Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 809 (2004) 105–110
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

# Liquid chromatography determination of the anti-androgen vinclozolin and its metabolites in rat serum

Adolfo Sierra-Santoyo [a,b], Hugh A. Barton [a], Michael F. Hughes [a,*]

[a] *Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, MD B143-01, Research Triangle Park, NC 27711, USA*
[b] *Toxicology Section, CINVESTAV-IPN, Mexico City, Mexico*

Received 22 January 2004; received in revised form 24 May 2004; accepted 8 June 2004

Available online 2 July 2004

**Abstract**

The objective of this study was to develop a chromatographic method for the analysis of the anti-androgen vinclozolin (V) and its metabolites 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1), 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) and 3,5-dichloroaniline (M3) in rat serum. V, M1–M3 were resolved using an HPLC gradient program with a mobile phase consisting of 60–75% methanol:acetonitrile (70:30) and 0.05 M monobasic sodium phosphate buffer pH 3.3 at 1 ml/min, a C18 column, and monitored at 212 nm. Incubates of 0.01 M monobasic potassium phosphate buffer (PB) pH 7.4 and rat serum were spiked with V and its metabolites and processed by diluting samples (1:4) with 0.1 M PB pH 3.3, to limit methodological hydrolysis of analytes, followed by addition of acetonitrile. Recoveries of V, M1 and M2 ranged from 85 to 105%, whereas recovery of M3 was <25%. V was hydrolyzed to M1 and M2 after incubation in PB pH 7.4 and rat serum, with M1 the predominant metabolite. This method was successfully applied in the analysis of V and its metabolites in the serum of a male rat after oral administration of V (100 mg/kg).


*Keyword:* Vinclozolin

## 1. Introduction

Recent concern for the possible effects of endocrine-disrupting chemicals on humans and wildlife [1] has resulted in considerable interest in environmental contaminants, such as several pesticides, that effect aspects of reproduction and early development. Vinclozolin (V), a pesticide used to treat fungal infections in plant-derived foodstuffs, plants and grasses, causes abnormal male sexual development in rats [2–5]. As a result of its widespread use, the analysis of V on food samples has been incorporated into the US Food and Drug Administration Pesticide Residue Monitoring Program [6].

V can undergo chemical hydrolysis, photolysis or metabolism by mammals and bacterial systems. Szeto et al. [7] isolated three hydrolysis products from aqueous buffers which were identified as 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1), 3′5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) and 3,5-dichloroaniline (M3) (Fig. 1). They proposed that the opening of the 2,4-oxazolidine ring of V occurs by hydrolysis to yield either M1 or M2. The hydrolysis of V to M1 is pH dependent and reversible. The conversion of V to M1 is favored at basic pH, while the reaction of M1 to V is preferred at acidic pH [7,8]. The hydrolysis of V to M2 involves the loss of $CO_2$. The breakdown of an amide bond in M1 and M2 forms M3. Photolysis of V in aqueous solutions also leads to the opening of the 2,4-oxazolidinedione ring producing 3,5-dichlorophenyl isocyanate and M3 [9].

While specific metabolic pathways have been proposed for V [10], information on the in vivo disposition of V is limited, particularly in the target organs of mammalian species. In serum of rats administered repeated oral doses of V, M1 was the major metabolite detected. Only trace levels of V and M2 were detected in these animals [3,11]. In other species, such as fathead minnows,

* Corresponding author. Fax: +1 919 541 4284.
*E-mail address:* hughes.michaelf@epa.gov (M.F. Hughes).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.06.002

