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Note

Simultaneous determination of 2-(*p*-chlorophenyl)pyrazolo[4,3-c]quinoline-3(5H)-one and its 6-, 7- and 8-hydroxy metabolites in plasma, urine and bile by high-performance liquid chromatography

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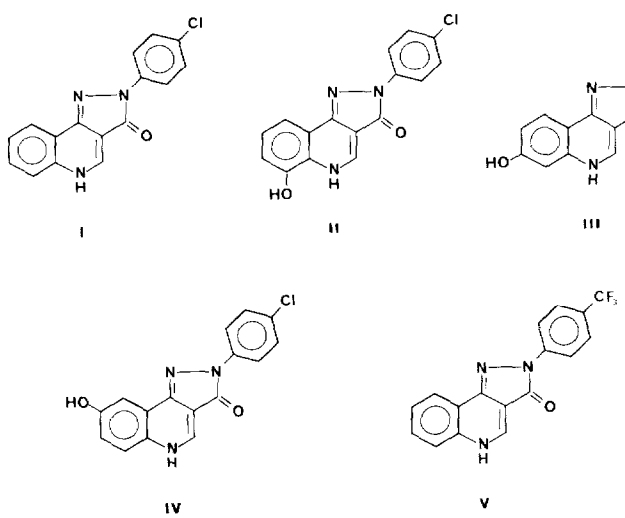
2-(*p*-Chlorophenyl)pyrazolo[4,3-c]quinoline-3(5H)-one (I, Fig. 1) is a novel compound exhibiting anxiolytic properties similar to the benzodiazepines



I II III IV V

Fig. 1. Molecular structures of the parent compound (I), three of its identified metabolites (II—IV) and the internal standard (V).

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[1–3]. Initial studies in rats, monkeys and humans indicated the necessity of measuring I plasma and urine concentrations in the low nanogram range. The primary route of elimination is via the bile and consequently less severe analytical criteria are required for bile determinations. In order to support pharmacokinetic and bioavailability studies, development of a high-performance liquid chromatographic (HPLC) method was undertaken. The HPLC procedure described in this report is capable of simultaneously measuring I and its identified metabolites, the 6-hydroxy (II), 7-hydroxy (III) and 8-hydroxy (IV) adducts of I (Fig. 1).

EXPERIMENTAL

Preparation and sources of reagents

Ethyl acetate (MCB, Gibbstown, NJ, U.S.A.), absolute methanol and water (Burdick & Jackson, Muskegon, MI, U.S.A.) were HPLC grade. All other chemicals and reagents were reagent grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Compounds I (CGS 9896), II (CGS 15995), III (CGS 15994), IV (CGS 15489) and the internal standard, V (CGS 12967), were supplied by Ciba-Geigy (Summit, NJ, U.S.A.).

Citrate–phosphate buffer, pH 4, was prepared by dissolving 2.6 g/l (0.012 *M*) citric acid monohydrate and 1.7 g/l (0.012 *M*) dibasic anhydrous sodium phosphate in water. The mobile phase was N,N-dimethylformamide–citrate-phosphate buffer–methanol (10:35:55). The degassed mobile phase was filtered through a 0.45-μm polyvinylidene fluoride filter (Millipore, Bedford, MA, U.S.A.).

Preparation of standard solutions and calibration standards

Standard stock solutions of I–V were prepared by dissolving each compound in N,N-dimethylformamide to concentrations of 2 and 0.2 μg/ml (I–IV) and 5 μg/ml (V). Standard solutions were stored refrigerated at 4°C and remained stable for at least three months.

Calibration standards were prepared by spiking pooled plasma, urine or bile with the standard solutions.

