Journal of Chromatography, 414 (1987) 235–241
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3438

Note

Chromatographic analysis of the new antiestrogen zindoxifene and its metabolites in biological material

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(First received August 4th, 1986; revised manuscript received September 25th, 1986)

Endocrine pharmacotherapy with antiestrogens has become an established method in the management of estrogen receptor positive breast cancer [1]. Up to now, only one agent, tamoxifen citrate, has been available for this purpose. Recently, we reported on the development of a new drug, zindoxifene [5-acetoxy-2-(4-acetoxyphenyl)-1-ethyl-methylindole] (Fig. 1) for treatment of hormone-dependent malignancies [2]. This drug is presently undergoing phase I clinical trials. For the determination of blood levels of zindoxifene and its metabolites, we established an assay using high-performance liquid chromatography (HPLC) with fluorimetric detection. This method is sensitive enough to detect the drug in tissue specimens as well.

EXPERIMENTAL

Material and reagents

Zindoxifene and other 2-phenylindole derivatives necessary for comparison were synthesized in the author's laboratory [3]. As internal standard 5-hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-propylindole [3] was used. The synthesis of ^{14}C-labelled zindoxifene (7 mCi/mmol) has been described previously [4]. All chemicals were analytical grade and the water used for HPLC was purified by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

Chromatography

The system consisted of an LKB-2150 pump, an LKB-2152 LC controller, a Merck 250×4.6 mm I.D., 7-μm LiChrosorb RP-18 column (E. Merck, Darm-

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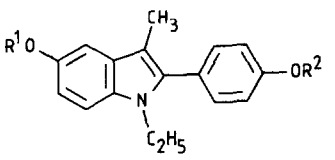

$R^1, R^2 = COCH_3$: 1a, Zindoxifene
$R^1 = COCH_3, R^2 = H$: 1b
$R^1, R^2 = H$: 1c
$R^1, R^2 = SO_3Na$: 1d

Fig. 1. Chemical structures of zindoxifene and its metabolites.

stadt, F.R.G.) at ambient temperature, a Merck–Hitachi fluorescence detector (E. Merck), with excitation at 300 nm and emission at 370 nm, or a Uvikon 720 LC UV detector (Kontron, Eching, F.R.G.) at 300 nm. Peak integration was performed with a Chromatopac C-R1A (Shimadzu). Fractions of chromatographic runs with ^{14}C-labelled compounds were counted in a Beckman LS 1800 liquid scintillation counter using Quickszint 212 (Zinsser) as scintillation cocktail. The mobile phase, methanol–water (65:35, v/v) was delivered at 1.2 ml/min.

Sample preparation

Female Sprague–Dawley rats of 200–250 g (Ivanovas, Kisslegg, F.R.G.) were administered by gavage a single dose of 10 mg of zindoxifene per kg body weight, dissolved in 1 ml of olive oil. Blood samples were obtained by heart puncture of anaesthesized animals. Before centrifugation (2000 *g*; 10 min, 4 °C), blood (1 ml)

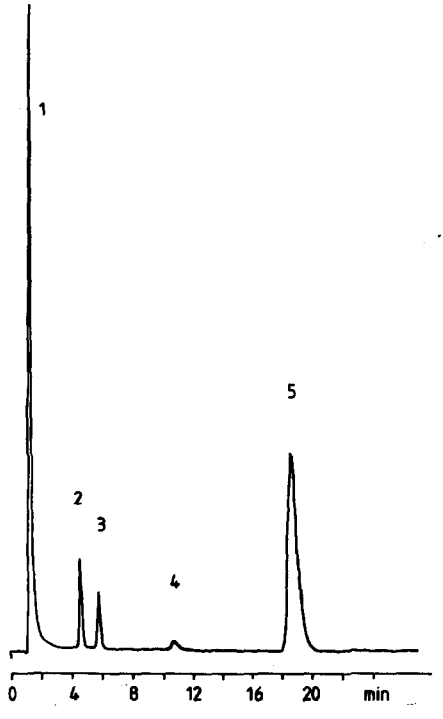

Fig. 2. Chromatogram of a standard solution of 1d (1), 1c (2), internal standard 2 (3), monoacetate 1b (4), and zindoxifene (5).