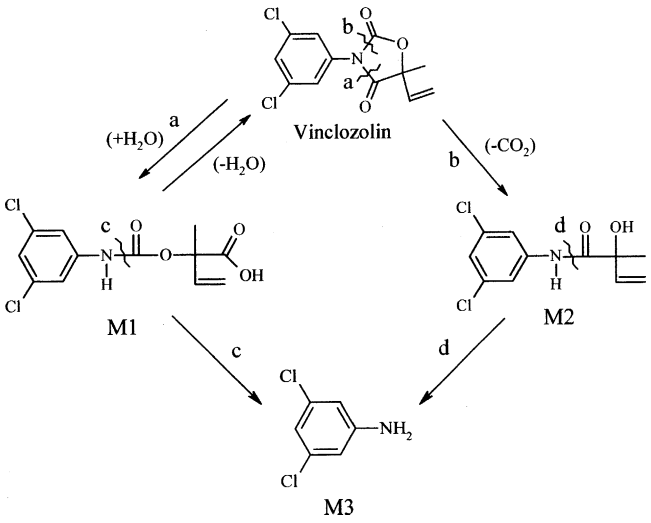


Fig. 1. Conversion of vinclozolin (V) by chemical hydrolysis. Hydrolysis of V to M1 requires addition of water (a), while M2 formation involves the loss of $CO_2$ (b). M3 generation is due to hydrolysis of the ester bond of M1 and M2 (c and d).

biotransformation of V appears to be different from that observed in rats. V was the main chemical detected in minnow tissues and females accumulated significantly more V than males after 21 days of exposure [12]. Isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide have been observed as metabolites of V in *Cunninghamella elegans* [13] and hens [14].

The two hydrolysis products of V, M1 and M2, are competitive antagonists of the androgen receptor [11,15]. The antagonism by M1 and M2 of this receptor can ultimately decrease androgen-dependent gene expression [16], which in developing, pubertal and adult male rats may result in adverse effects. Administration of V during the critical period of sexual differentiation results in sexual abnormalities expressed later in the adult male rat [2–5]. Pregnant rats exposed to V at specific gestational stages had male offspring with a significant degree of morphological feminization and demasculinization [2,4,5].

The analysis of V and its metabolites M1 and M2 in biological samples, such as serum, has received limited attention. This is due in part to analytical difficulties such as instability of V in aqueous media [7]. The purpose of this study was to optimize a method for the analysis of V and these metabolites by HPLC in rat serum.

## 2. Experimental

### 2.1. Reagents and standards

Vinclozolin (96% purity, lot 80818) was purchased from Crescent Chemical Co. (Augsburg, Germany). M1 and M2 were gifts from Elizabeth Makynen (US EPA, Duluth, MN, USA). M3 was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade water, acetonitrile and methanol were purchased from Burdick and Jackson, Inc. (Muskegon, MI, USA). All other chemicals used were of reagent grade and the highest purity available.

A stock standard solution (1 mg/ml) of V (3.5 mM) was prepared in acetonitrile, while those of M1 (3.3 mM), M2 (3.8 mM) and M3 (6.2 mM) were prepared in methanol. These solutions were stored in the dark at 4 °C.

### 2.2. Liquid chromatography

V and its metabolites were analyzed by injecting samples into a liquid chromatograph equipped with a binary pump and a UV–vis diode array detector (HP 1100, Agilent Technologies, Palo Alto, CA, USA). The detector wavelength was set at 212 nm and the reference wavelength was 550 nm. A Nucleosil (Deerfield, IL, USA) 100-5 $C_{18}$ AB column (5 μm particle size; 4.6 × 250 mm i.d.) and a Perisorb (Upchurch Scientific, Oak Harbor, WA, USA) RP-18 guard column (30–50 μm particle size; 20 × 1.5 mm i.d.) were used. System solvents consisted of methanol:acetonitrile (70:30) (A) and 0.05 M monobasic sodium phosphate pH 3.3 (B). The pH of solvent B was adjusted by adding 1 M HCl. The solvents were filtered and vacuum degassed before use. The initial solvent conditions were set at 60% A:40% B flowing at a rate of 1 ml/min at room temperature. After sample injection (20 μl), there was a 20 min linear gradient change to 70% A:30% B, followed by a 5 min linear gradient change to 75% A:25% B. Initial conditions were then reestablished by a step gradient and the column was equilibrated for 5 min. The identity of V and its metabolites was confirmed by retention time ($t_{ret}$) and UV–vis spectra of standards.