Extraction procedure

A 1-ml volume of plasma (urine, bile) was transferred to a 15-ml glass centrifuge tube to which 30 μl (150 ng) of the internal standard (V) solution were added. The plasma was then washed with 8 ml hexane containing 1% acetone by shaking for 5 min on a horizontal shaker. After centrifugation (10 min at 500 *g*) the organic layer was aspirated and discarded. The washed plasma was extracted with 5 ml ethyl acetate by shaking on a horizontal shaker for 10 min. The mixture was subsequently centrifuged (10 min at 500 *g*) and 4 ml of the ethyl acetate layer (top layer) were transferred to a clean tube and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 100 μl of 10% N,N-dimethylformamide in mobile phase by vortexing for 1 min and centrifuging (1 min at 500 *g*) to insure adequate dissolution of the residue and drainage from the tube wall. The entire 100-μl aliquot was transferred to automatic injection vials from which 20 μl were injected on-column.

Chromatographic conditions and instrumentation

The chromatographic system consisted of a Beckman 112 HPLC pump, a Waters 710B autoinjector, an endcapped octadecyldimethylsilane bonded phase on a 3-μm spherical silica column (10.0 cm × 4.6 mm), C_{18} Short-One (Rainin, Woburn, MA, U.S.A.), a Kratos Spectroflow 773 variable-wavelength UV detector and a Spectra-Physics SP4100 computing integrator. The mobile phase was delivered at a flow-rate of 0.9 ml/min. The column temperature was ambient. The detection wavelength for all compounds was monitored at 310 nm (0.002 a.u.f.s.).

RESULTS AND DISCUSSION

Chromatography and specificity

Figs. 2–4 show the chromatograms corresponding to the extract of 1 ml plasma and urine (human) and 0.1 ml bile (rat). The retention times are 4.1 min (IV), 5.8 min (III), 7.6 min (II), 9.4 min (I) and 15.3 min (V). Blank control samples showed no significant interferences.

Accuracy, precision and linearity

The results of within-day accuracy and precision for the determination of I–IV in separately prepared spiked plasma samples (1 ml) are presented in Table I. Quantitation of the concentrations found was made by comparison to an independent calibration curve ranging from 2 to 200 ng/ml and calculated by linear least-squares regression. Similar results were obtained in urine and bile (Table II).

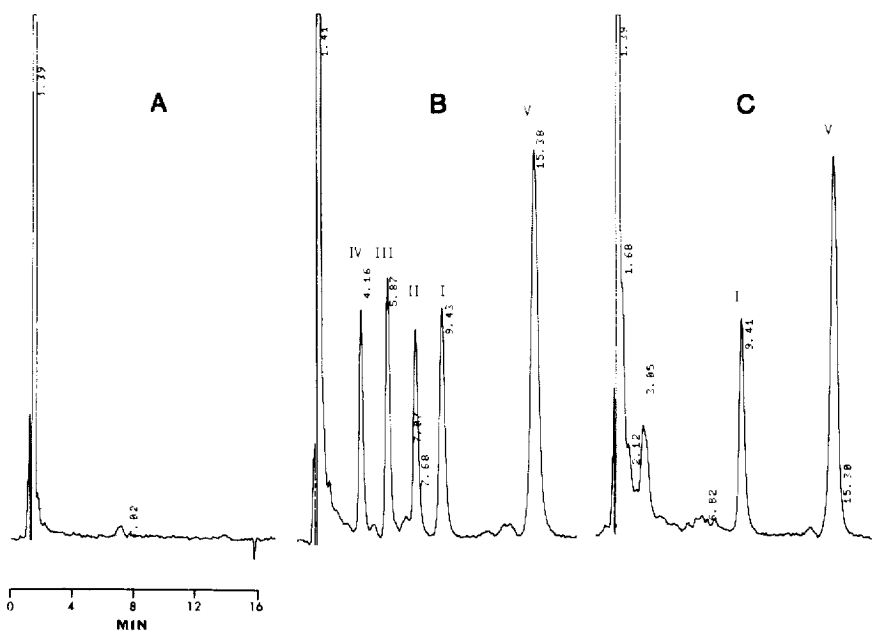

Fig. 2. Chromatograms obtained from the extraction of 1 ml human blank plasma (A), 1 ml of human plasma spiked with 40 ng/ml I–IV (B) and an authentic plasma sample (1 ml) from a subject receiving I (C).

