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High-performance liquid chromatography of the aromatase inhibitor, letrozole, and its metabolite in biological fluids with automated liquid–solid extraction and fluorescence detection

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Abstract

An analytical method for the determination of letrozole (CGS 20 267) in plasma and of letrozole and its metabolite, CGP 44 645, in urine is described. Automated liquid–solid extraction of compounds from plasma and urine was performed on disposable 100-mg C_8 columns using the ASPEC system. The separation was achieved on an ODS Hypersil C_{18} column using acetonitrile–phosphate buffer, pH 7, as the mobile phase at a flow-rate of 1.5 ml/min. A fluorescence detector was used for the quantitation. The excitation and emission wavelengths were 230 and 295 nm, respectively. The limits of quantitation (LOQ) of letrozole in plasma and in urine were 1.40 nmol/l (0.4 ng/ml) and 2.80 nmol/l, respectively. The respective mean recoveries and coefficient of variation (C.V.) were 96.5% (9.8%) in plasma and 104% (7.7%) in urine. The LOQ of CGP 44 645 in urine was 8.54 nmol/l (2 ng/ml). The mean recovery was 108% (6.3%). The compounds were well separated from co-extracted endogenous components and no interferences were observed at the retention times of compounds. The sensitivity of this method for letrozole in plasma should be sufficient for kinetic studies in humans with single doses of 0.5 mg and possibly less.

Keywords: Letrozole

1. Introduction

Letrozole (CGS 20 267) is a potent aromatase inhibitor under development as a drug for the treatment of oestrogen-dependent diseases, such as breast cancer in post-menopausal women.

A high-performance liquid chromatography (HPLC) method for the determination of letrozole in biological fluids has been described previously [1], but it appeared that it was not sensitive enough to measure the unchanged compound after administration of low doses (0.5 mg). An enzyme immunoassay (EIA) [1] more sensitive than this HPLC method (0.7 versus 28 nmol/l, respectively) was developed. Its application to clinical samples revealed that it was not specific enough. A strong cross-reactivity of the antibodies with the metabolite CGP 44 645 (I) has been observed with all urine samples and several plasma samples from patients under repeated administration [1].

This report describes an HPLC method with a fully automated liquid–solid extraction and fluorescence detection offering improved sensitivity for the

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determination of letrozole in plasma and of letrozole and its metabolite, I, in urine.

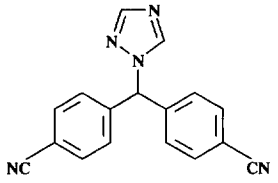
2. Experimental

2.1. Chemicals and reagents

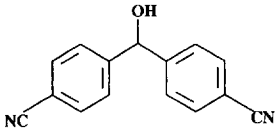
Letrozole, its metabolite, I, and CGP 47 645, used as the internal standard (I.S.) (Fig. 1), were supplied by Ciba (Basle, Switzerland).

All the chemicals were of analytical grade.

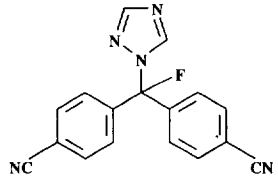
Methyl alcohol, acetonitrile and potassium dihydrogenphosphate were obtained from Carlo Erba (Rueil-Malmaison, France). 0.1 *M* HCl, 0.1 *M* NaOH and disodium hydrogenphosphate were obtained from Merck (Nogent sur Marne, France).



Letrozole (CGS 20 267)
mol. wt. : 285.31 ; $C_{17}H_{11}N_5$



Metabolite I (CGP 44 645)
mol. wt. : 234.26 ; $C_{15}H_{10}N_2O$



Internal standard CGP 47 645
mol.wt. : 303.301 ; $C_{17}H_{10}FN_5$

Fig. 1. Chemical structures of letrozole, its metabolite and the internal standard.

0.01 *M* phosphate buffer, pH 7, was prepared by dissolving 2.72 g of KH_2PO_4 and 2.84 g of Na_2HPO_4 in 2 l of distilled water.

Disposable extraction columns (DECs) of 1-ml capacity containing 100 mg of C_8 reversed-phase (Bond-Elut, Analytichem International, Varian, Les Ulis, France) were used for liquid–solid extraction.

