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# SENSITIVE GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF OXICONAZOLE IN PLASMA

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## SUMMARY

A specific and highly sensitive gas—liquid chromatographic method was developed for the determination of oxiconazole in rat, dog, and human plasma. The compound and its internal standard were extracted from plasma at basic pH with *n*-hexane—isoamyl alcohol (98:2, v/v), gas chromatographed on 3% SP-2250/Supelcoport (80—100 mesh) and quantified by means of an electron-capture detector.

Oxiconazole was extracted almost quantitatively from plasma in the concentration range 10—5000 ng/ml. The sensitivity limit was about 1 ng/ml, using a 1-ml specimen. The method was applied to 13-week tolerance studies in dogs and rats in order to follow oxiconazole concentrations in plasma after oral administration of the compound. The assay was sensitive enough to measure precisely the small amounts of unchanged compound in plasma after intravaginal application of labelled oxiconazole to human volunteers.

## INTRODUCTION

Oxiconazole nitrate (Ro 13-8996/001, Sgd 301-76) is being developed by Siegfried, Zofingen, Switzerland and F. Hoffmann-La Roche, Basle, Switzerland. The compound, synthesized by Mixich and Thiele [1], is a new member of a series of 1,4-imidazole derivatives with antimycotic properties [2]. Oxiconazole has, in vitro and in vivo, a broad antifungal spectrum which includes yeasts and dermatophytes [3]. Topical application forms against dermatomycoses and vaginal candidiases are now under clinical evaluation.

Chemically, oxiconazole nitrate is the *Z*-isomer of 2′,4′-dichloro-2-(im-

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idazole-1-yl) acetophenone-O-(2,4-dichlorobenzyl)-oxime nitrate (Fig. 1). Several methods for the determination of imidazole derivatives with antimycotic activity are described in the literature, including photometry [4], microbiology [4—6], thin-layer densitometry [4] and high-performance liquid chromatography [7, 8].

We describe a specific and highly sensitive gas—liquid chromatographic (GLC) method with electron-capture detection for the determination of oxiconazole in dog, rat, and human plasma. The assay was sensitive enough to measure precisely the small amounts of unchanged oxiconazole in plasma after topical application of the compound.

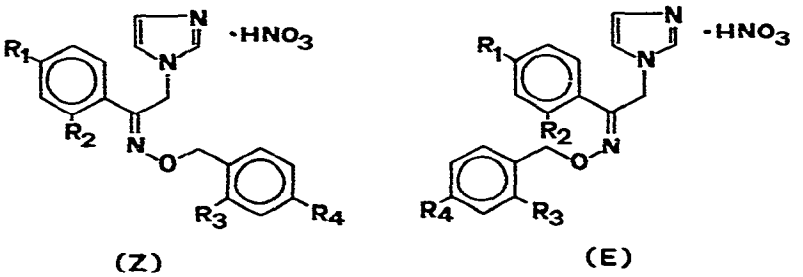


Fig. 1. Chemical structures of oxiconazole nitrate and of some analogues used in the assay.

| Compound | Isomer | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| Oxiconazole nitrate | *Z* | Cl | Cl | Cl | Cl |
| Ro 13-8997/001 | *E* | Cl | Cl | Cl | Cl |
| Ro 14-1023/001 | *Z* | Cl | Cl | H | Cl |
| Sgd 149-76 | *Z* | I | H | Cl | Cl |

## EXPERIMENTAL

### *Materials*

*n*-Hexane (for pesticide residue analysis), isoamyl alcohol (p.a.), ethyl acetate (p.a.), ethanol (p.a.), methanol (p.a.) and potassium hydroxide (p.a.) were purchased from E. Merck, Darmstadt, G.F.R. SP-2250 (3%) on Supelcoport (80—100 mesh) was used as stationary phase (Supelco, Bellefonte, PA, U.S.A.).

Oxiconazole nitrate, Ro 13-8997/001, Ro 14-1023/001 and Sgd 149-76 were supplied by Siegfried, Zofingen, Switzerland.

The priming solution was prepared by extraction of human blank plasma (5 ml) with ethyl acetate (15 ml) on a rotating shaker (15 rpm, 10 min; Heidolph). An aliquot (10 ml) of the organic phase was transferred to a clean glass bottle.

