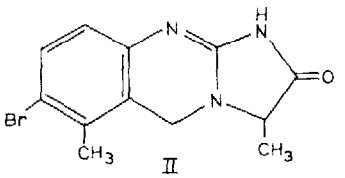
Standard solutions

A stock solution of I in methanol was prepared by dissolving 20 mg of the compound in 100 ml of methanol by ultrasonication. This methanolic solution can be stored at −20°C for more than three months without degradation. Appropriate quantities of the methanolic stock solution were diluted with water to give solutions within the range of 7.5 to 0.05 $\mu g\ ml^{-1}$ of I. These solutions were used as plasma standards by diluting 0.5 ml with blank plasma to 25 ml, covering the concentration range of 150 ng ml^{-1} to 1 ng ml^{-1}.

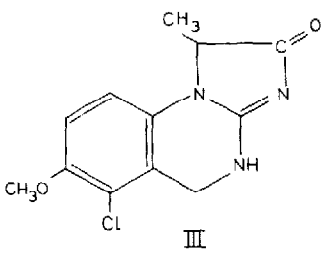
A stock solution of the internal standard was prepared by dissolving 5 mg of



I



II



III

Fig 1. I = Ro 13-6438 = D-(—)-6-chloro-1,5-dihydro-3-methylimidazo(2,1-*b*)quinazolin-2(3H)-one (Europäische Patentschrift No. 718). II = *rac*-7-bromo-1,5-dihydro-3,6-dimethylimidazo(2,1-*b*)quinazolin-2(3H)-one (Europäische Patentanmeldung No. 21338 A1). III = *rac*-6-chloro-4,5-dihydro-7-methoxy-1-methylimidazo(1,2-*a*)quinazolin-2(1H)-one (Europäische Patentanmeldung No. 46267 A1). The synthesis of compounds I—III is described in ref. 12.

III as the hydrochloride in 10 ml of methanol by ultrasonication. This solution could be stored at −20°C, although crystallization occurred. Prior to use, redissolution was effected by ultrasonication.

Two methanolic solutions of the internal standard were prepared, one containing 100 μg ml^{-1}, the other 25 μg ml^{-1}, 1 ml of each of these solutions was diluted with the extraction mixture to 1000 ml giving the internal standard solution 1 containing 100 ng ml^{-1}, and giving the internal standard solution 2 containing 25 ng ml^{-1}.

The chromatographic system was tested daily by injecting mixtures of 5, 10 and 20 ng of I and 100 ng of III in 1 ml of 0.5% perchloric acid.

Equipment and chromatography

The following system was used for HPLC: an Altex Model 110 A pump (Altex Scientific, Berkeley, CA, U.S.A.); a Rheodyne 7125 valve injector with a 1000-μl loop (Rheodyne, Berkeley, CA, U.S.A.), or a Kontron MSI 660 automatic sample injector with a 1000-μl loop (Kontron, Zürich); a Waters 440 fixed-wavelength detector, wavelength 254 nm (Waters, Milford, MA, U.S.A.); a W + W recorder, Model 1100 or 1200 (Kontron, Zürich); and a Spectra-Physics SP 4100 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

The column for chromatography was a 120 mm × 4.6 mm I.D. stainless-steel column (Dr. H. Knauer, Bad Homburg, F.R.G.) packed with Hypersil ODS, 5-μm particle size (Shandon Southern, Astmoore, Cheshire, U.K.). The mobile phase was 155 g of buffer pH 6.8 (0.05 *M*) mixed with 35 g of acetonitrile, degassed by ultrasonication. Using a flow-rate of 2 ml min^{-1}, a pressure of 150—250 bar was obtained. The retention times were approximately 3.9 min for the internal standard III and 4.5 min for I; injection volume was 1000 μl.

Sample preparation

Plasma or urine (1 ml) and the extraction mixtures (5 ml) containing an appropriate amount of the internal standard according to Table I were mixed for 5 min on a tumbler extractor (REAX II, Heidolph Elektra, Keilheim, F.R.G.) at 15 rpm and then centrifuged at 1200 *g* for 5 min. Then 4.5 ml of the organic phase were transferred to a tapered glass tube; 5 ml of *n*-hexane and 1.5 ml of 0.5% perchloric acid were added and mixed for 5 min on a tumbler extractor. After centrifuging for 5 min at 1200 *g*, the organic phase was aspirated and discarded. A portion of the remaining perchloric acid extract was then chromatographed. About 1200 μl were needed to fill the injection valve loop completely (nominal volume 1000 μl).