2.2. Equipment

The chromatographic equipment used was a Model 305 programmable pump and a Model 805 manometric module (Gilson, Villiers-le-Bel, France), an ASPEC (automatic sample preparation with extraction columns) system (Model XL, Gilson) which performed extractions and injections, a fluorescence detector (Perkin-Elmer LS 30) to determine the letrozole spectrum (Fig. 2), a fluorescence detector (Hitachi 1080, Merck) with excitation and emission wavelengths set at 230 nm and 295 nm, respectively; a data station connected to the detector equipped with Millennium software that performed integration, recording, calculations and storage of the data (Waters, Saint Quentin en Yvelines, France). The analytical pre-packed column was a stainless tube filled with ODS Hypersil C_{18}, 5 μm, 200×4.6 mm I.D. (Ref. No. 79916OD-574, Hewlett-Packard). To protect this column, a guard-column, stainless-tube, 20×4.6 mm I.D., pre-packed with Supelguard LC 18, 5 μm (Ref. No. 45-9564, Supelco, Saint Quentin Fallavier, France) was installed. The mobile phase (0.01 *M* phosphate buffer, pH 7–acetonitrile, 70:30, v/v) was used at a flow-rate of 1.5 ml/min.

2.3. Calibration and validation samples

Working solutions containing 0.4 to 200 ng of each compound in 100 μl of water were prepared from methanolic stock solutions. Appropriate aliquots were added to 1 ml of plasma or to 0.5 ml of urine diluted with 0.5 ml of water. The extraction procedure on DEC C_8 was automatically performed by the ASPEC system as described in Section 2.4.

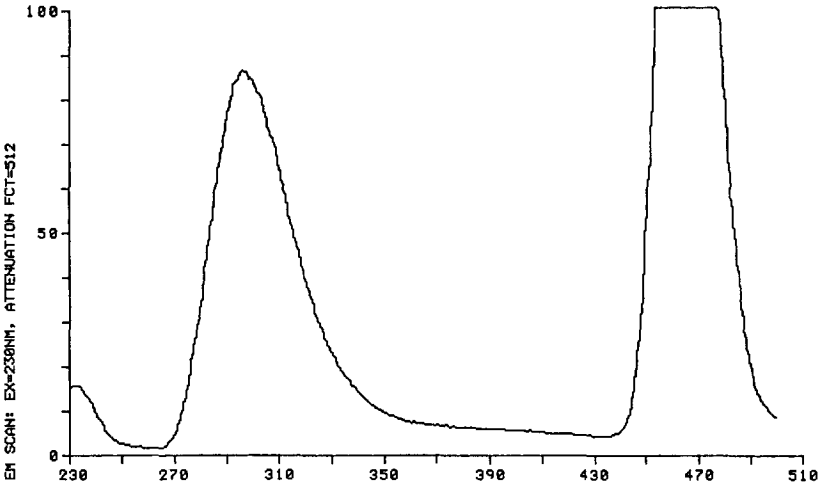


Fig. 2. Letrozole spectrum.

2.4. Extraction procedure

The plasma or urine samples were placed on the rack of the ASPEC system. At every step, the elution through the C_8 DEC was performed by pressurising air dispensed above the packing, to induce the desired flow-rate through the DEC.

1. — DEC conditioning: 1 ml of methanol and 1 ml of 0.1 *M* HCl were successively dispensed on the DEC (dispensing flow-rate, 6 ml/min; air volume, 0.025 ml/min).
2. — I.S. addition and sample dilution: 0.1 ml of I.S. solution and 1 ml of 0.1 *M* HCl were successively added to the sample (dispensing flow-rate, 6 and 10 ml/min, respectively) and were bubbled with 2 ml of air.
3. — Sample transfer: 2.2 ml of diluted plasma sample were transferred to the DEC (dispensing flow-rate, 0.5 ml/min; air volume, 1 ml/min).
4. — Column washing: 2 ml of buffer, pH 7, and 0.5 ml of buffer, pH 7–CH_3CN (80:20, v/v) were successively dispensed on the DEC (dispensing flow-rate, 6 and 3 ml/min, respectively; air volume, 1 ml/min).
5. — Elution: 2 ml of buffer, pH 7–CH_3CN (60:40, v/v) were dispensed on the DEC (dispensing flow-rate, 6 ml/min; air volume, 1 ml/min) and the eluate was bubbled with 2 ml of air.
6. — Injection: 300 μl of the eluate were dispensed through the 100-μl injection loop.

After each transfer, the needle was rinsed with 1 ml of H_2O–MeOH (70:30, v/v). As already observed for another drug [2], the compounds were partially adsorbed on the needle of ASPEC. Therefore, a preliminary washing of the needle before each preparation was necessary to avoid cross-contamination. The washing was with 4 ml of 0.1 *M* NaOH followed by 4 ml of methanol.