### 2.3. Recovery of V and its metabolites

V and its metabolites were recovered from rat serum and 0.01 M monobasic potassium phosphate (PB) pH 7.4 by acidifying 100 μl aliquots of samples to pH 5.6 by adding 400 μl 0.1 M PB pH 3.3. In preparation of PB, the pH was adjusted by adding either 1 M HCl or 1 M NaOH. In a preliminary study, the pH of the 0.01 M PB pH 7.4 and serum, that was acidified with 0.1 M PB pH 3.3, was determined using an Accumet pH meter (model 805 MP) with an accuTupH electrode (Fisher Scientific, Pittsburgh, PA, USA). After acidification of the samples, 5 ml of acetonitrile was then added. The solution was vortexed for 1 min and centrifuged (1650 × *g*) for 10 min at 4 °C. The acetonitrile was used to precipitate proteins in the serum. The supernatants were dried using anhydrous sodium sulfate and evaporated under a stream of $N_2$ at room temperature. Residues were dissolved in 200 μl methanol and stored at 4 °C until analyzed by HPLC.

Recovery was evaluated in samples of PB pH 7.4 and rat serum spiked with 35 μM V, 33 μM M1, 38 μM M2 and 62 μM M3. These samples were processed for analysis as described above. Precision of the assay was evaluated by calculating the relative standard deviation (coefficient of

variation) and accuracy was evaluated by determining the relative error (absolute error divided by the actual concentration).

The limit of detection was determined by injecting a very low concentration of standard, that had been spiked into serum, and processed as described above (seven replicates for each standard). The standard deviation of these replicates was determined and multiplied by 3.29, which yielded the limit of detection [17]. Limits of quantification were defined as the lowest concentration of the calibration curve which could be measured with a precision and accuracy below 30% [18].

### 2.4. Stability studies

V (35 μM) was incubated in 0.01 M PB pH 7.4 or rat serum in the dark in a shaking water bath at 37 °C for 24 h. In a direct analysis assay, 20 μl aliquots of PB pH 7.4 incubate were injected directly into the HPLC at selected time points for determination of V and its metabolites. Stability assays of M1 and M2 by direct injection were also carried out in PB pH 7.4, using similar experimental conditions to those of V. In assays where samples were acidified and acetonitrile was added, aliquots of 100 μl of PB pH 7.4 or rat serum incubate were collected at different times and processed for analysis. The experiments of direct analysis of samples were carried out in duplicate while assays with sample acidification and addition of acetonitrile were carried out in triplicate.

### 2.5. In vivo study

A 70-day-old male Long Evans rat (Charles River, Raleigh, NC, USA) was administered an oral dose of V (100 mg/kg) in corn oil. Four h later, the animal was sacrificed by cardiac puncture under $CO_2$-induced anesthesia. Serum was obtained from the blood and processed and analyzed for V and metabolites as described in Section 2.3.

## 3. Results and discussion

### 3.1. Chromatography

With the chromatographic conditions established in this study, V and three metabolites were resolved within 19 min. The absolute $t_{ret}$ were 10.3, 12.8, 15.4 and 18.1 min for M3, M1, M2 and V, respectively (Fig. 2). The $t_{ret}$ for V and each metabolite were constant for either standards in acetonitrile, PB, or spiked serum from non-treated rats (Fig. 2).