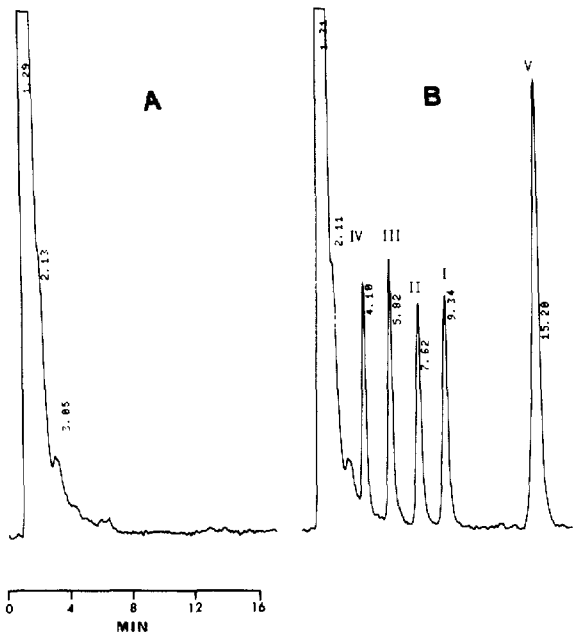

Fig. 3. Chromatograms obtained from the extraction of 1 ml human blank urine (A) and 1 ml of human urine spiked with 40 ng/ml I—IV (B).

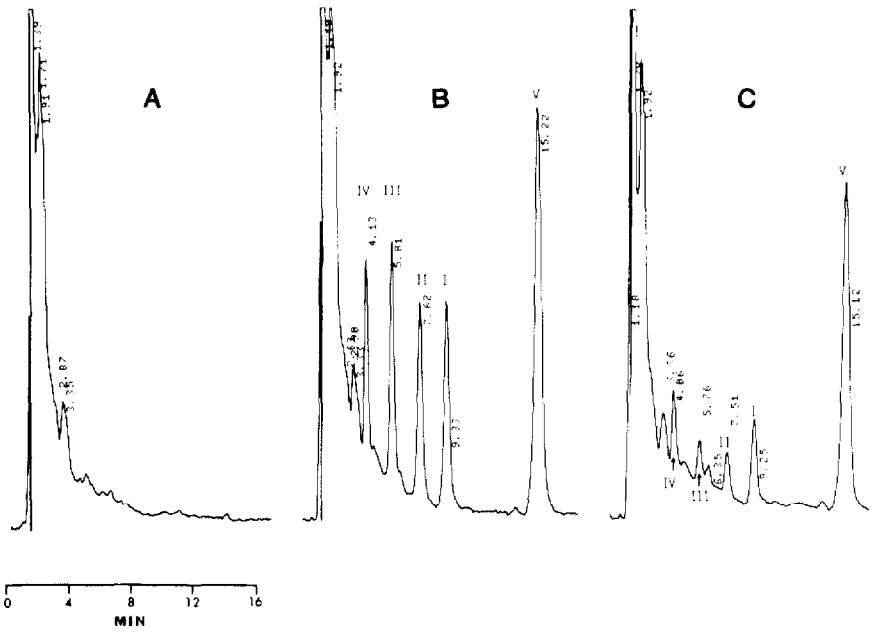

Fig. 4. Chromatograms obtained from the extraction of 0.1 ml rat blank bile (A), 0.1 ml of rat bile spiked with 400 ng/ml I—IV (B) and 0.1 ml of authentic bile recovered from a rat with a bile duct cannula and receiving I (C).

TABLE I

WITHIN-DAY ACCURACY AND PRECISION FOR THE DETERMINATION OF I—IV IN PLASMA

$\bar{X}$ = mean found; C.V. = coefficient of variation; n = number of samples.