Each sample was prepared separately during the chromatography of the previous sample.

2.5. Calibration curves

Calibration standard samples at seven or eight different concentrations (a single sample per concentration) were prepared each week.

For letrozole in plasma, the range was 1.40 to 140 nmol/l. For letrozole and I in urine, the range was 2.80 to 280 and 8.54 to 342 nmol/l, respectively.

The calibration curves were generated by Millennium software from the peak-height ratio of letrozole

or I to I.S. versus the concentration of either compound in the sample. The equation was calculated using weighted [1/(concentration)^2] linear least-squares regression as the mathematical model. The calibration curve data are shown in Table 1. The correlation coefficients were between 0.9988 and 0.9997. Individual fit of the calibration standards to the curve was assessed from the relative error (RE in %; 100×[(back calculated concentration from the regression line equation)−(nominal concentration)]/(nominal concentration). As shown in Table 1, the differences for back calculated concentrations were

Table 1
Data on calibration curves

	Letrozole in plasma		Letrozole in urine		I in urine	
	Slope (n=5)	C.C.[a]	Slope (n=3)	C.C.[a]	Slope (n=3)	C.C.[a]
Mean	0.0517	0.9997	0.028	0.9988	0.0120	0.9992
S.D.	0.0013	0.0001	0.0037	0.0007	0.0013	0.0006
C.V. (%)	2.49	0.011	13.0	0.072	11.3	0.0609

[a]C.C.=correlation coefficient.

Accuracy and precision of calibration curves using back-calculated concentrations

Letrozole in plasma

	Concentrations (nmol/l)							
	1.40	2.80	3.5	7.00	17.5	35.0	70.0	140
Mean	1.41	2.79	3.47	7.05	17.7	35.7	69.8	137
S.D.	0.013	0.037	0.094	0.055	0.251	0.344	1.38	1.52
C.V. (%)	0.919	1.33	2.71	0.780	1.42	0.966	1.98	1.10
Mean recovery (%)	101	100	99	101	101	102	100	98
Mean RE[b] (%)	0.713	1.06	1.55	0.889	1.31	1.65	1.69	1.92

Letrozole in urine

	Concentrations (nmol/l)							
	2.80	5.60	7.00	14.0	35.0	70.0	140	280
Mean	2.79	5.98	7.00	14.1	34.7	69.6	137	269
S.D.	0.134	0.309	0.185	0.212	1.08	0.404	1.15	–
C.V. (%)	4.82	5.17	2.64	1.51	3.12	0.580	0.845	–
Mean recovery (%)	100	107	100	101	99	99	98	–
Mean RE[b] (%)	3.14	6.53	2.03	1.07	2.60	0.529	2.24	4.02

I in urine

	Concentrations (nmol/l)						
	6.83	8.54	17.1	42.7	85.4	171	342
Mean	6.77	8.30	17.4	42.6	87.1	171	342
S.D.	0.130	0.255	0.849	0.854	2.15	6.03	–
C.V. (%)	1.92	3.07	4.88	2.01	2.47	3.52	–
Mean recovery (%)	99	97	102	100	102	100	–
Mean RE[b] (%)	1.44	3.19	3.38	1.32	2.32	2.37	2.17

[b]RE (%): absolute value of RE (%): 100 [(back-calculated concentration from the curve)−(nominal concentration)]/(nominal concentration).
–: only one value available.

between 0.53 and 6.5% from the theoretical values and indicated a good fit of the regression model over the range of the calibration curve. The data of the calibration curves were collected over a ten week period. The results showed good reproducibility and precision for the calibration curves.

3. Results and discussion

3.1. Specificity

Representative chromatograms are shown in Fig. 3 for drug-free human plasma and plasma spiked with letrozole and I.S., in Fig. 4 for drug-free human urine and urine spiked with letrozole, its metabolite, I, and I.S.

The compounds were well separated from co-extracted endogenous components and no interferences were observed at the retention times of compounds (11.3, 12.3 and 16.7 min for letrozole, I and I.S., respectively).

3.2. Limits of quantitation

The limit of quantitation (LOQ) was the lowest concentration whose mean recovery (predicted/nominal in %) was within 80–120% of the expected value and C.V. did not exceed 20%. The lowest concentration (1.40 and 2.80 nmol/l for letrozole in plasma and urine, respectively, and 8.54 nmol/l for I in urine), whose accuracy and precision were within the proposed criteria, was defined as the LOQ.