TABLE I

PLASMA STANDARDS AND AMOUNTS OF INTERNAL STANDARD TO BE USED FOR THE ASSAY

Expected concentration range of I in the unknown samples (ng ml^{-1})	Plasma standards for calibration (ng ml^{-1})	Amount of internal standard III added with the extraction mixture (ng)	Range of the UV detector
150—50	150, 100, 75, 50	500*	0.02
80—5	75, 50, 25, 10, 5	500*	0.01
30 to detection limit	25, 10, 5, 2, 1	125**	0.01

*5 ml of the internal standard solution 1.
**5 ml of the internal standard solution 2.

Calculation

Four to five plasma standards covering the expected concentration range were processed as described above and analysed as calibration samples alongside the unknown samples.

A calibration curve was obtained by least-squares regression of the peak height ratios of I to the internal standard against the concentration of I. This calibration curve was then used to calculate the concentration of I in the unknown samples. The SP 4100 computing integrator was programmed for this calculation.

RESULTS

Characteristics of method

Selectivity. I and the internal standard III were well separated from the main endogenous plasma interferences. Many human blank plasmas were tested and

only in a few were interferences encountered; the levels, however, were insignificant (Fig. 2).

In human urine, the internal standard III was not separated from endogenous urine constituents. Compound II could be used as internal standard (Fig. 1, retention time approximately 9.6 min). However, since only a small fraction of the dose was excreted renally as unchanged drug in man, no attempt was made to modify the assay for urine, and external calibration was applied.

