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Note

High-performance liquid chromatography of the oral anti-asthmatic agent pirquinozol and its active metabolite

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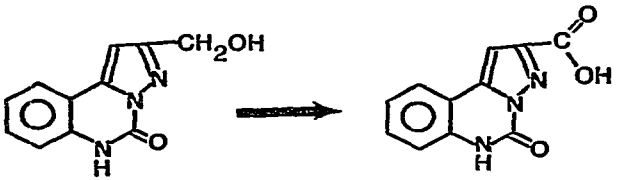
The compound pirquinozol [2-(hydroxymethyl)pyrazolo[1,5-*c*]-quinazolin-5-(1H)-one[1], (E. R. Squibb, SQ13,847)] I, possesses anti-asthmatic and antiinflammatory properties[2–4]. *In vivo*, the drug is metabolized to the acid, II, which is the active agent[4].



I II

High-performance liquid chromatographic (HPLC) methods were devised for bulk and formulated drug and for unchanged drug and its active metabolite in plasma, serum and urine.

EXPERIMENTAL

Chromatography

A modular HPLC system was assembled using a pump (Altex or Waters Assoc.) capable of maintaining a constant flow-rate between 200 and 3000 p.s.i.g. Prior to the precision loop injector (Rheodyne or Chromatronix, 20-μl nominal volume) is a saturator column (250 × 4.6 mm I.D.) packed with 37-μm silica, to extend the life of the column.

For analyzing bulk and formulated compound I, the column is a 10-μm, reversed-phase prepacked octadecylsilane column (Partisil, LiChrosorb or μBondapak). Detection is at 254 nm (Altex or Chromatronix), using a compatible 25-cm strip chart recorder (Linear or Curken). The mobile phase is 20% acetonitrile and 80% water.

To analyze body fluids for compounds I and II, a prepacked column containing 10-μm strong cation exchange (SCX) particles (ES or Partisil) is used. Prior to the SCX column is a guard column packed with silica to trap any remaining particles

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and proteins in the body fluid. Detection involves native fluorescence, with excitation at 315 nm and emission at 345 nm. These close wavelengths necessitated using such dual grating instruments as a Perkin-Elmer Model 204 fluorescence spectrophotometer, equipped with a HPLC flow-cell, or a Perkin-Elmer Model 650-10LC fluorescence spectrophotometer. In addition, ultraviolet absorbance at 254 nm was monitored; both detectors providing inputs into one dual pen or two single pen, 25-cm recorders (Linear or Curken). The mobile phase is 0.03 *M* aqueous ammonium sulfate and 0.2% acetonitrile, adjusted to pH 4.0. Flow-rates are 1 ml/min.

Standard and sample solutions

To assay bulk drug, a reference standard solution of 0.2 mg of I per ml of methanol (stable at 4°C for at least 2 weeks) is diluted 25-fold with aqueous acetonitrile mobile phase. This solution is used to assay other lots of bulk material at the same dilution, or for analyzing the content of the drug in tablets. Tablets containing 50 mg of I, in a formulation consisting mainly of Avicel and dicalcium phosphate, are weighed (*ca.* 400 mg), ground and mixed. About 20 mg (equivalent to *ca.* 2.5 mg of drug), accurately weighed, are extracted with 25 ml of methanol for 15 min with the aid of ultrasonication. After centrifugation at 2000 rpm (1100 *g*), 4.0 ml of the supernatant are diluted to 50 ml with mobile phase and then injected into the chromatograph; typically at a sensitivity of 0.04 absorbance units full scale (a.u.f.s.).