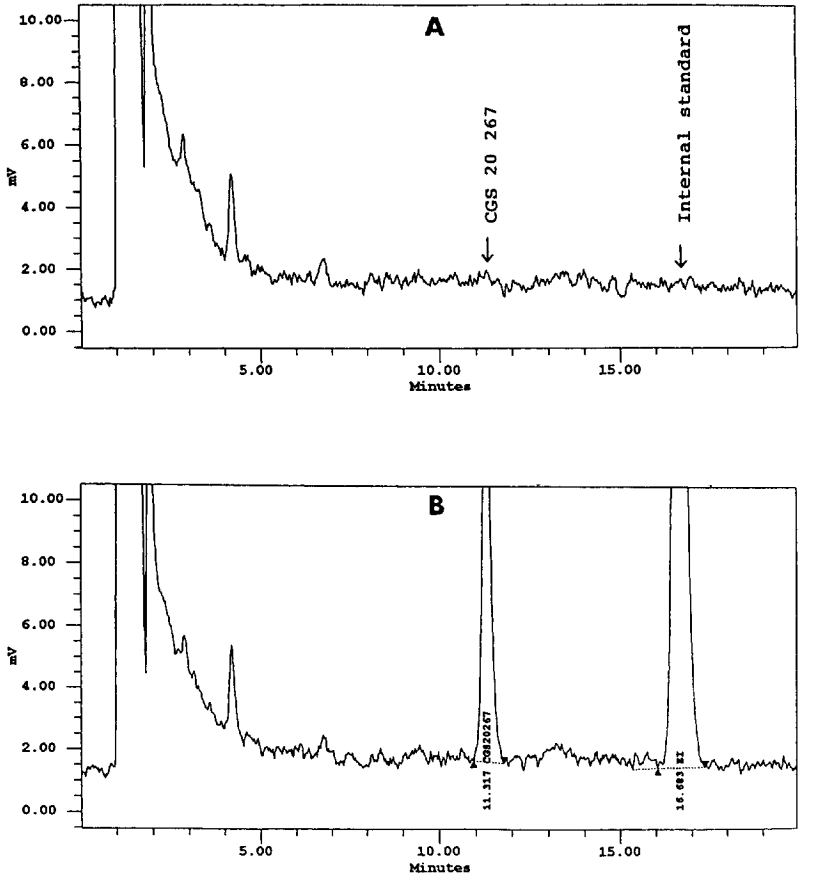


Fig. 3. Typical chromatograms of 1 ml of drug-free plasma (A) and 1 ml of plasma spiked with 7.00 nmol/l of letrozole and 0.033 nmol of internal standard (B).