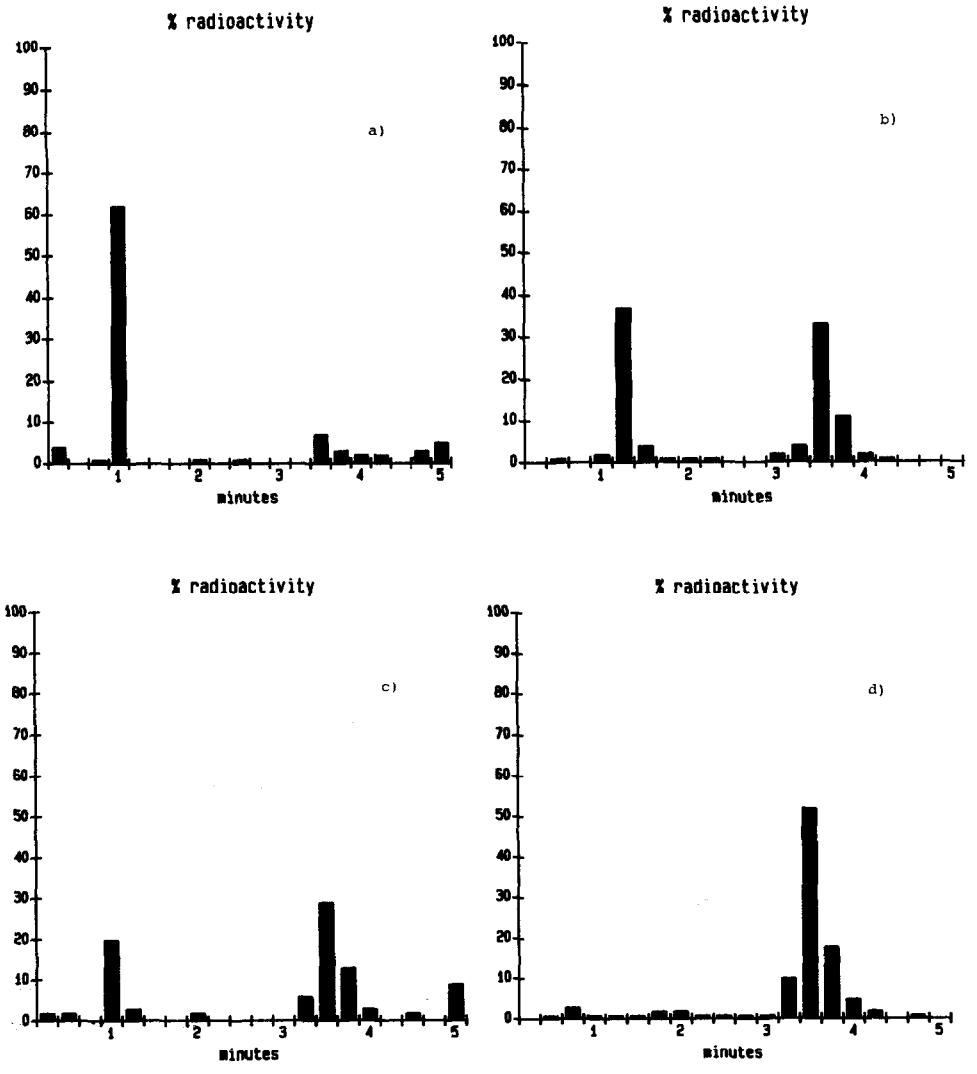

Fig. 3. Distribution of radioactivity in the HPLC eluate, 2 h after administration of 100 μCi of [^{14}C]zindoxifene per kg. Plasma samples were either untreated (a) or incubated with β-glucuronidase (b), aryl sulphatase (c) or both enzymes (d).