### *Plasma standards*

According to the wide concentration range of oxiconazole present in biological samples, three sets of plasma standards were prepared (see Table I). Using oxiconazole nitrate, a stock solution was prepared corresponding to 1 mg of the free base per ml in ethanol. The working solutions were obtained by dilut-

ing aliquots of the stock solution with methanol. The plasma standards were prepared by diluting 0.1 ml of the corresponding working solution with blank plasma to 10 ml.

The stock solution could be stored in a refrigerator for about four weeks. Working solutions were prepared prior to use. The plasma standards were divided into 2.5-ml aliquots and stored deep-frozen until required for analysis.

TABLE I

CONCENTRATIONS OF OXICONAZOLE IN WORKING SOLUTIONS AND PLASMA STANDARDS

| Calibration set 1 | | Calibration set 2 | | Calibration set 3 | |
|---|---|---|---|---|---|
| Working solutions (μg per 0.1 ml) | Plasma standards (ng/ml) | Working solutions (μg per 0.1 ml) | Plasma standards (ng/ml) | Working solutions (μg per 0.1 ml) | Plasma standards (ng/ml) |
| 0.025 | 2.5 | 0.4 | 40 | 10 | 1000 |
| 0.05 | 5 | 0.8 | 80 | 20 | 2000 |
| 0.1 | 10 | 1.6 | 160 | 40 | 4000 |
| 0.2 | 20 | 3.2 | 320 | 80 | 8000 |
| 0.4 | 40 | 6.4 | 640 | | |
| | | 12.8 | 1280 | | |

*Extraction procedure*

Depending on the expected plasma concentration of oxiconazole, an appropriate quantity of internal standard, potassium hydroxide solution (1%) and sample* was added to a ground-glass stoppered centrifuge tube (see Table II). After the addition of 5 ml of *n*-hexane—isoamyl alcohol (98:2, v/v) the sample was extracted by shaking for 15 min on a rotating shaker (15 rpm; Heidolph) and centrifuged for 10 min (10°C, 1700 *g*; Hettich). An aliquot of the organic layer (4 ml) was transferred to a tapered evaporation tube. The aqueous layer remaining was extracted again as described above and a second fraction (5 ml) of the upper phase was retained.

The combined organic phases were concentrated at 40°C by means of a gentle stream of pure nitrogen until only the isoamyl alcohol remained. After the addition of either 1 ml of ethyl acetate or 1 ml of priming solution (see Table II) 2 μl of the resulting solution were injected for GLC analysis.

*Gas—liquid chromatography*

Depending on the expected plasma concentration of oxiconazole two different gas chromatographic systems were used. Samples containing less than 40 ng oxiconazole per ml were analysed manually on a Packard Model 429 gas chromatograph with an electron capture detector (ECD) ($^{63}$Ni, 10 mCi) coupled to a Model SP 4100 (Spectra-Physics) computing integrator. All other samples were analysed on a Hewlett-Packard GLC system, consisting of a gas

*Either: Plasma standard (calibration), control plasma (quality control), biological sample (analyses), drug-free plasma (plasma blank) or bidistilled water (reagent blank).

TABLE II

QUANTITIES OF PLASMA SAMPLE AND REAGENTS TO BE USED IN THE ASSAY

| | Expected concentration range of oxiconazole (ng/ml) | | |
|---|---|---|---|
| | 2.5—40 | 40—1280 | 1000—8000 |
| Volume of sample (ml) | 1 | 0.5—1 | 0.1 |
| Amount of internal standard (ng) (dissolved in 20 μl of methanol) | 20 (Ro 14-1023) or 75 (Sgd 149-76) | 320 (Ro 14-1023) or 1000 (Sgd 149-76) | 1000 (Sgd 149-76) |
| Volume of 1% KOH (ml) | 0.5 | 0.25—0.5 | 0.05 |
| Type of reconstitution medium | Ethyl acetate | Ethyl acetate | Priming solution |

chromatograph with a 15-mCi $^{63}$Ni ECD (HP 5700 A), an automatic sampler (HP 7671 A) and a data system for integration and sampler control (HP 3352 B).

The column, a coiled glass tube 1 m × 3 mm I.D., was treated for 10 min with a solution of 2% dimethylchlorosilane in toluene. After having been washed with toluene, methanol and acetone the deactivated column was dried at 100°C and packed with 3% SP-2250 on 80—100 mesh Supelcoport.