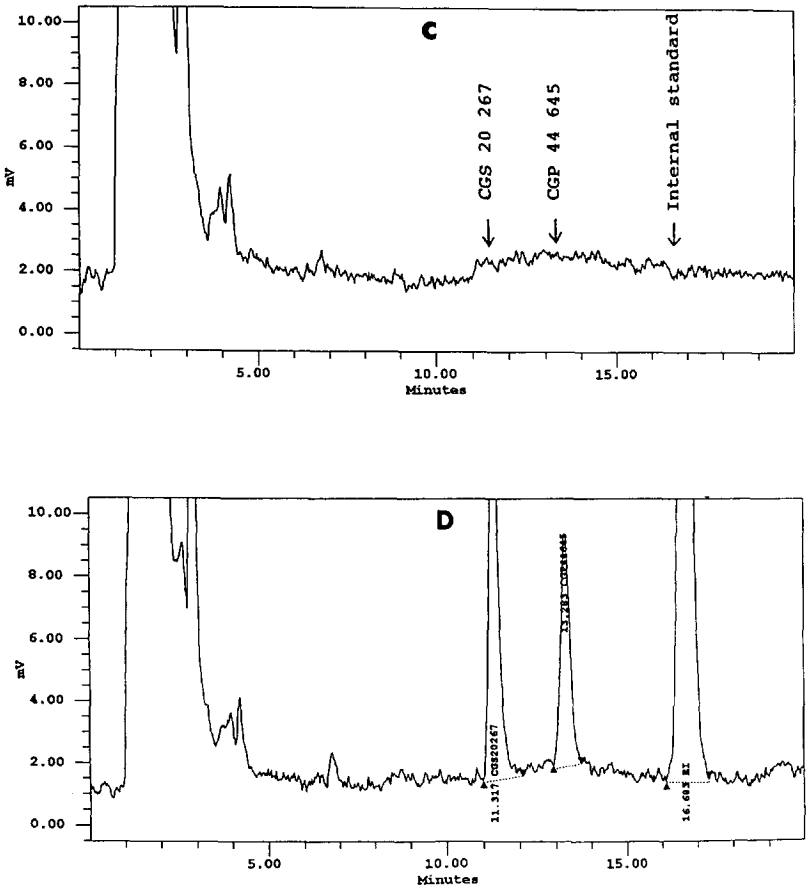


Fig. 4. Typical chromatograms of 0.5 ml of drug-free urine (C) and 0.5 ml of urine spiked with 7.00 nmol/l of letrozole and 8.54 nmol/l of CGP 44 645 and 0.033 nmol of internal standard (D).

3.3. Within-day precision and accuracy for letrozole in plasma

With the given concentrations of 1.40, 3.50, 7.00, 35.0, 70.0 and 140 nmol/l, mean recoveries ranged from 93.4 to 108% and the C.V. ($n=3$) ranged from 1.5 to 5.7%. For the LOQ, the mean recovery was 93.4% and the C.V. was 5.7% ($n=3$).

3.4. Day-to-day letrozole measurements in plasma

Concentrations of validation samples over the day-to-day measurements were calculated from the calibration curve established on day one. Individual and mean recoveries with coefficients of variation are presented in Table 2. Overall mean recoveries ranged from 96.5 to 106% and C.V. ranged from 2.7 to 9.8%. For the limit of quantitation, the mean recovery was 96.5% and the C.V. was 9.8%. This demonstrated that calibration curves can be used for sample determination over a period of at least one working week.

3.5. Within-day precision and accuracy in urine

3.5.1. Letrozole

With given concentrations of 2.80, 7.00, 14.0, 35.0, 140 and 280 nmol/l, mean recoveries ranged from 97.7 to 102% and the C.V. ($n=3$) from 0.99 to 9%. For the LOQ, the mean recovery was 101% and the C.V. was 9% ($n=3$).

Table 2
Day-to-day reproducibility and precision for letrozole in spiked human plasma

Concentration given (nmol/l)	Mean recovery (n=3) (%)				Overall mean recovery (%)
	Day 1	Day 2	Day 3	Day 6	
1.40	93.4 (5.7)	97.0 (9.9)	97.8 (18.9)	97.6 (3.3)	96.5 (9.8)
3.50	95.1 (3.5)	98.7 (4.7)	104.4 (6.8)	90.9 (1.8)	97.3 (6.7)
7.00	105 (2)	101 (5.3)	99.1 (1.6)	101 (3.3)	102 (3.7)
35.0	102 (2.5)	101 (1.7)	105 (1.5)	99.3 (0.8)	102 (2.7)
70.0	103 (1.5)	101 (1.9)	106 (0.9)	98.3 (2.4)	102 (3.2)
140	108 (2.4)	104 (2.0)	107 (4.1)	105 (5.3)	106 (3.6)

Values in parentheses are coefficients of variation.

3.5.2. Compound I

With given concentrations of 8.54, 17.1, 42.6, 171 and 342 nmol/l, mean recoveries ranged from 101 to 106% and the C.V. (n=3) ranged from 0.6 to 9.0%. For the LOQ, the mean recovery was 106% and the C.V. was 9.0% (n=3).

3.6. Day-to-day measurements in urine

Concentrations of validation samples over the day-to-day measurements were calculated from the calibration curve established on day one.

3.6.1. Letrozole

Individual and mean recoveries with coefficients of variation are presented in Table 3. Overall mean recoveries ranged from 99.9 to 104% and C.V. from 2.2 to 7.7%. For the LOQ, the mean recovery was 104% and the C.V. was 7.7%. This demonstrated that calibration curves can be used for sample determination over a period of at least one working week.

3.6.2. Compound I

Individual and mean recoveries with coefficients of variation are presented in Table 4. Overall mean recoveries ranged from 102 to 108% and C.V. ranged from 1.2 to 6.3%. For the LOQ, the mean recovery was 108% and the C.V. was 6.3%. This demonstrated that calibration curves can be used for sample determination over a period of at least one working week.