was incubated with 4 μg of the internal standard for 30 min with gentle shaking. Plasma was treated either with β-glucuronidase (75 μl; 4 U/ml; Sigma) or with arylsulphatase (100 μl; 20 U/ml; Sigma), or a standard mixture of both enzymes (25 μl; 12 and 60 U/ml; Merck) for 16 h at 37°C. Protein was precipitated by addition of 2 ml of acetonitrile per ml plasma and removed by centrifugation at 3500 g (10 min). For chromatographic separation and UV detection or determination of radioactivity, acetonitrile was removed in vacuo and the aqueous phase (1 ml) was extracted with ethyl acetate (6 ml) by means of an extraction column (Extrelut, Merck). After evaporation of the solvent, the residue was taken up in

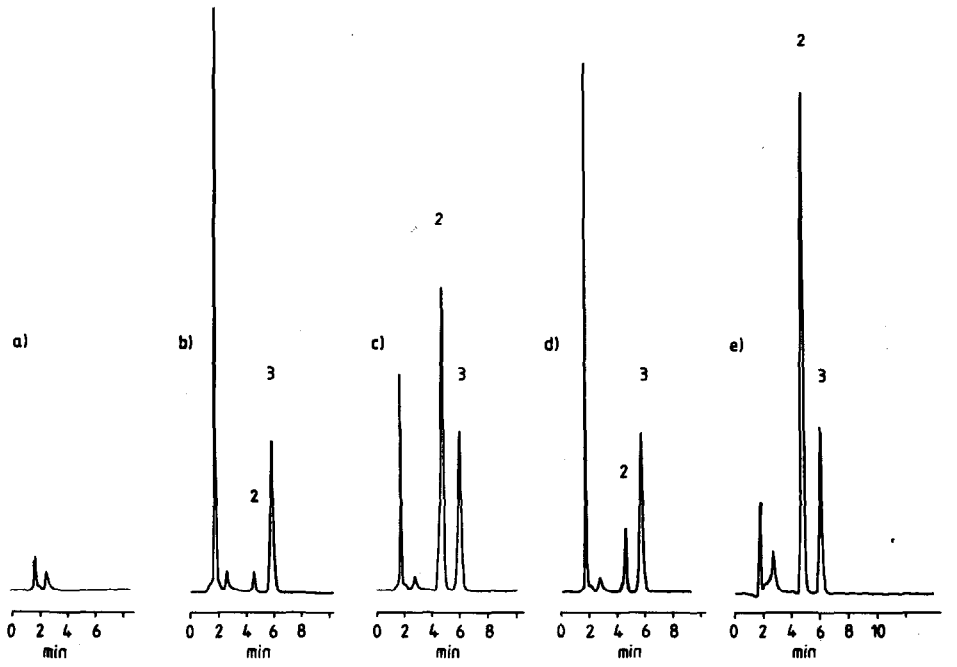

Fig. 4. Chromatograms of (a) a blank plasma sample, (b) an untreated plasma sample 30 min after administration of 10 mg of zindoxifene per kg to rats, and of plasma samples incubated with (c) β-glucuronidase, (d) arylsulphatase or (e) both enzymes. Signals were obtained by fluorescence detection at 370 nm. Peak 2 corresponds to the dihydroxy metabolite 1c, and peak 3 to the internal standard.

