Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 7, pp. 675–685, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

Stability indicating assay for fetindomide (NSC 373965), a potential prodrug of mitindomide (NSC 284356), employing high-performance liquid chromatography

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Abstract: A stability indicating assay for fetindomide using reversed-phase high-performance liquid chromatography (HPLC) has been developed. The chromatography was performed on a MOS Hypersil (C_8) column and was optimized by investigating the effects of methanol (7.5–20%), acetonitrile (5–7%), tetrabutylammonium hydrogen-sulphate (TBA, 0–90 mM) and acetate buffer (pH 3.5, 60–100 mM) on the retention of fetindomide and mitindomide. The optimum mobile phase was 5% acetonitrile in an aqueous solution containing 100 mM acetate buffer (pH 3.5) and 40 mM TBA. Complete resolution of fetindomide, and its degradation products, phenylalanine, the two isomers of *N*-phenylalanyloxymethylmitindomide and mitindomide was achieved within 10 min at a flow rate of 2 ml/min. The method is linear, accurate and precise for the determination of fetindomide and mitindomide and was applied to study the stability of fetindomide in various aqueous media.

Keywords: *Fetindomide; mitindomide; reversed phase high performance liquid chromatography; stability indicating assay.*

Introduction

Fetindomide (NSC 373965, *N,N'*-diphenylalanyloxymethylmitindomide dihydrochloride hemihydrate) (I, Fig. 1) is a water soluble prodrug of the antineoplastic agent, mitindomide (NSC 284356) (IV, Scheme 1). Mitindomide itself has been shown to be active against a variety of tumour models [1]. However, it is exceptionally insoluble in water (less than 1 μg/ml) and most pharmaceutically acceptable solvents. The National Cancer Institute have prepared the disodium salt of mitindomide which has adequate solubility in water for formulation as an injectable product. However, when the freeze-dried preparation is reconstituted in an aqueous diluent to 20 mg/ml, the pH is unacceptably high (>10) and the solution is relatively unstable at 25°C (half life ≃ 4 h) [2].

Fetindomide was synthesized in an attempt to improve the solubility of the parent compound so that a pharmaceutically acceptable formulation could be developed. The

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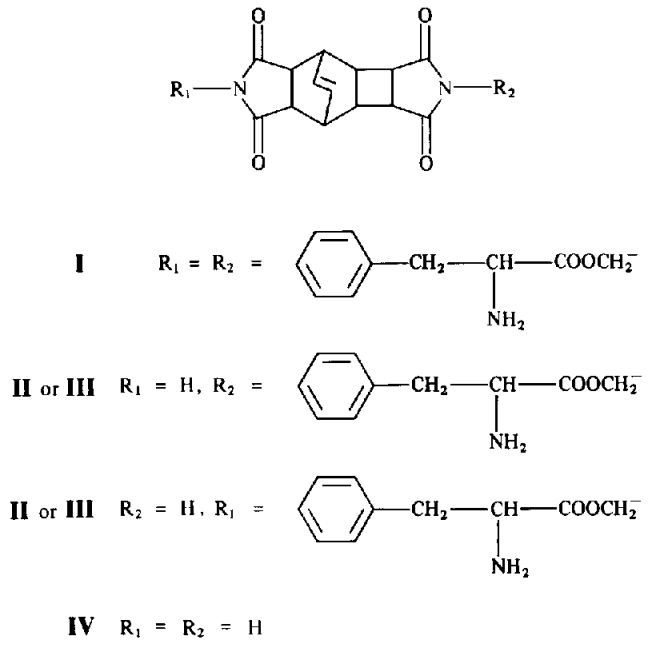
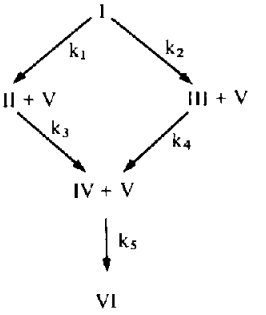


Figure 1
Chemical structures of fetindomide (I), the isomers of *N*-phenylalanyloxymethylmitindomide (II and III) and mitindomide (IV).