The packed column was conditioned for about 10 min at 60°C with a nitrogen flow-rate of 40 ml/min, then "baked" for 3 h at 340°C under "no flow" conditions [9] and then, finally, held at 310°C for 2 days with a nitrogen flow-rate of 40 ml/min.

The instrumental parameters used in this assay were: carrier gases, nitrogen (Packard 429), argon—methane (9:1) (HP 5700 A); carrier gas flow-rates, 40 ml/min; column oven temperature, 270—290°C; injector temperature, 300°C; detector temperature, 350°C.

To maintain the chromatographic system in a deactivated state and, therefore, achieve reproducible chromatography, it was necessary to change the septum, the liner, and the silanized glass-wool at the top of the column every three days.

*Calibration and calculation*

Only biological samples containing oxiconazole levels within the ranges 2.5—40, 40—1280 or 1000—8000 ng/ml were analysed in the same run. Along with these samples, the corresponding calibration set 1, 2 or 3 consisting of 4—6 plasma standards with appropriate oxiconazole concentrations was carried through the procedure (see Table I). A calibration curve was obtained by a least-squares regression of the peak-height ratios of oxiconazole/internal standard versus oxiconazole concentrations.

This internal standard curve was then used to interpolate unknown concentrations of oxiconazole in the biological samples from the ratio of measured peak heights of the compound and the internal standard.

## RESULTS AND DISCUSSION

### *Specificity and choice of internal standard*

The method was developed for the determination of oxiconazole in plasma of three different species; namely, rats, dogs and human volunteers.

Several blank plasma samples from different rats, dogs and human subjects were analysed as described before. In all cases clean plasma extracts were obtained, indicating specificity of the assay with respect to other endogenous components present in plasma (see Fig. 3).

A different metabolic pattern was observed in biological samples depending on the type of species investigated and the quantity of oxiconazole administered. In no case was interference of co-extracted metabolites with the parent compound observed.

Depending on type and amount of co-extracted metabolites, two internal standards with different retention characteristics were used (see Figs. 1 and 2). Whenever possible, Ro 14-1023 was preferred because a short chromatography time (7 min) was obtained even at a column temperature of 270°C (see Figs. 4 and 5). The retention time of Sgd 149-76* was longer than that of Ro 14-1023. The former compound was only used as an internal standard when co-extracted metabolites interfered with Ro 14-1023 (see Fig. 6). An acceptable run time (9 min) was obtained by chromatographing the plasma extracts

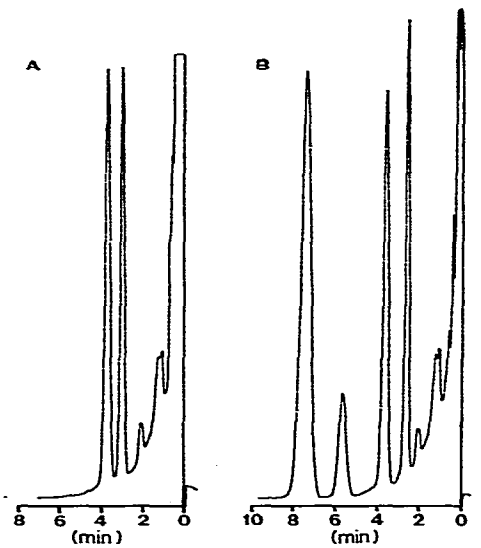


Fig. 2. Chromatograms of spiked blank plasma samples containing: (A) 50 ng of oxiconazole nitrate and 50 ng of Ro 13-8997/001 per ml of plasma; (B) 50 ng of oxiconazole nitrate, 50 ng of Ro 14-1023/001 and 125 ng of Sgd 149-76 per ml of plasma. Oven temperature, 280°C; range, 4; attenuation, 10. Retention times (min): Ro 14-1023, 2.6; Ro 13-8997, 3.0; oxiconazole, 3.7; impurity, 5.7; Sgd 149-76, 7.5.

*Sgd 149-76 contained an impurity which was well separated from the other peaks and did not affect the reproducibility of the assay (see Fig. 2B).