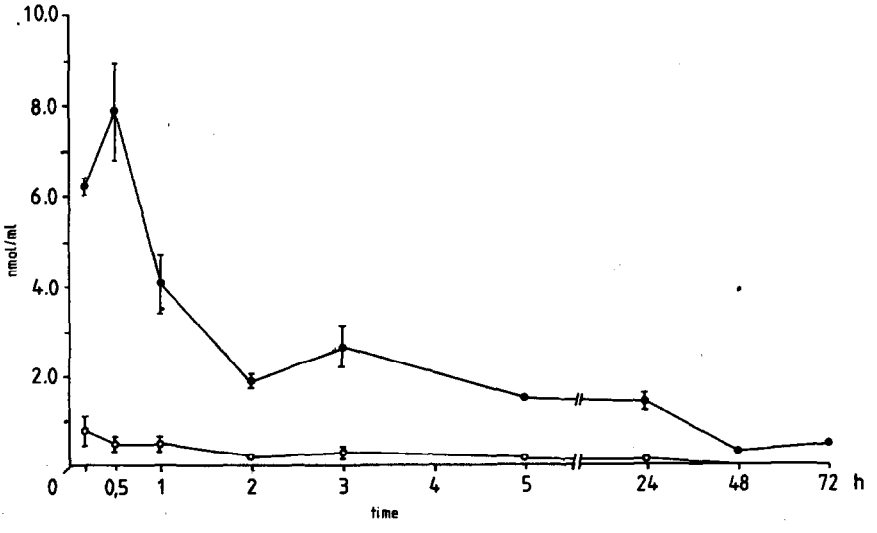

Fig. 5. Plasma concentrations of free 1c (○) and free plus conjugated 1c (●) following oral administration of zindoxifene (10 mg/kg) to rats.

TABLE I

TISSUE CONCENTRATIONS OF METABOLITES OF ZINDOXIFENE IN RATS

Administration of 10 mg/kg per os, determined as 1c after complete enzymic hydrolysis

Time★	Concentration★★ (nmol/g)			
	Liver	Kidney	Uterus	Muscle
10 min	73.2±24.8	11.9±6.1	0.92±0.2	0.3 ±0.3
30 min	49.2±10.0	8.3±1.8	1.33±0.45	0.28±0.2
1 h	40.3±11.0	6.2±1.7	0.85±0.22	0.15±0.05
2 h	14.3±1.8	3.1±0.2	0.61±0.28	0.13±0.05
3 h	20.3±8.3	3.9±1.1	0.70±0.17	0.21±0.1
5 h	22.5±6.7	3.5±0.3	0.79±0.1	<0.1
24 h	18.0±9.4	2.8±0.4	0.39±0.09	<0.1

★Time when animals were killed.

★★Mean ± S.D. of three animals; two samples were taken from each.

200 μl of methanol. Aliquots of 20 μl were used for HPLC. For fluorescence detection the aqueous acetonitrile solution was used directly.

Drug concentrations in tissues were determined after homogenization of tissue samples (1–2 g) in 0.9% saline at 4°C. After centrifugation (2000 *g*) (15 min), the supernatant was worked up in the same way as the plasma samples.

RESULTS AND DISCUSSION

Chromatographic conditions were elaborated under the assumption that the ester functions of zindoxifene are rapidly cleaved in vivo affording the free hydroxy derivative 1c (Fig. 1) that is believed to be the active metabolite. For quantitative determinations of 1c an exact amount of the internal standard 2 was added prior to separation. The close analogy of 2 to 1c makes it likely that the two compounds will behave in an identical fashion during work-up of the plasma. Baseline separation of the two compounds was achieved with a 7-μm LiChrosorb RP-18 column and methanol–water (65:35) as mobile phase. Fig. 2 presents the chromatogram of a standard solution containing zindoxifene, the monoacetate 1b, the hydroxy derivative 1c, the internal standard 2 and the disulphate 1d as one of the potential conjugates.

Chromatographic analysis of untreated plasma showed no UV absorption at retention times corresponding to zindoxifene and the monoacetate (1b) and only a minor peak at 4.4 min. When ^{14}C-labelled zindoxifene (100 μCi/kg) was administered, radioactivity was mainly found in the first fractions between 1.0 and 2.0 min due to polar metabolites (Fig. 3a). Treatment of plasma either with arylsulphatase or β-glucuronidase gave rise to a second maximum of radioactivity in the expected region (Fig. 3b and c). Application of both enzymes led to a single peak at 4.3 min (Fig. 3d). Total radioactivity (100%) of a single chromatographic run was between 300 and 700 dpm, therefore values below 5% cannot be differentiated from the background. The UV spectrum of this peak confirmed the structure of 1c [λ_{max} (ϵ): 232 (27 000); 297 (17 000)]. The limit of detection for 1c using the absorption at 300 nm is ca. 1 nmol/ml of plasma.