Scheme 1
Proposed mechanism of the chemical degradation of fetindomide (I) to give the isomers of *N*-phenylalanyloxymethylmitindomide (II and III), mitindomide (IV), phenylalanine (V) and the degradation products of mitindomide (VI).

prodrug was designed so that the active principle is released *in vivo* by the loss of two molecules of phenylalanine and formaldehyde (Scheme 1). It is reasonable to assume that the release of mitindomide from the prodrug occurs *via* two parallel pathways involving two isomeric intermediates of *N*-phenylalanyloxymethylmitindomide (II and III, Scheme 1). Without authentic samples it is not possible to assign the actual structures to II and III and the designations made here are based on their elution order from the chromatography column.

The objective of the present study was to develop a stability indicating assay for I and IV in the presence of II, III and V (phenylalanine) since these are the most likely degradation products of fetindomide in aqueous solution (Scheme 1). In addition, the

degradation products (VI, Scheme 1) of mitindomide itself may also be present in aqueous solutions. The degradations of mitindomide in alkaline solutions has been characterized by Vishnuvajjala and Cradock [2].

Experimental

Chemicals and reagents

The samples of mitindomide and fetindomide dihydrochloride hemihydrate were provided by the National Cancer Institute, Bethesda, MD, USA and were used as received. All the aqueous buffers were prepared from reagent grade salts and were obtained from a variety of sources. HPLC grade methanol and acetonitrile were obtained from Fisher Scientific, Fair Lawn, NJ, USA and the water was purified using the Mega-pure system (Model MP-1, Corning, Buffalo, NY, USA).

Liquid chromatography

The liquid chromatograph was constructed from a Beckman 110B pump (Beckman Instrument Company, Berkeley, CA, USA), a Rheodyne model 7125 injector (fitted with a 20 μl loop, Rheodyne, Cotati, CA, USA), a Spectroflow 757 detector (set at 254 nm, Kratos Analytical Instruments, Ramsey, NJ, USA) and a Shimadzu C-R3A integrator (purchased from Delta Instrument Company, Overland Park, KS, USA). Chromatography was performed on an 5-μm MOS Hypersil Column (150 × 4.6 mm i.d.) (Shandon Southern, distributed by Anspec Company Inc., Ann Arbor, MI, USA). The column was packed in an upward direction using chloroform as the suspending solvent (2.3 g of packing in 60 ml) and methanol as the packing solvent. The chromatographic conditions were optimized by investigating the effects of methanol (7.5–20%, v/v), acetonitrile (5–7%, v/v), acetate buffer (pH 3.5, 60 mM in mobile phases containing methanol and 100 mM in mobile phases containing acetonitrile) and tetrabutylammonium hydrogensulphate (TBA) (0–90 mM) on the retention of I and IV. The capacity ratios (k') of I and IV and the selectivity factor (α) were calculated using equations (1) and (2), respectively:

$$k' = (t_r - t_o)/t_o, \tag{1}$$

$$\alpha = k'(\mathrm{I})/k'(\mathrm{IV}), \tag{2}$$

where t_r and t_o are the elution times of the compound of interest (I or IV) and an unretained compound (water), respectively. The optimum mobile phase for the analysis of aqueous solutions of fetindomide was 5% acetonitrile in an aqueous solution containing 40 mM tetrabutylammonium hydrogensulphate (TBA) and 100 mM acetate buffer (pH 3.5).