To assay for I and II in plasma or serum, 10 mg of the reference standard of each compound (in one flask) are first dissolved in 100 ml of methanol (stable at 4°C for at least 2 weeks). Dilutions are prepared with mobile phase to give several standard solutions containing 20–200 ng of each agent per ml. These standards are then diluted 1 ml to 4 with 0.033 *M* sodium acetate buffer, pH 4.0. Plasma and serum samples are stored at −20°C until needed. After thawing, 2.0 ml of sample and 2.0 ml of acetonitrile (to precipitate proteins) are pipetted into a 15-ml conical, glass-stoppered, centrifuge tube. After shaking the tube for 5 min, and centrifuging it at 2000 rpm (1100 *g*) for 10 min, 2.0 ml of the clear supernatant are diluted with 2.0 ml of 0.033 *M* sodium acetate buffer, pH 4.0. After recentrifugation, the supernatant is injected into the chromatograph. Urine is diluted with mobile phase and analyzed using standard solutions containing appropriate concentrations of both I and II, 20–200 ng per ml of mobile phase.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of I. The specificity of the reversed-phase HPLC system was tested by injection of methanolic solutions of the synthetic intermediates[1]. As seen from the relative retention times (RRT) given in Fig. 2, all of the precursors can be resolved from I. A series of repetitive injections of 8 μg of I per ml, on three successive days, using two different modular chromatographs, gave relative standard deviations of less than 1%. Another series of injections using concentrations of I from 0.2 to 16 μg/ml, yielded a linear response passing through the origin, with a relative standard deviation of the response, corrected for dilution, of 0.2%.

Prior to assaying tablets containing I, placebo formulation to which I was added, gave an average recovery of 99.9% ($n = 8$, relative standard deviation = 1.4%) after being carried through the recommended procedure. Placebo formulations,

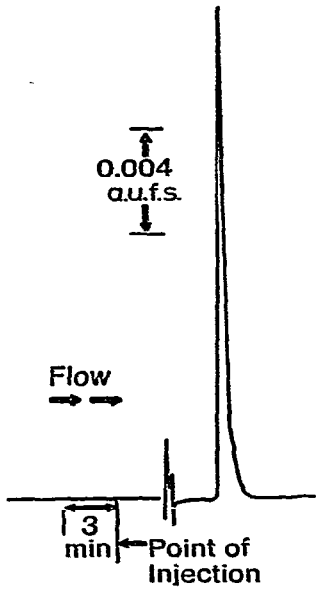


Fig. 1. High-performance liquid chromatogram of pirquinozol (I). See text for details of the reversed-phase system and chromatography.

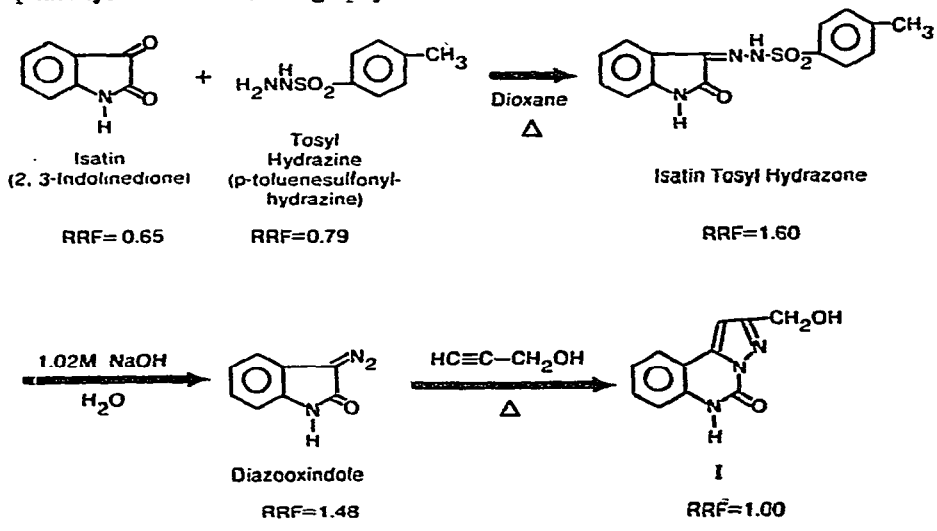


Fig. 2. Relative retention times (RRT) of the synthetic precursors of pirquinozol.

after being carried through the recommended procedure, show no ultraviolet absorbing material eluting in the region where I appears. The assay of 10 representative, individual, 50-mg tablets gave results of 49.0, 50.3, 50.1, 49.8, 51.3, 49.0, 49.7, 49.4, 50.0, and 49.2 mg. The average is 49.8 mg, with a relative standard deviation of 1.4%.