For clinical drug monitoring it was necessary to increase the sensitivity of this assay without using radiolabelled compounds. Therefore, we considered the use of fluorescence detection. Excitation of 1c in methanol at a wavelength of 300 nm gives rise to an intense fluorescence with emission at $\lambda_{max} = 372$ nm and an excellent quantum yield of 0.61. Quantum yields are 0.62 for the internal standard and 0.13 for zindoxifene. These values make the fluorimetric determination of the drug the method of choice. No concentration steps are required to reach a detection limit of 100 pmol/ml of plasma. Quantification of 1c in plasma samples was made from the ratio 1c/2. Since the quantum yields of these compounds differ somewhat, all values found were corrected by calibration curves. Multiple-point calibration (two to four points) was performed using peak areas covering the previously determined range of analyses. Calibration coefficients and their standard errors were calculated by the least-squares method. Linearity was checked in the particular ranges of concentration for which calibration curves were established (0.2–1.0 ng; 1.0–5.0 ng; 5.0–20.0 ng), and it was found to be very accurate for either region concerned, but not for the whole range.

All calculations and statistical analyses were performed by means of our own FORTRAN program running on an Olivetti M24 PC. The precision of analysis is 3%, accuracy is within 1.3% for the range 0.5–20 ng. The lower limit of quantitation is at least 0.2 nmol/ml 1c (signal-to-noise ratio greater than 10).

Fig. 4 shows chromatograms of blank plasma (a), untreated plasma sample (b) and samples treated either with β-glucuronidase (c) or arylsulphatase (d) or both enzymes (e). Blood was collected from rats 30 min after administration of 10 mg zindoxifene per kg body weight. The relative amounts of non-conjugated 1c calculated from these chromatograms are 4% in Fig 4b, 67% in Fig. 4c, and 14% in Fig. 4d (in Fig. 4e, 1c = 100%). Since the total amount of 1c in Fig. 4b–d is only 85%, it can be assumed that mixed conjugates (sulphate–glucuronide) are formed as well.

The HPLC assay with fluorescence detection was used to determine blood levels of 1c and conjugates over a period of 72 h after administration of a single dose of 10 mg/kg zindoxifene (Fig. 5). After a few minutes, the maximum concentration was reached. Between 2 and 24 h, the blood level remained rather stable at 1.8 nmol/ml. Obviously, the phase II metabolites of zindoxifene are not rapidly excreted.

The sensitivity of this assay allows the determination of drug concentrations in various tissues (Table I). As expected, the metabolites are accumulated in liver and kidney. The amount of 1c found in the uterine tissue was about five times higher than in the muscle. That may be due to the high binding affinity of 1c for the uterine estrogen receptor [3]. This assay will be used for therapeutic monitoring of zindoxifene metabolites in the plasma of breast cancer patients.

ACKNOWLEDGEMENTS

The authors thank Mrs. A. Schmitz-Seitz for technical asistance and the Bundesministerium für Forschung und Technologie, the Deutsche Forschungsge-

meinschaft (SFB 234) and the Fond der Chemischen Industrie for financial support.

REFERENCES

1 B. Henningsen, F. Linder and C. Steichele, Endocrine Treatment of Breast Cancer, Springer, Berlin, 1980.
2 E. von Angerer, J. Prekajac and M. Berger, Eur. J. Cancer Clin. Oncol., 21 (1985) 531.
3 E. von Angerer, J. Prekajac and J. Strohmeier, J. Med. Chem., 27 (1984) 1439.
4 J. Engel, A. Bork, E. von Angerer and H. Schönenberger, Chem. Ztg., 108 (1984) 407.