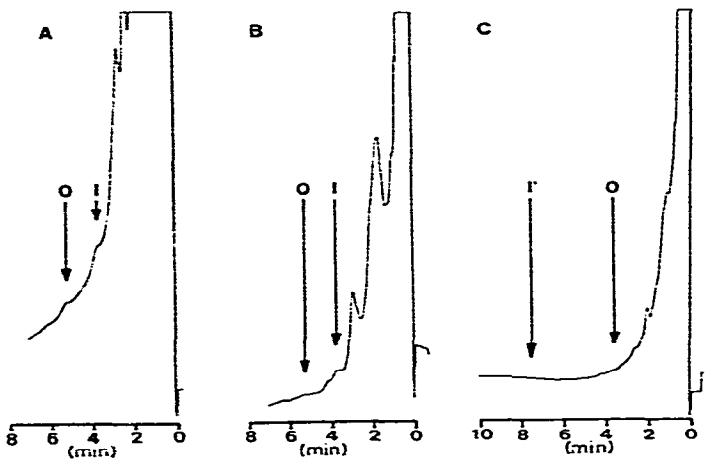


Fig. 3. Chromatograms of blank plasma samples from rat (A), man (B) and dog (C). Oven temperature, 270°C (A and B), 280°C (C); range, 2; attenuation, 10. The arrows indicate the retention times of oxiconazole and the internal standards. O = oxiconazole, I = Ro 14-1023, I' = Sgd 149-76.

at a higher column temperature (280—290°C). Further increase of the temperature (> 290°C) led to decreased deactivation of the chromatographic system in a short time which, in turn, led to deterioration of the peak shape and a decrease of the linear range.

Ro 13-8997, a stereoisomeric form of oxiconazole was tried as an internal standard. Separation from the oxiconazole peak only occurred on well-deactivated columns with high efficiencies (see Fig. 2A). For this reason, further trials with Ro 13-8997 were not continued.

*Limit of detection*

The limit of detection, defined by a signal-to-noise level of approx. 3:1, was 1 ng of oxiconazole per ml of plasma using a 1-ml specimen. This minimum detectable concentration was equivalent to an absolute amount of 2 pg of oxiconazole per injection, indicating an excellent ECD response of the compound.

The intra-assay precision for the detection limit was determined by analysing four different spiked plasma samples on the same day containing 1 ng/ml oxiconazole. A relative standard deviation (C.V.) of ± 7.3% was obtained.

*Linearity*

As already mentioned, plasma levels of oxiconazole varied over a wide range (2—2500 ng/ml) when the compound was administered orally to rats and dogs in 13-week tolerance studies. Three linear subranges with different extraction and calibration procedures (see Tables I and II) were established for the following reasons:

(1) Limitation of the absolute amount of oxiconazole injected on to the column in order to prevent "memory effects" (reversible and irreversible adsorption of oxiconazole to the glass wool) and saturation of the ECD.