In vivo, the drug is oxidized to the acid, II. HPLC of II, using the method recommended for analyzing I, gave a broad peak for the acid. Diminishing the acetonitrile content of the mobile phase to 10% and adding 0.1% concentrated ammonium hydroxide (to convert II to one ionic species) caused II to elute as a sharp

peak, completely resolved from I, but too close to the void volume. The HPLC system was therefore redeveloped, resulting in a procedure using a strong cation exchange column and a mobile phase of 0.03 *M* aqueous ammonium sulfate–0.2% acetonitrile. Essentially one ionic species of II is found at pH 4.0.

Fig. 3 shows a chromatogram of sample and standard of I and II using a fluorescence detector (315 nm/345 nm). A repetitive series of injections of standard solution at a concentration of 10 ng of I per ml gave a mean peak height of 2.65 cm ($n = 4$), with a relative standard deviation of 1.7%. At higher concentrations, the error diminishes as expected; *e.g.* at 40 ng of II per ml, the peak height is 13.34 cm ($n = 4$) and the relative standard deviation is 1.2%. At concentrations of I and II between 50 and 1800 ng/ml, the response is linear and passes through the origin, with a relative standard deviation, corrected for dilution, of *ca.* 1%. No difference was found between the chromatographic properties of I and II in plasma or serum using the recommended procedure. The limit of detection with an older light source is *ca.* 2 ng/ml, with a signal to noise ratio of 4. A newer lamp and a refocused cell permits the detection of 0.5 ng/ml.

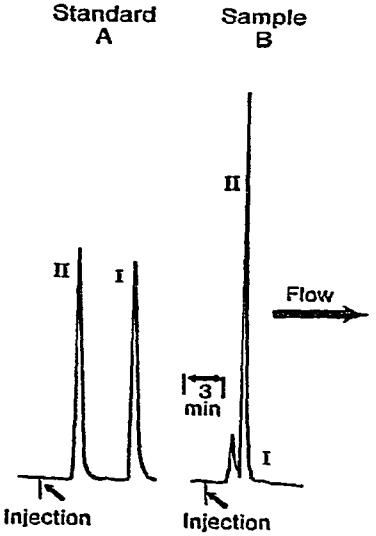


Fig. 3. High-performance liquid chromatogram of pirquinozol (I) and its metabolite (II); A, a standard solution of I and II; B, a sample of plasma containing I and II. A fluorescent detector was used (315/345 nm); see text for details of the ion exchange system, sample preparation and chromatography.

Table I shows that recoveries of I and II, added to human serum, average 98.3% (relative standard deviation = 0.9%) and 99.5% (relative standard deviation = 2.6%), respectively. There are no significant differences between the subgroups of concentrations. Fig. 4 shows the average concentration of I and II in plasma collected from five subjects from 0 to 72 h after the administration of the parent compound, I. The acid, II, predominates over the parent alcohol. Placebo serum, plasma and urine show no peaks appearing where either I or II is expected to elute.

Fig. 5 shows a typical chromatogram of I and II in diluted urine, and an appropriate standard solution containing both compounds. The peak eluting prior

TABLE I

PERCENTAGE RECOVERY OF ADDED DRUG FROM HUMAN SERUM

Concentration (ng/ml)	*II*	*I*
1800	99.6	97.7
	96.6	101.0
	97.9	100.5
500	98.9	95.9
	99.1	98.6
	99.0	99.4
50	97.6	103.1
	97.4	—
	98.3	102.9
	98.2	96.6
Mean	98.3	99.5
Relative standard deviation (%)	0.9	2.6