Calibration solutions ($n = 7$) were prepared by diluting stock solutions of fetindomide dihydrochloride hemihydrate (20 mg/ml) and mitindomide (5 mg/ml) in dimethylsulphoxide (DMSO) with mobile phase. The accuracy and precision of the method were determined by diluting the stock solutions of fetindomide and mitindomide with mobile phase six times so that their nominal concentrations fell within the linear regions of the respective calibration curves which were 0.15–1.5 mg/ml for fetindomide and 0.023–0.23 mg/ml for mitindomide. Sample solutions containing fetindomide were also diluted with mobile phase prior to assay, so that they fell within the linear region of the

calibration curves. All solutions were injected at least twice onto the liquid chromatograph and the mean peak areas were used for quantification.

Formulation of fetindomide

A pilot formulation of fetindomide suitable for intravenous administration was prepared. An aqueous solution containing 20 mg/ml fetindomide dihydrochloride hemihydrate and 20 mg/ml mannitol was filtered through 0.2 μm Metricel filters (Type GA-8, Gelman Scientific Inc., Ann Arbor, MI, USA) under aspectic conditions and 10 ml of the solution transferred to glass injection vials. The solvent was removed by freeze-drying and the vials sealed with rubber closures.

Stability studies

All the stability studies were conducted at 25 ± 0.1°C in 10 ml volumetric flasks. The temperature of the solutions was maintained by placing the flasks in a model 2095 Forma Scientific circulating water bath (Forma Scientific, Marietta, OH, USA). In all cases, aliquots (200 μl) were withdrawn at predetermined intervals, diluted if necessary with mobile phase and injected onto the liquid chromatograph. The progress of the reactions was followed for 3–4 half lives and each experiment was conducted in duplicate.

The stability of the pilot formulation of fetindomide was studied following reconstitution with 10 ml of 0.9% sodium chloride injection USP (normal saline, NS). The reconstituted formulation was also diluted further to nominal concentrations of 0.5 and 1.0 mg/ml with NS and 5% dextrose injection USP (D5W). The stability of these solutions was then monitored.

The stability of fetindomide was also studied at 25 ± 0.1°C and various pH values. The pH of the solutions was maintained with the following buffers: pH 1–2.5 (HCl); pH 7.5 (50 mM phosphate); pH 8.5 (50 mM tris); pH 10–11.5 (80 mM phosphate). The initial concentrations of fetindomide were *ca* 400 μg/ml (pH 1–2.5 and 10.5–11.5), 50 μg/ml (pH 8.5) or 25 μg/ml (pH 7.5). These solutions were prepared by adding the appropriate aliquots of the 20 mg/ml stock solution of fetindomide in DMSO to the buffer solutions. The total concentration of DMSO in the reaction solutions was maintained at 4%. The pH values of the solutions were measured initially and at the end of the experiments and did not change by more than 0.1.

Results and Discussion

Assay development

Early in the assay development, a classical example of the general elution problem [3] was encountered. Fetindomide did not elute from an MOS Hypersil column using a mobile phase of 20% methanol in acetate buffer (60 mM, pH 3.5) which is suitable for the analysis of mitindomide (Table 1). Since fetindomide is considerably more soluble in water than mitindomide, interaction between the residual silanols and the protonated amino groups [4, 5] of fetindomide was suspected as being a major contributor to the retention of this compound on the column. Accordingly, a competing ion, tetrabutylammonium (TBA), was added to the mobile phase in an attempt to reduce the affinity of fetindomide for the column (Table 1) [4, 5]. This approach was successful and the retention of fetindomide decreased with increasing concentration of TBA (Table 1). Unfortunately, addition of TBA also reduced the retention of mitindomide (Table 1).