The quantification of peaks was carried out by an external standard method, which included using measurements of peak areas with a six-point calibration curve. Calibration graphics were made from spiked rat serum samples that were processed for analysis as described in Section 2.3. Linear relationships ($r^2 > 0.9946$) were observed in a range of 5–150 ng for all analytes. Equations obtained by least-squares linear regression of analyte mass versus peak area were: V, $y = (3.494 \pm 0.127)x + (16.570 \pm 9.827)$; M1, $y = (5.189 \pm 0.082)x + (3.484 \pm 6.339)$; M2, $y = (6.131 \pm 0.036)x + (2.482 \pm 2.778)$; M3, $y = (5.288 \pm 0.053)x + (4.078 \pm 4.053)$. The limits of detection for V, M1, M2 and M3 were 0.745, 0.766, 1.505 and 0.564 μM, respectively, while limits of quantification were 2.240, 1.430, 1.664 and 1.961 μM, respectively. Compared with existing methods [3,4,7], these conditions may allow the identification and quantification of V, M1, M2, and M3 and other possible metabolites from biological and environmental samples.

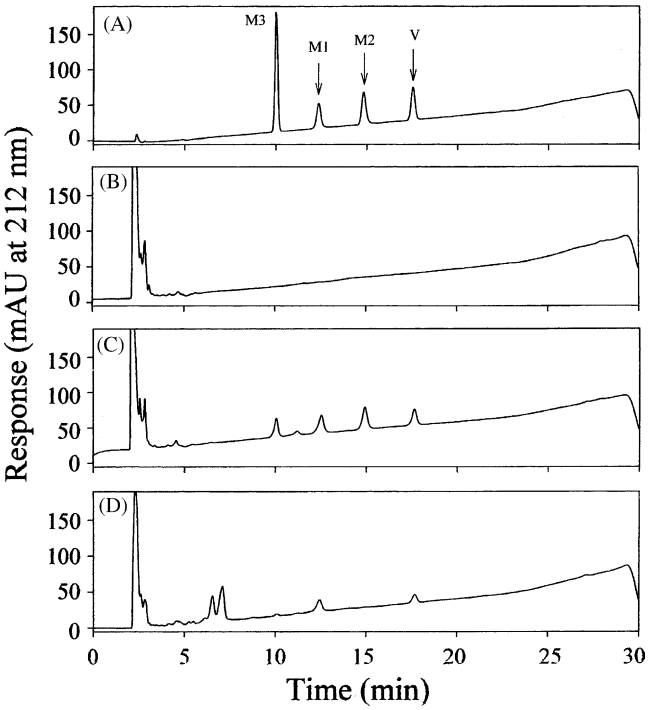


Fig. 2. HPLC chromatograms of: (A) standard solution of V (35 μM), M1 (33 μM), M2 (38 μM) and M3 (62 μM) in acetonitrile; (B) non-treated rat serum; (C) non-treated rat serum spiked with V (35 μM), M1 (33 μM), M2 (38 μM) and M3 (62 μM); (D) serum from a rat administered 100 mg/kg V by oral gavage. Serum samples were acidified to pH 5.6 by addition of 0.1 M monobasic potassium phosphate, pH 3.3 and 5 ml of acetonitrile was added. The solution was taken to dryness under a stream of $N_2$, and the residue was reconstituted in 200 μl of methanol.

### 3.2. Stability and recovery of V and metabolites

The analysis of V and its metabolites from biological and environmental samples have previously involved adjusting the sample to a low pH and extraction with an organic solvent [12,16]. The pH of the samples were adjusted to improve the extraction of M1, which has an ionizable carboxylic acid moiety. In initial experiments (data not shown), high levels of M3 were observed after extraction of V, M1 and M2 from incubations in PB pH 7.4 or rat serum after lowering the sample pH < 2 and adding acetonitrile at pH 2.5.