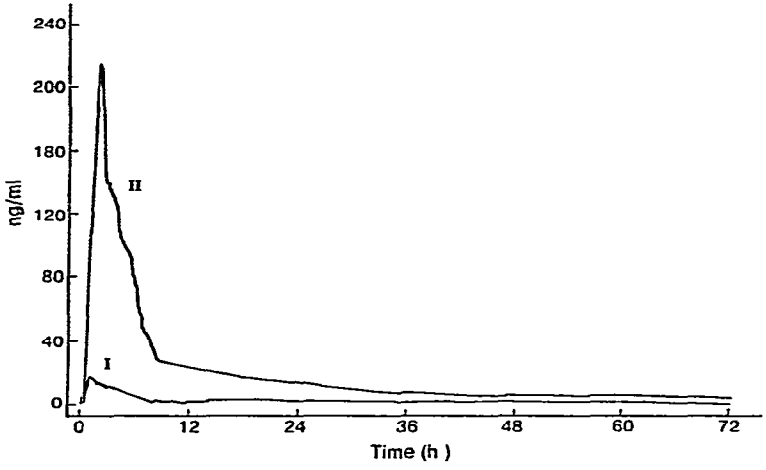
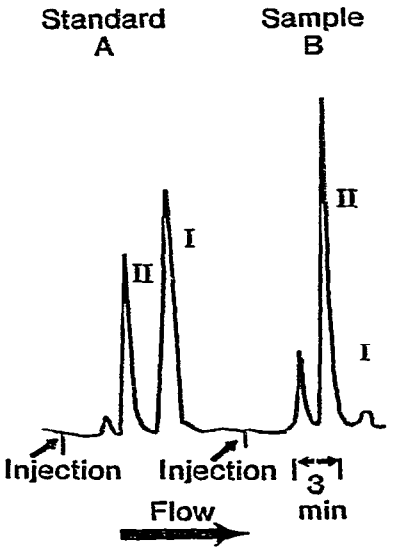


Fig. 4. High-performance liquid chromatogram of plasma from five subjects carried through the recommended procedure. Shown are the average contents of the alcohol pirquinozol (I) and the acid metabolite (II), 0–72 h after the administration of I.

to II is present in the urine of all five subjects studied, as well as that of other volunteers. It may be due to the presence of one or more vitamins superimposed on a small peak found in some lots of acetonitrile. Recoveries of 100 ng of added I and II per ml of control urine are $100.5\% \pm 1.7\%$ and $97.3\% \pm 3.2\%$ ($n = 4$), respectively. Fig. 6 depicts the contents of I and II in urine, from 0 to 24 h after administration of I to a human subject. Again, the metabolite, II, predominated.

CONCLUSIONS

The assay of the antiinflammatory, anti-asthmatic agent pirquinozol in bulk or formulated as tablets, is easily performed by reversed-phase HPLC. Sample



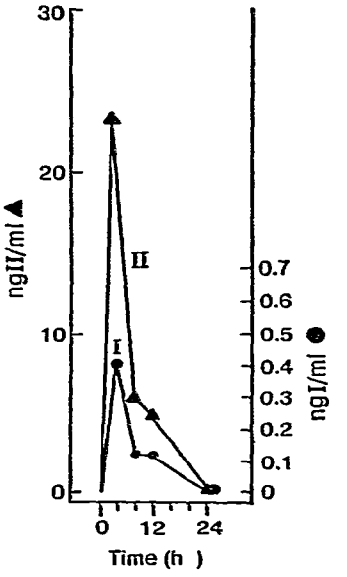


Fig. 5. High-performance liquid chromatogram of I and II in human urine; A, solution of standards, and B, diluted urine containing I and II.

Fig. 6. Contents of pirquinozol (I) and its metabolite in the urine from a human subject, 0–24 h after the administration of I.

preparation, involving only dissolution in methanol, is simple and rapid. Pirquinozol and its active metabolites in body fluids are easily determined using ion exchange liquid chromatography. The data show that the alcohol, I, is rapidly converted to the acid, II.

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