A typical chromatogram of a spiked plasma with 50 ng ml^{-1} I is shown in

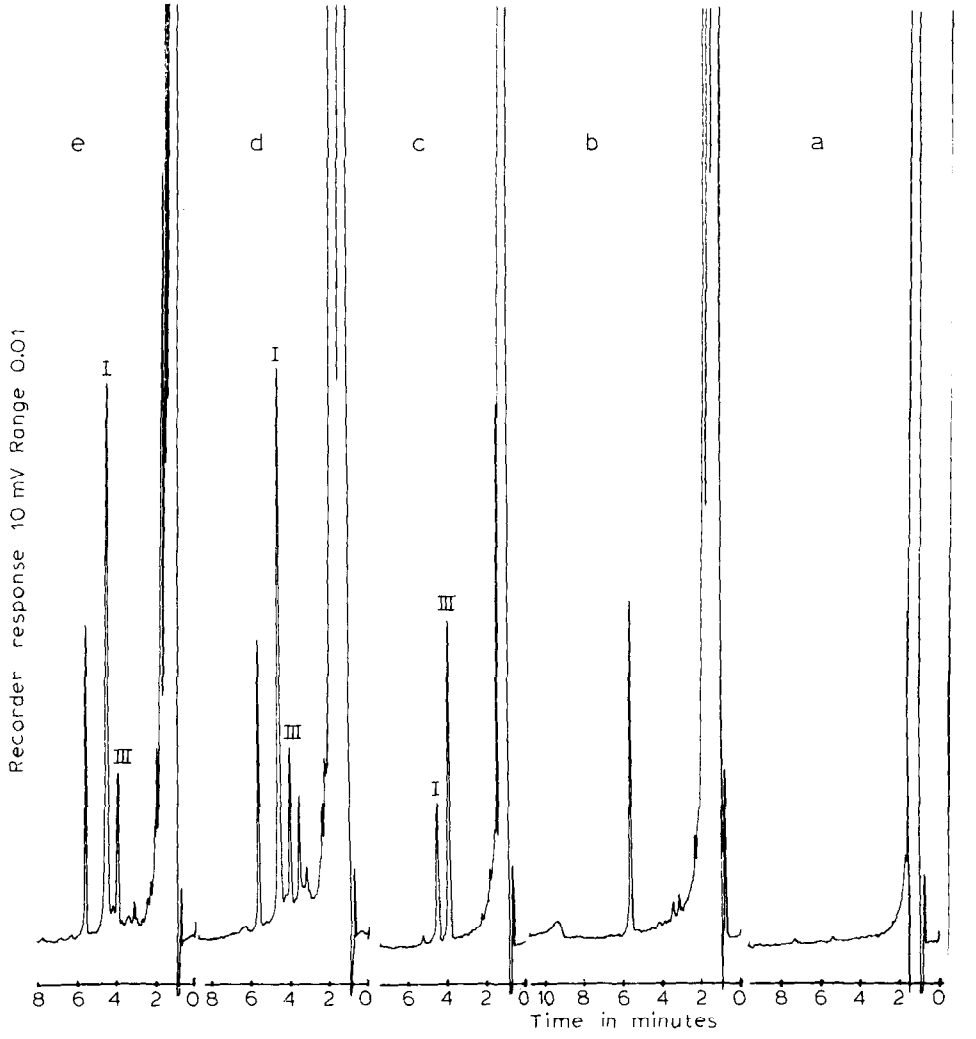


Fig. 2. (a) Chromatogram of 1000 μl of 0.5% perchloric acid. (b) 1000 μl of plasma blank extract. (c) 1000 μl of 0.5% perchloric acid with 5 ng ml^{-1} of I and 100 ng ml^{-1} of III (internal standard). (d) Chromatogram of 1000 μl of perchloric acid extract of plasma standard with 50 ng ml^{-1} of I added. (e) Chromatogram of the extract of a plasma sample of a volunteer taken 30 min following an i.v. dose of 7.5 mg of I. Column: 120 × 4.6 mm, Hypersil ODS, 5 μm. Mobile phase: 155 g of buffer pH 6.8 + 35 g of acetonitrile. Flow-rate: 2.0 ml min^{-1}. UV detection: 254 nm.

Fig. 2, together with a chromatogram of plasma from a volunteer 30 min after administration of a 7.5 mg intravenous (i.v.) dose.

Linearity. A linear response of the UV detector was obtained for up to 2000 ng of I injected in 1000 μl.

Recovery. The recovery of I from plasma was found to be 75% (standard deviation 8%) and was independent of the concentration.

Accuracy. The accuracy of the method, defined as the difference between the amount added to blank plasma and the amount found expressed as a percentage of the amount added, is summarized in Table II.

Precision. The precision of the method is represented by the relative standard deviation of the mean of replicate assays of the same sample. The precision of the method for spiked plasma samples is shown in Table III. For unknown clinical plasma samples, a mean precision of about 2% was found for the concentration range 5—185 ng ml^{-1} [8]; in the range of 5 ng ml^{-1} down to the detection limit the precision was about 8%.

Detection limit. The detection limit for I was dependent on the quality and the age of the column used. Based on a signal-to-noise ratio of 3:1, 0.5 ng of I injected in 1000 μl was detectable. This corresponds to a detection limit in

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OF I IN SPIKED HUMAN PLASMA SAMPLES

Amount added (ng ml^{-1})	Amount found (ng ml^{-1})	*n* replicates*	Amount found expressed as percentage of amount added (%)	Coefficient of variation of the determinations (%)
90	92.3	11	103	3.2
60	62.6	11	104	2.7
30	32.0	9	107	4.4
15	15.7	5	105	6.2
7.5	8.2	14	109	8.5
3	3.1	13	103	8.3

*Replicates analysed on separate days over a period of eight weeks.

TABLE III

PRECISION DATA FOR I FROM CLINICAL TRIALS

Coefficient of variation was calculated from duplicates with each determination carried out on separate days; number of duplicates = *n*.

	Concentration range (ng ml^{-1})		
	< 5 (n = 17)	5—185 (n = 56)	10—35 (n = 10)
Plasma	8%	2%	—
Urine	—	—	4%

TABLE IV

STABILITY OF I IN HUMAN PLASMA

Five replicates in all cases. Statistical calculations are based on logarithms of the data [11].

Storage condition	Amount added (ng ml^{-1})	Amount found (ng ml^{-1})	Percentage difference between amount found and amount added (%)	Confidence interval for the difference between the means of the stored and freshly prepared sample (%)
One day at room temperature	40	39.8 (99.4)*	−0 6	−2.5 to +1.2
	10	9.87 (98.7)	−1.3	−3.4 to +1.0
Three months at −20°C	40	40.3 (101)	+1	−0.4 to +2.0
	10	9.8 (98)	−2	−7.2 to +3.5
Five months at −20°C	40	41.6 (104)	+4	+1.8 to +6.4
	10	9 96 (99.6)	−0.4	−2.9 to +2.3

*In parentheses: percentage of amount added.

plasma of about 1 ng ml^{-1}. With new, high-quality columns, this limit could be reduced to about 0.5 ng ml^{-1}.

Due to endogenous interferences, the detection limit in urine was about 10 ng ml^{-1}.