Direct HPLC injection of aliquots of PB pH 7.4 incubate (37 °C) containing V showed that V hydrolyzed to yield M1

and M2 (Fig. 3). However, M3 was not detected as a product up to 24 h of incubation under these conditions. The relative concentration of V decreased fairly rapidly with time. At 8 h of incubation only about 10% remained as parent compound. A half-life of 2.5 h was calculated for V under this experimental condition, which is in agreement to that reported by Szeto et al. [7] who incubated V in PB pH 7.5 at 35 °C ($t_{1/2} = 2.8$ h). The concentrations of M1 and M2 increased steadily in different proportions until 8 h. M1 was the major metabolite detected and represented approximately 82% of the total at 24 h with M2 about 17% and the remainder V (Fig. 3).

Direct HPLC injection of PB pH 7.4 (37 °C) incubate containing M1 and M2 showed these metabolites were very stable up to 72 h. M1 and M2 did not hydrolyze or convert to other metabolites under these conditions (data not shown). These results clearly showed that M1 and M2 were stable in a buffer with a pH similar to that of serum. Overall, these results suggest that M3 was formed from acid-catalyzed hydrolysis of V, M1 or M2 in our initial procedure with the sample pH was adjusted to <2. However, M3 has been detected after photolysis of V [9] and in long-term incubations of V at alkaline pH [7].

To efficiently recover M1, it was necessary to lower the pH of the incubate before addition of acetonitrile. The incubate pH was adjusted to 5.6 by the addition of PB pH 3.3 to the samples (dilution 1:4) before addition of acetonitrile. Less than 10% of V was hydrolyzed by this method. Recoveries for V, M1 and M2 from PB and rat serum ranged from 84 to 105% (Table 1). The precision and accuracy of the method ranged from 3 to 13% and 6 to 16% for V, M1 and M2, respectively (Table 1). Other solvents with different polarity were also evaluated. The results with methanol and ethyl acetate were comparable to those of acetonitrile (data not shown). However, peaks that did not correspond to those of V and its metabolites were formed with methanol and ethyl acetate (data not shown). A low recovery (<33%) for M3 with acetonitrile, ethyl acetate and methanol was observed.

Table 1
Recovery, precision and accuracy of phosphate buffer (PB) and rat serum assay for V and metabolites

| Chemical | Media | *N* | Recovery | Precision | Accuracy |
|---|---|---|---|---|---|
| V | PB | 5 | 84.4 ± 9.3 | 11.0 | 15.6 |
| | Serum | 6 | 86.3 ± 2.3 | 2.7 | 12.4 |
| M1 | PB | 5 | 102.6 ± 10.1 | 9.9 | 9.9 |
| | Serum | 5 | 104.7 ± 12.9 | 12.9 | 13.8 |
| M2 | PB | 5 | 94.9 ± 5.1 | 5.4 | 5.6 |
| | Serum | 5 | 95.0 ± 8.5 | 9.0 | 7.6 |
| M3 | PB | 5 | 4.7 ± 2.0 | 43.5 | 93.8 |
| | Serum | 5 | 24.6 ± 3.5 | 14.1 | 74.3 |

Aliquots (100 μl) of chemical (10 μg/ml) spiked phosphate buffer pH 7.4 and rat serum were acidified to pH 5.6 by the addition of 400 μl of phosphate buffer pH 3.3 followed by addition of 5 ml of acetonitrile. The solution was processed for HPLC analysis as described in Section 2.3. Recovery represents (mean ± S.D.) %, of the mass of chemical detected in the spiked samples. The precision represents the coefficient of variation of the assay. The accuracy represents the % relative error of the assay.

V was incubated in PB pH 7.4 at 37 °C over 24 h. Aliquots were removed and processed with addition of PB pH 3.3 and acetonitrile for HPLC analysis. V was hydrolyzed to M1 and M2 and its rate of hydrolysis was similar to that observed when the incubation medium was injected directly into the HPLC (Fig. 4). M3 was not detected in the samples. A half-life of 2.9 h was calculated for hydrolysis of V and a similar relative concentration for M1 and M2 was determined with this method. The results indicated that addition of PB pH 3.3 and acetonitrile to the samples may be a good approach for analysis of V, M1 and M2 in rat serum.