The chromatographic conditions were optimized by systematically varying the

Table 1
Effect of mobile phase composition on the capacity ratio (k') and the selectivity factor (α) or fetindomide (I) and mitindomide (IV) on MOS Hypersil

Mobile phase				k'*		
Type	Modifier (%)	Acetate‡ (mM)	TBA§ (mM)	I	IV	α†
Methanol	20	60	0	¶	<0.5	—
	10	60	10	33.4	2.1	15.9
	15	60	10	17.4	1.2	14.5
	15	60	35	10.8	1.2	9.0
	15	60	50	7.2	0.82	8.8
	15	60	60	5.6	0.60	9.3
	12	60	70	5.8	0.82	7.1
	10	60	70	7.4	1.1	6.7
	7.5	60	90	8.3	1.2	6.9
Acetonitrile	7	100	40	3.6	0.73	4.9
	5	100	40	6.3	1.0	6.3

*$k' = (t_r - t_o)/t_o$ [equation (1)].
†$\alpha = k'$ (I)/k'(IV) [equation (2)].
‡Total concentration of acetate buffer (pH 3.5) in the aqueous component of the mobile phase.
§Total concentration of tetrabutylammonium hydrogensulphate (TBA) in the aqueous component of the mobile phase.
¶Did not elute from the column after 35 min.

concentrations of methanol, acetonitrile, TBA and acetate buffer (pH 3.5) (Table 1) with the overall objective of minimizing the selectivity factor (α) while maintaining adequate retention of mitindomide ($k' > 1$) and an overall analysis time of less than 10 min. These objectives were realised with a mobile phase of either 10% methanol in an aqueous solution of 60 mM acetate buffer containing 70 mM TBA or 5% acetonitrile in an aqueous solution of 100 mM acetate buffer (pH 3.5) containing 40 mM TBA (Table 1). The latter conditions were preferred due to the slightly shorter analysis time and the lower pressure drop (2000 psi compared with 3000 psi). The conditions developed permitted the quantitative determination of both fetindomide (I) and mitindomide (IV) in degraded solutions of the parent compound (Scheme 1 and Fig. 2).

Figure 2 shows a chromatogram of a partially hydrolysed sample of fetindomide and reveals two partially resolved peaks (II and III) which were attributed to the two isomers of *N*-phenylalanyloxymethylmitindomide (II and III). The rate of appearance of these two peaks was consistent with them being intermediates in the formation of mitindomide from fetindomide (Scheme 1, Figs 3 and 4). This aspect will be discussed in more detail in the applications section.

The relationship between the peak areas and concentration injected was linear ($r > 0.999$) for both fetindomide and mitindomide over the ranges 0.15–1.5 mg/ml (0.2–2 mM) and 0.02–0.2 mg/ml (0.07–0.7 mM), respectively. The slopes (response factors, RF) of the linear relationships between peak area and concentration injected were 304.5 and 245.0 μm^{-1} for fetindomide and mitindomide, respectively. It should be noted that calibration curves should be freshly prepared due to the instability of fetindomide and injected immediately after preparation. In addition, calibration solutions of the two drugs should not be prepared as mixtures since traces of

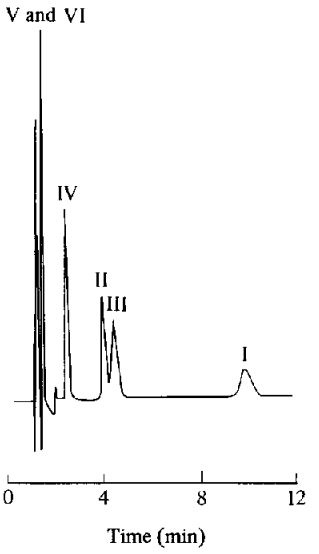


Figure 2
Optimum separation of fetindomide (I) from its degradation products (II–VI, Fig. 1 and Scheme 1). Stationary phase: MOS Hypersil (150 × 4.6 mm i.d., 5 μm); mobile phase: 5% (by volume) in 40 mM TBA.HSO_4, 100 mM acetate buffer (pH 3.5). Flow rate: 2.0 ml/min; detector wavelength: 254 nm.

mitindomide derived from fetindomide can result in significant positive intercepts for the calibration curve of mitindomide. The precision and accuracy of the method for fetindomide and mitindomide were obtained by diluting the stock solutions to concentrations of 1.04 and 0.17 mg/ml, respectively. This procedure was repeated six times for both drugs and the diluted solutions injected into the column. The precision (%RSD) was 0.4% for fetindomide and 2.8% for mitindomide. These values correspond to 95% confidence intervals (%RSD × $t/n^{1/2}$) of ±0.33% for fetindomide and ±2.3% for mitindomide. The accuracies of the procedures were 99.1% for fetindomide and 102.8% for mitindomide.