Stability. Plasma samples spiked with 10 and 40 ng ml^{-1} were stored for 24 h at room temperature and for three and five months at −20°C. The results of these stability tests are compiled in Table IV. The data indicate that I is stable in plasma.

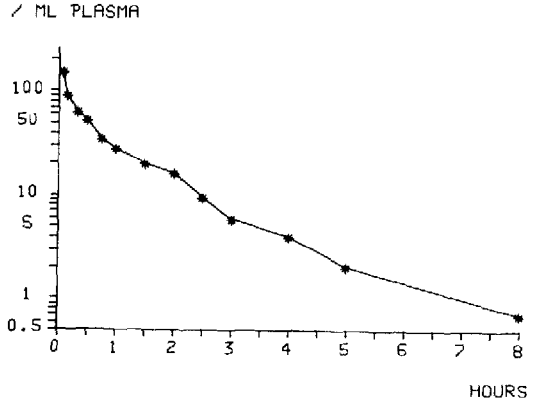


Fig. 3. Plasma concentration—time profile of I of a volunteer (S.H.) following a single i.v. dose of 7.5 mg of the drug as a microsuspension.

A 0.5% perchloric acid solution of I was stored at 4°C for three months, and no measurable degradation was observed. The internal standard III was less stable in 0.5% perchloric acid and fresh solutions should be prepared weekly.

Analysis of plasma and urine samples

The drug I was given to volunteers as a single i.v. dose of 7.5 mg as a microsuspension. The plasma levels of the unchanged drug were determined with the method described. The data for one volunteer are shown in Fig. 3. The corresponding urine data of the volunteer are given in Table V.

TABLE V

CONCENTRATIONS OF I IN URINE OF A VOLUNTEER (S H.) FOLLOWING A SINGLE i v. DOSE OF 7.5 mg OF THE COMPOUND AS A MICROSUSPENSION

Collection period (h)	Quantity of urine (ml)	Urine levels of I (ng ml^{-1})
0—1	500	25.6
1—2	108	10.1
2—3	68	<10

In general, following an i.v. dose of 7.5 mg, plasma levels could be determined up to 6—8 h post medication. The assay allowed the calculation of the most important pharmacokinetic parameters.

DISCUSSION

The main problem in developing an assay for I was to find suitable C_{18} reversed-phase material. Several 5-μm particle size materials were tried; for example, Polygosil C_{18}, Nucleosil C_{18}, and Spherisorb ODS 2. These products had good separation properties but there were large batch-to-batch variations which required a modification of the mobile phase for each new batch. Up to the present time, more than five different batches of Hypersil ODS 5-μm particle size have been used and no modification of the mobile phase has been necessary. Compared to normal-phase columns, the lifetime of ODS material is limited. After some days of operation, tailing peaks and a significant decrease in sensitivity were observed. By turning the column (inlet to outlet; replacement of filters) the original conditions were restored. This procedure could be repeated several times. It is interesting to note that no voids at the tops of the columns were observed using Hypersil ODS.

The pH of the buffer used for the mobile phase was reduced from 7.8 to 6.8 but the influence on the lifetime of the column was not significant [9].

The complex extraction procedure developed was necessary due to the polarity of I. Isobutanol was used at first for the extraction mixture, but negative interfering peaks appeared. With 1-hexanol instead of isobutanol, these interferences were eliminated. To improve the extraction yields, *n*-hexane was added to 0.5% perchloric acid for back-extraction. Although the recovery showed some variation from day to day, this did not influence the precision and accuracy of the assay.

The sensitivity of the method depended mainly on the quality of the column. As mentioned previously, detection was possible down to 0.5 ng ml^{-1}, although this limit could not always be attained.

The sensitivity of this assay for I is adequate for pharmacokinetic studies following a single i.v. dose of 7.5 mg of the drug.

To study the elimination phase of I in man a more detailed method which has a 5—10 times lower sensitivity limit than the present assay would be helpful. This limit cannot be reached by HPLC with UV detection.

Other approaches, such as derivatization prior to or post chromatography would have to be tried. It is questionable whether the sensitivity could be significantly improved with gas chromatography—mass spectrometry.

The assay of I was improved using the internal-standard technique [10]. The quantitative consequences of the variation in separation characteristics of a column during a series of determinations could be eliminated by this approach.

ACKNOWLEDGEMENTS

The authors are grateful to Miss R. Hartenbach for her conscientious technical assistance and to Dr. D. Dell for correction of the manuscript.

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