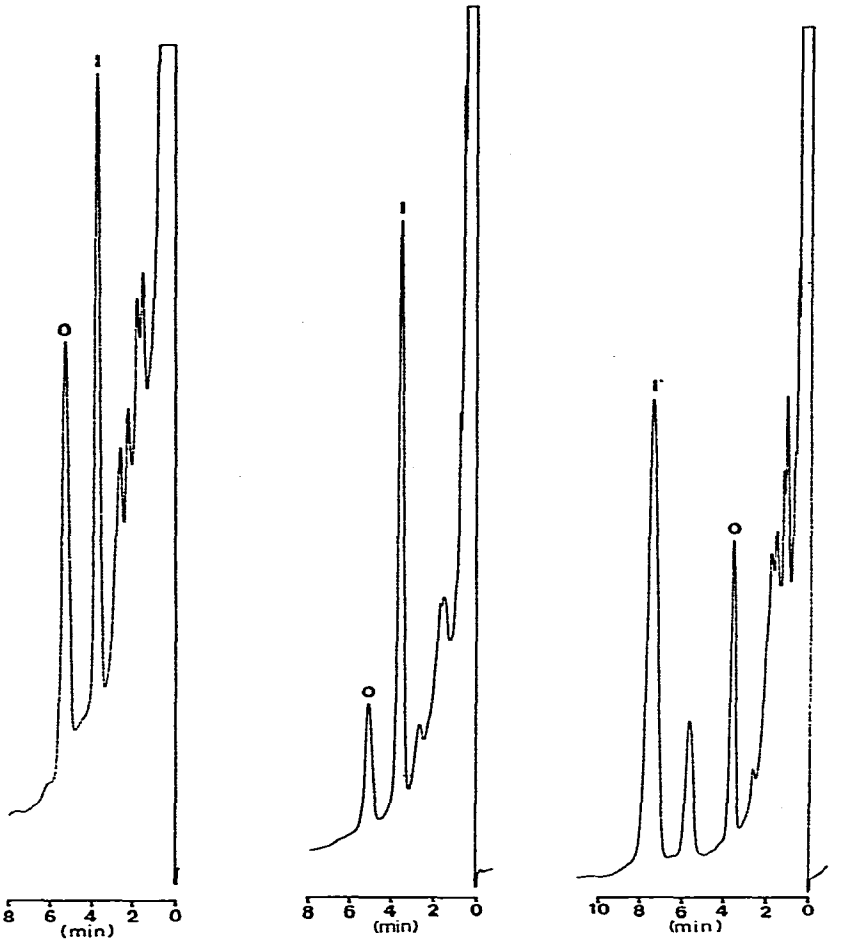


Fig. 4. Chromatogram of a rat plasma sample collected four weeks after daily oral administration of 35 mg/kg oxiconazole nitrate; oxiconazole concentration = 51 ng/ml. O = oxiconazole, I = Ro 14-1023 (internal standard); oven temperature, 270°C; range, 2; attenuation, 10.

Fig. 5. Chromatogram of a female human plasma sample collected 24 h after a single intravaginal application of 150 mg of $^{14}$C-labelled oxiconazole nitrate (107 $\mu$Ci); concentration of unchanged oxiconazole = 32 ng/ml. O = oxiconazole, I = Ro 14-1023 (internal standard); oven temperature, 270°C; range, 2; attenuation, 10.

Fig. 6. Chromatogram of a dog plasma sample collected three days after daily administration of 150 mg/kg oxiconazole nitrate; oxiconazole concentration = 52 ng/ml. O = oxiconazole, I' = Sgd 149-76 (internal standard); oven temperature, 280°C; range, 2; attenuation, 10.

(2) Adjustment of the amount of internal standard added to the sample to achieve reasonable values for oxiconazole/internal standard peak height ratios.

(3) Maintenance of linearity over the complete concentration range.

In each subrange an acceptable linear correlation between the peak height ratios of oxiconazole/internal standard versus concentrations of oxiconazole was observed. Table III indicates that the coefficient of determination ($r^2$) showed a good day-to-day reproducibility. The intercepts of the calibration curves did not differ significantly from zero.

Oxiconazole was very sensitive to adsorption sites in the chromatographic system: direct chromatography of methanolic oxiconazole solutions resulted, after a few days, in tailing peaks and non-linear calibration curves. Only in the presence of column deactivating plasma components (lipids, lecithin, etc.) were gaussian peak shapes, and thus linearity between detector response and oxiconazole concentration, obtained ("priming effect").

Even the amount of priming agents co-extracted together with oxiconazole from plasma was critical in the assay: when small plasma volumes (0.1 ml) were analysed, a non-linear standard curve was sometimes obtained when the plasma extract was reconstituted in ethyl acetate. Priming of the chromatographic system before and between the analysis by repeated injections of either lecithin [10], phospholipids [11] or plasma extracts [12] did not overcome the problem completely. Therefore, in the case of small plasma volumes, sufficient amounts of deactivating plasma components were added to the sample *after* extraction by use of a priming solution as reconstitution medium.

TABLE III

REPRODUCIBILITY OF THE COEFFICIENT OF DETERMINATION ($r^2$) ESTABLISHED ON FIVE DIFFERENT DAYS COVERING A PERIOD OF ABOUT SIX WEEKS

| Day | Concentration range of oxiconazole | | |
|---|---|---|---|
| | 2.5—40 ng/ml | 40—1280 ng/ml | 1—8 μg/ml |
| 1 | 0.9998 | 0.9997 | 0.9995 |
| 2 | 0.9998 | 0.9994 | 0.9998 |
| 3 | 0.9996 | 0.9999 | 1.0000 |
| 4 | 0.9987 | 0.9998 | 0.9999 |
| 5 | 0.9999 | 0.9998 | 0.9999 |
| Mean ± S.D. | 0.9996 ± 0.00049 | 0.9997 ± 0.00019 | 0.9998 ± 0.00019 |

*Recovery*

The recovery (extraction yield) was determined from the difference between the peak height ratio when oxiconazole was added to the plasma (the internal standard being added to the final extract) and the peak height ratio when both were added to the final extract of blank plasma.