Applications

A lyophilized formulation of fetindomide (200 mg) containing 200 mg mannitol was successfully prepared and was easily reconstituted with 10 ml of 0.9% sodium chloride (NS). The stabilities of the reconstituted formulation (20 mg/ml) and further dilutions of the original reconstruction in NS and 5% dextrose injection (D5W) were studied (Table 2). The degradation of all these formulations was first order over at least three half lives. The degradation of fetindomide did not appear to be concentration dependent in NS or D5W, although the degradation does appear to be slightly faster in D5W. Nevertheless, the pilot formulation of fetindomide does appear promising and the shelf life of the reconstituted product and dilutions thereof appear acceptable (t_{90} > 7 h) at room temperature (25 ± 0.1°C) (Table 2).

Preliminary data for the stability of fetindomide in buffers of various pH were generated and the results are given in Table 3. The degradation of fetindomide was first order over 3–4 half lives and independent of pH over the range 1–2.5 (Table 3). Representative chromatograms showing the disappearance of fetindomide and the appearance of its degradation products (II–V) with time are presented in Fig. 3. The rate of decomposition of fetindomide was markedly increased above pH 7.5 and was too rapid to measure above pH 10.5 (Fig. 5). Figure 5 shows that fetindomide is rapidly

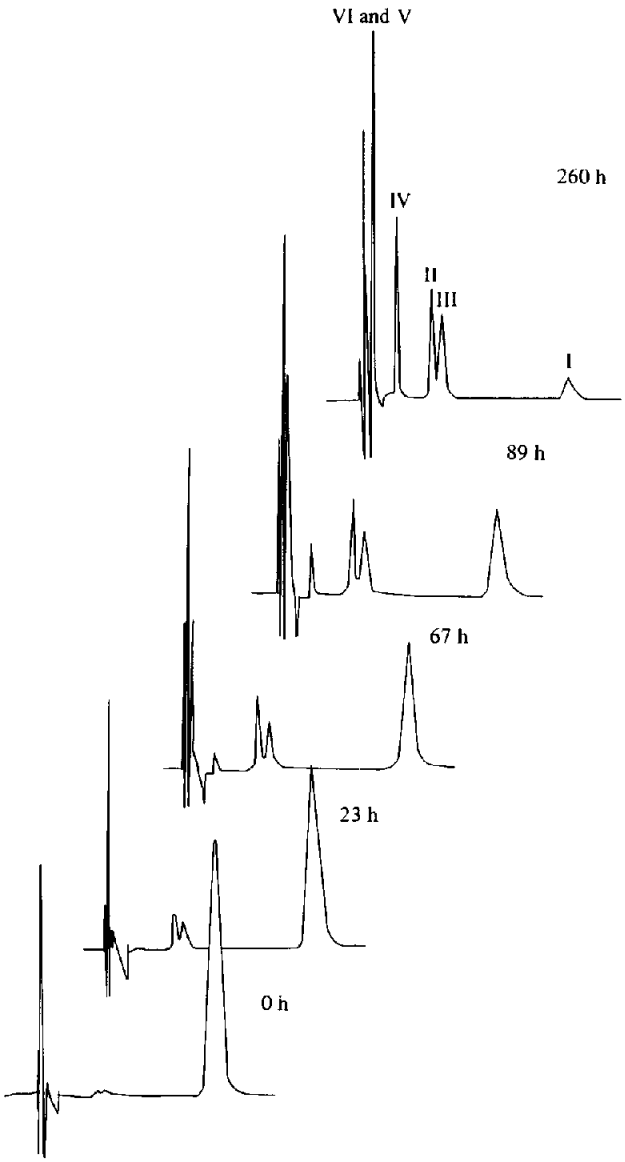


Figure 3
Chromatograms of fetindomide (I) showing the loss of the parent compound and the appearance of its degradation products (II–V) as a function of time in 0.1 M HCl (μ = 0.5, 25 ± 0.1°C). Chromatographic conditions as in Fig. 2.