This process was used to evaluate the stability of V in rat serum at 37 °C over 24 h. A half-life of 0.5 h was calculated

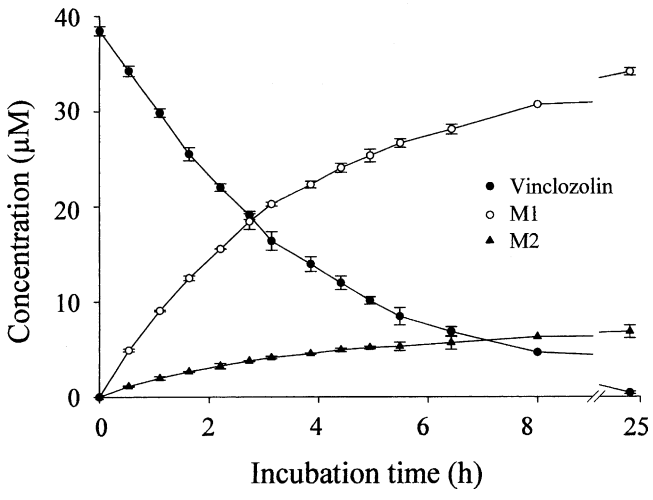


Fig. 3. Hydrolysis of vinclozolin in phosphate buffer pH 7.4 at 37 °C. The incubate was directly injected into the HPLC for analysis of V and its metabolites. Values represent mean ± S.D. ($n = 2$).

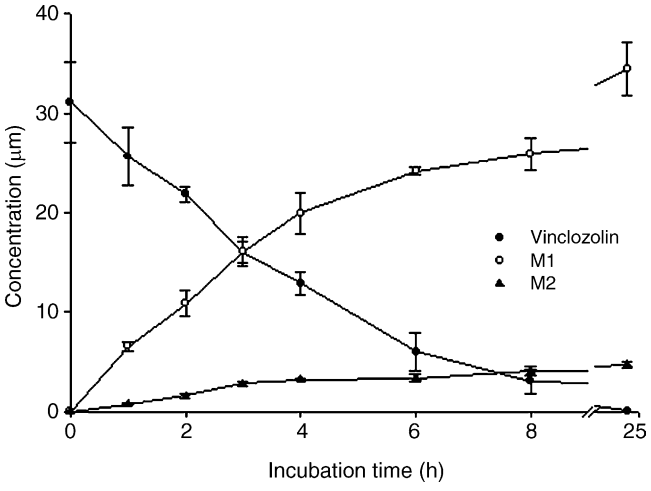


Fig. 4. Hydrolysis of vinclozolin in phosphate buffer pH 7.4 at 37 °C. Aliquots of buffer were removed and diluted with PB buffer, pH 3.3, 5 ml of acetonitrile was added and the solution was processed for HPLC analysis of V and metabolites. Values represent mean ± S.D. ($n = 3$).

for V when it was incubated in rat serum at 37 °C. In this medium, V was hydrolyzed faster to M1 and M2 than in PB pH 7.4 (Fig. 5). The relative concentration of V decreased over 90% in 2 h and then stabilized. M1 and M2 were the only products of V hydrolysis, as M3 was not detected. M1 was the major product of hydrolysis. This metabolite reached its maximal relative concentration at 4 h and represented approximately 80% of metabolites. Formation of M2 reached its maximum relative concentration at 2 h and remained constant until 24 h. The relative concentration of M2 represented about 20% of V products.