Added (ng/ml)	I			II			III			IV		
	$\bar{X}$ (ng/ml)	C.V. (%)	n	$\bar{X}$ (ng/ml)	C.V. (%)	n	$\bar{X}$ (ng/ml)	C.V. (%)	n	$\bar{X}$ (ng/ml)	C.V. (%)	n
2	1.9	16.1	5	2.1	25.6	5	2.0	10.8	4	2.0	18.7	4
4	3.7	12.1	8	3.6	17.7	4	4.3	8.2	5	4.6	16.0	4
6	5.9	14.7	6	6.0	6.1	5	6.6	6.1	5	5.7	9.6	4
10	10	5.7	10	9.5	7.8	4	11	7.8	4	11	5.3	6
20	20	6.0	15	21	10.6	4	21	6.0	4	20	8.4	5
50	52	4.0	13	53	7.3	4	54	8.6	3	50	7.8	3
100	98	5.1	7	104	4.7	3	101	4.1	3	98	2.8	3
200	200	2.5	5	217	4.1	4	201	2.7	4	191	9.9	4

TABLE II

WITHIN-DAY ACCURACY AND PRECISION FOR THE DETERMINATION OF I—IV IN URINE AND BILE

n = 4.

Added (ng/ml)	I		II		III		IV	
	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	C.V. (%)
Urine (human)								
6	7.3	4.9	7.0	1.4	7.3	1.2	6.4	3.8
50	50	1.9	50	2.5	50	2.3	50	2.3
Bile (rat)								
500*	508	2.7	509	2.4	506	1.9	512	6.6
2000	2002	1.1	1967	2.1	1969	0.8	1972	2.4

*Actual volume analyzed was 100 µl.

TABLE III

DAY-TO-DAY PRECISION FOR THE DETERMINATION OF I- IV IN PLASMA

Day-to-day variability is demonstrated for a period greater than two months. C.V. = coefficient of variation; r = linear correlation coefficient.

Compound	Mean regression intercept	Mean regression slope	C.V. (n = 9) (%)	Mean r	C.V. (%)
I	0.01331	0.009171	8.1	0.998	0.2
II	0.001141	0.008277	9.5	0.999	0.2
III	0.003542	0.01010	9.0	0.999	0.2
IV	0.003135	0.009052	9.9	0.999	0.1

Calibration curves were reproducibly linear for I—IV in the concentration range 2—200 ng/ml. All standard curves were calculated by linear least-squares regression of the peak-height ratio versus the concentration. Day-to-day variability is demonstrated for a period in excess of two months by the change

in slope of the standard curves (Table III). The coefficients of variation of 8—10% indicate the necessity of daily standard curves.

Extraction efficiency

The extraction efficiencies (mean ± S.D.) for six spiked plasma samples with concentrations of 10 and 200 ng/ml were 98.3 ± 6.04%, 90.3 ± 3.27%, 94.6 ± 6.97% and 102.3 ± 11.88% for I–IV, respectively. The mean extraction efficiency (mean ± S.D.) for three spiked plasma samples with 150 ng/ml internal standard (V) was 86.7 ± 4.96%. The extraction efficiency was linear in the indicated concentration range.

Stability

The stability of I–IV in plasma was determined under accelerated conditions by incubating at 37°C two plasma pools containing concentrations of 10 and 200 ng/ml in a water bath and sampling the pools over a seven-day period. Control samples were immediately analyzed. At day 1 the concentration (mean ± S.D.) relative to the control was 1.00 ± 0.044 (n = 6) for I; at day 3 the relative concentrations were 1.04 ± 0.049 (n = 6), 0.98 ± 0.071 (n = 5), 0.97 ± 0.116 (n = 5) and 0.94 ± 0.076 (n = 6) for I—IV, respectively; at day 7 the relative concentrations were 0.99 ± 0.061 (n = 6), 0.96 (n = 2), 0.96 ± 0.087 (n = 5) and 1.03 ± 0.180 (n = 4) for I–IV, respectively. No difference in relative concentrations was observed between the 10 and 200 ng/ml pools.

Plasma (n = 5) and urine (n = 5) samples spiked with 5 and 200 ng/ml I stored at −10°C for 26 weeks resulted in recoveries (mean ± S.D.) of 98.4 ± 7.06% and 97.8 ± 6.91%, respectively. These data indicate the stability of I–IV in plasma and I in urine.

CONCLUSION

A sensitive and specific analytical procedure for the determination of I and its hydroxy metabolites in plasma, urine and bile has been developed. This assay is suitable for use in bioavailability and pharmacokinetic studies.

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