Table IV indicates that the compound is extracted nearly quantitatively from human and dog plasma in the concentration range investigated. The extraction yield for high concentrations of oxiconazole (> 1000 ng/ml) is enhanced because in this case a 0.1-ml instead of a 1-ml plasma sample was extracted according to the extraction procedure described above (see Table II).

TABLE IV

EXTRACTION YIELD OF OXICONAZOLE FROM DOG AND HUMAN PLASMA

| Concentration (ng/ml) | Human plasma ($n$ = 3) | | Dog plasma (n = 3) | |
|---|---|---|---|---|
| | Recovery (%) | C.V. (%) | Recovery (%) | C.V. (%) |
| 5000 | 96.3 | 1.7 | 97.5 | 1.0 |
| 250 | 89.5 | 2.1 | 90.5 | 1.0 |
| 50 | 90.3 | 4.4 | 90.9 | 2.1 |
| 10 | 92.7 | 3.1 | 92.2 | 1.7 |

*Stability of oxiconazole in human plasma*

Oxiconazole was added to blank plasma at three different concentrations (250, 50 and 10 ng/ml) and stored at different temperatures for different time intervals (five months at −20°C, one day at +20°C). A set of five freshly prepared control samples was analysed together with five stored samples of the same concentration.

Table V illustrates that in no case was a significant difference detectable between the results of stored and control samples.

TABLE V

STABILITY OF OXICONAZOLE IN HUMAN PLASMA ($n$ = 5)

| Sample | Conc. (ng/ml) | C.V. (%) | Difference from control (%) |
|---|---|---|---|
| Control | 250 | 1.4 | |
| 1 day, 20°C | 248 | 0.5 | −0.8 |
| 5 months, −20°C | 248 | 2.4 | −0.8 |
| Control | 50 | 1.9 | |
| 1 day, 20°C | 50.2 | 3.9 | +0.4 |
| 5 months, −20°C | 50.3 | 2.8 | +0.6 |
| Control | 10 | 3.5 | |
| 1 day, 20°C | 9.74 | 1.4 | −2.6 |
| 5 months, −20°C | 9.70 | 5.5 | −3.0 |

*Reproducibility*

The reproducibility of the internal standard method was evaluated over a concentration range of 10—5000 ng of oxiconazole per ml of plasma.

The intra-assay reproducibility was obtained by analysing five specimens from each concentration on one day. The inter-assay reproducibility was determined by analysing one specimen from each concentration over five days within a period of about six weeks.

The data presented in Table VI indicate that the precision (C.V. of replicate analyses) and accuracy (difference between found and expected concentration) were acceptable over the concentration range investigated.

TABLE VI

REPRODUCIBILITY ($n$ = 5)

| Conc. added (ng/ml) | Conc. found (ng/ml) | C.V. (%) | Difference between found and added conc. (%) |
|---|---|---|---|
| *Inter-assay reproducibility* | | | |
| 5000 | 4897 | 1.4 | −2.1 |
| 250 | 242.6 | 1.2 | −3.0 |
| 50 | 50.7 | 6.8 | +1.4 |
| 10 | 10.1 | 6.7 | +1.0 |
| *Intra-assay reproducibility* | | | |
| 5000 | 4829 | 2.0 | −3.4 |
| 250 | 246.8 | 4.3 | −1.3 |
| 50 | 50.8 | 3.7 | +1.6 |
| 10 | 9.6 | 5.5 | −4.0 |

*Application of the method to biological samples*

The method described was used for the analyses of dog and rat plasma samples in 13-week tolerance studies and for the determination of the parent compound in plasma after intravaginal application of labelled oxiconazole to female human volunteers. Figs. 4—6 show typical chromatograms from these studies, demonstrating the validity of the new assay.

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