The pattern of metabolites from hydrolysis of V in PB pH 7.4 and rat serum was similar, however, in the latter matrix the hydrolysis of V was enhanced. These results indicate that V is very susceptible to hydrolysis in basic media as has been reported previously [7] and the hydrolysis is enhanced in presence of the biological molecules and ions of rat serum. Rat serum that had been filtered through a membrane with a MW cutoff > 3000 was incubated with V to determine the enzymatic contribution of its hydrolysis. The rate of hydrolysis in filtered serum was greater than in nonfiltered serum (data not shown). The pH of the filtered serum was measured and found to be >8.5. The higher pH of the filtered serum most likely accelerated the hydrolysis reaction. Therefore, the presence of M1 and M2 in biological samples from in in vivo experiments with V must be interpreted cautiously, because they may not be a product of enzyme-dependent V metabolism.

The analysis of serum from a V-treated rat (100 mg/kg) 4 h post-exposure showed the presence of V, M1, M3 and two unidentified metabolites that eluted around 7 min (Fig. 2D). The concentration of V and M1 detected were 19.8 and 23.1 μM, respectively. Preliminary results suggest that the larger of the two peaks around 7 min corresponds to 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide. The definitive identification of this peak is a subject of further investigation. V, M1 and M2 have previously been identified in the serum of rats administered repeated oral doses of V [3,11]. M3 has been detected as a V metabolite where biological systems are involved [12,19], however, there are other studies where its presence was not reported [3,13]. The presence of M3 in biological samples after exposure to V could be due to the acidic conditions used during analysis or production from V, M1 and M2 by the action of esterases. Definitive information about the biotransformation of V and its metabolites in the intact animal is limited in the peer-reviewed literature. In assays with bacteria and fungus present in soil and cultured in anaerobic and aerobic conditions, V was hydrolyzed to M1, M2 and M3 [19–21]. In addition, Pothuluri et al. [13] characterized two other metabolites from V, *N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3,5-dichlorophenyl-1-carbamic acid and the 3*R*- and 3*S*-isomers of 3′,5′-dichloro-2,3,4-trihydroxy-2-methylbutyranilide in *C. elegans* cultures. The latter metabolite was also detected in the liver of hens administered an oral dose of V [14].

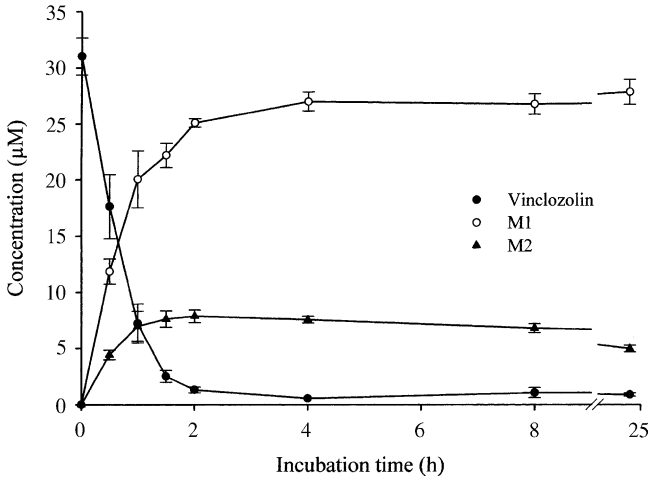


Fig. 5. Hydrolysis of vinclozolin in rat serum. V was incubated in rat serum at 37 °C. Aliquots of the incubate were removed, diluted with PB pH 3.3, 5 ml of acetonitrile was added and the solution was processed for HPLC analysis of V and its metabolites. Values represent mean ± S.D. ($n = 3$).

## 4. Conclusions

The results of this study indicate that the analytical method developed maintains V, M1 and M2 in their original form. V, M1 and M2 were recovered efficiently after sample acidification to pH 5.6 using PB pH 3.3 and addition of acetonitrile. The results also confirm that V is chemically hydrolyzed to M1 and M2 in physiological conditions. There is limited information about the in vivo metabolism of V. Because M1 and M2 are competitive antagonists of the androgen receptor, it is important to determine the tissue dosimetry of V and these metabolites. This analytical method may be useful for the kinetic analysis of V and its metabolites in serum of V-exposed rats. The results would assist in the development of a physiologically-based pharmacokinetic model, which would provide critical insight into the tissue dosimetry of this anti-androgenic toxicant.