3.7. Stability in plasma samples

The stability of letrozole in plasma samples on the rack of the ASPEC system was tested. Freshly spiked plasma samples (3.50, 17.5 and 70.0 nmol/l, n=3 for each concentration) were extracted immediately after preparation and other samples stayed on the rack of the ASPEC at room temperature for 16 h. The results demonstrated that letrozole in diluted plasma samples kept at room temperature is stable for at least 16 h (recovery between 89.4 to 103%).

Table 3
Day-to-day reproducibility and precision for letrozole in spiked human urine

Concentrations given (nmol/l)	Mean recovery (n=3) (%)			Overall mean recovery (%)
	Day 1	Day 6	Day 7	
2.8	101 (9)	99.1 (5.1)	111 (4.1)	104 (7.7)
7.0	100 (2.4)	101 (2.3)	101 (2.8)	101 (2.2)
14.0	101 (1.2)	100 (2.1)	104 (3.3)	102 (2.7)
35.0	97.7 (3.9)	99.0 (1.2)	103 (1.9)	99.9 (3.3)
140	102 (0.6)	97.9 (1.5)	104 (1.5)	101 (2.7)
280	101 (0.99)	98.3 (0.41)	104 (1.5)	101 (2.5)

Values in parentheses are coefficients of variation.

Table 4
Day-to-day reproducibility and precision for I in spiked human urine

Concentrations given (nmol/l)	Mean recovery (n=3) (%)			Overall mean recovery (%)
	Day 1	Day 6	Day 7	
8.54	106 (9)	108 (7.7)	110 (3.3)	108 (6.3)
17.1	101 (2.3)	100 (2.0)	108 (5.4)	103 (4.6)
42.6	101 (0.99)	101 (1.6)	105 (2)	102 (2.6)
171	103 (0.6)	101 (1.2)	103 (1.5)	102 (1.3)
342	102[a]	101 (1.1)	102 (1.7)	102 (1.2)

Values in parentheses are coefficients of variation.
[a] only two values available.

3.8. Discussion and application

Pfister et al. [1] reported EIA and HPLC methods in the same publication. The HPLC method was manual liquid–liquid extraction, UV detection and was distinctly less sensitive (30 nmol/l). The present method used an automated liquid–solid extraction, fluorescence detection with a LOQ of 1.40 nmol/l.

Nine spiked plasma samples (1.40 to 1860 nmol/l) were prepared and analysed blindly by this method. For the high concentrations (>140 nmol/l), a plasma dilution was done before the final analysis. The mean recovery was 99.8%.

This method was applied to plasma samples from one subject given a single oral dose of letrozole (2.5 mg) under fasting and fed conditions. The results are depicted in Fig. 5. The C_{max} (highest observed plasma concentration) was higher (142 nmo/l) under fasting conditions than under fed conditions (92.3 nmol/l). The corresponding t_{max} (time at which C_{max} occurred) were 1 and 2.5 h, respectively.

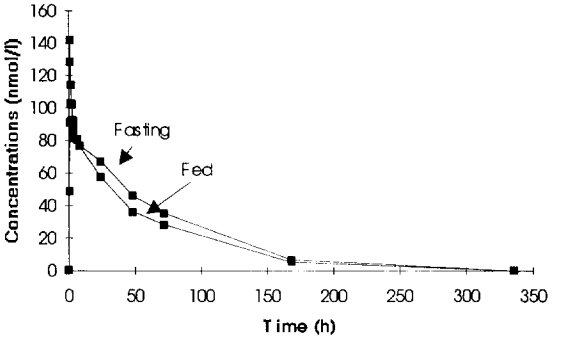


Fig. 5. Plasma concentration profile of letrozole in one subject under fasting and fed conditions.

This method was also applied to urine samples from two subjects who were given 2.5 mg of [^{14}C]letrozole orally. The letrozole urinary excretion in percent of the dose over 336 h was 2.2 and 3.9%, respectively.

The metabolite, I, was not detected as free in these urine samples. Compound I is excreted in urine mainly in a conjugated form. The described method using fluorescence detection was found to be unsuitable for determining conjugated I in urine.

During analysis of clinical samples, validation samples were extracted and chromatographed across the entire run to attest the good progress of the run.

4. Conclusions

The present HPLC method allows the determination of letrozole in plasma, and of letrozole and its free metabolite, I, in urine, with suitable reproducibility and accuracy. The LOQ for letrozole in plasma, which is two-fold higher than that of the EIA method (1.40 versus 0.7 nmol/l), should be sufficient for kinetic studies in humans with single doses of 0.5 mg and possibly less.

References

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