## Acknowledgements

A. Sierra-Santoyo was supported by a cooperative agreement (CR828790) between the National Research Council and the US EPA. This article has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The authors gratefully acknowledge the technical assistance of Brenda C. Edwards.

## References

[1] J. Miyamoto, J. Burger (Eds.), Pure Appl. Chem. 75 (11–12), 2003.
[2] L.E. Gray Jr., J.S. Otsby, W.R. Kelce, Toxicol. Appl. Pharmacol. 129 (1994) 46.
[3] E. Monosson, W.R. Kelce, C. Lambright, J. Ostby, L.E. Gray Jr., Toxicol. Ind. Health 15 (1999) 65.
[4] L.E. Gray Jr., J. Ostby, E. Monosson, W.R. Kelce, Toxicol. Ind. Health 15 (1999) 48.
[5] C.J. Wolf, G.A. LeBlanc, J.S. Ostby, L.E. Gray Jr., Toxicol. Sci. 55 (2000) 152.
[6] US Food and Drug Administration, FDA Pesticide Program Residue Monitoring, 1993–2001, http://www.cfsan.fda.gov/~dms/pesrpts.html (2001).
[7] S.Y. Szeto, N.E. Burlinson, J.E. Rahe, P.C. Oloffs, J. Agric. Food Chem. 37 (1989) 523.
[8] S.Y. Szeto, N.E. Burlinson, J.E. Rahe, P.C. Oloffs, J. Agric. Food Chem. 37 (1989) 529.
[9] B. Schick, P.N. Moza, K. Hustert, A. Kettrup, Pestic. Sci. 55 (1999) 1116.
[10] A. Vanni, R. Gamberini, A. Calabria, V. Pellegrino, Chemosphere 41 (2000) 453.
[11] W.R. Kelce, E. Monosson, M.P. Gamcsik, S.C. Laws, L.E. Gray Jr., Toxicol. Appl. Pharmacol. 126 (1994) 276.
[12] E.A. Makynen, M.D. Kahl, K.M. Jensen, J.E. Tietge, K.L. Wells, G. Van Der Kraak, G.T. Ankley, Aquat. Toxicol. 48 (2000) 461.
[13] J.V. Pothuluri, J.P. Freeman, T.M. Heinze, R.D. Beer, C.E. Cerniglia, J. Agric. Food Chem. 48 (2000) 6138.
[14] G.M. Dean, D. Kirkpatrick, J. Riseborough, S.R. Biggs, D.R. Hawkins, W. Kohl, R. Huber, Brighton Crop Protection Conference—Pest and Diseases 2 (1988) 693.
[15] C.-I. Wong, W.R. Kelce, M. Sar, E.M. Wilson, J. Biol. Chem. 270 (1995) 19998.
[16] W.R. Kelce, C.R. Lambright, L.E. Gray, K.P. Roberts, Toxicol. Appl. Pharmacol. 142 (1997) 192.
[17] L.M. Baker, in: L.S. Clesceri, A.E. Greenberg, A.D. Eaton (Eds.). Standard Methods for the Examination of Water and Wastewater, Washington, DC, 1998, p. 1.
[18] K. Wittke, H. Hajimiragha, L. Dunemann, J. Begerow, J. Chromatogr. B 755 (2001) 215.
[19] A. Banerjee, S. Padhi, T.K. Adhya, Pestic. Sci. 55 (1999) 1177.
[20] L.A. Golovleva, Z.I. Finkelstein, A.V. Polyakova, B.P. Baskunov, M.Y. Nefedova, J. Environ. Sci. Health B26 (1991) 293.
[21] C. Mercadier, D. Vega, J. Bastide, J. Agric. Food Chem. 46 (1998) 3817.