Table 2
Preliminary stability data for a lyophilized formulation of fetindomide after reconstitution with 0.9% sodium chloride injection (NS) and further dilution of the reconstituted formulation with NS and 5% dextrose injection (D5W), at 25 ± 0.1°C

Initial concentration (mg/ml)	Diluent	k_{obs}* (h^{-1}) × 10^3	t_{90}† (h)	r*
20.0‡	NS	9.19	11.4	0.999
		8.99	11.7	0.999
1.0§	NS	10.4	10.1	0.992
		10.5	10.1	0.999
0.5¶	NS	10.8	9.7	0.999
		10.4	10.1	0.999
1.0§	D5W	12.7	8.3	0.998
		12.2	8.6	0.998
0.5¶	D5W	14.4	7.3	0.995
		14.0	7.5	0.995

*Obtained by fitting the data to $\ln [I] = \ln [I]_0 - k_{obs}t$, by least squares linear regression, where [I], $[I]_0$ and k_{obs} are the concentrations of fetindomide at time t, the initial concentration of fetindomide and the pseudo-first order rate constant, respectively. The values for two determinations are given. r = correlation coefficient.

†Shelf life, $t_{90} = 0.105/k_{obs}$.

‡Lyphilized product containing 200 mg fetindomide and 200 mg mannitol, reconstituted with NS (10 ml).

§1:20 dilutions of the original reconstituted solution.

¶1:40 dilutions of the original reconstituted solution.

Table 3
Preliminary stability data for fetindomide in various buffers at 25 ± 0.1°C (μ = 0.5)

pH	Buffer (mM)	k_{obs}* (h^{-1}) × 10^3	$t_{1/2}$† (h)
1.0	HCl (100)	8.20	84.6
1.5	HCl (31.6)	8.00	86.6
2.0	HCl (10)	8.31	85.3
2.5	HCl (3.16)	8.39	82.7
7.5	Phosphate (50)	1278	0.54
8.5	Tris (50)	2496	0.28
10.5	Phosphate (80)	‡	‡
11.0	Phosphate (80)	‡	‡
11.5	Phosphate (80)	‡	‡

*Mean of two determinations except pHs 10, 11 and 11.5 which were single determinations. See Table 2 for further explanation.

†$t_{1/2} = 0.693/k_{obs}$.

‡Degradation of fetindomide was too rapid to be determined (see Fig. 5).

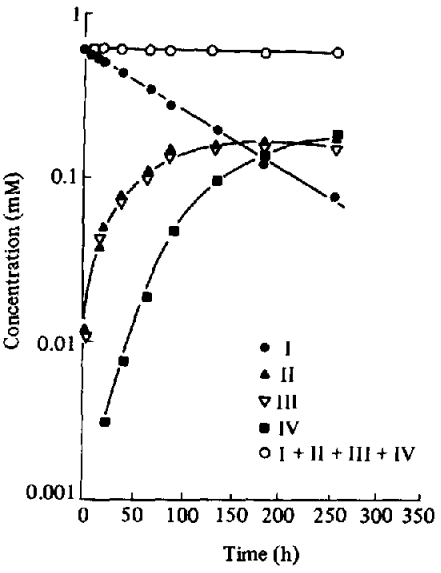


Figure 4
Loss of fetindomide (I) and the appearance of its degradation products (II–IV) as function of time in 0.1 M HCl (μ = 0.5, 25 ± 0.1°C, $[I]_o$ = 580 μM). Chromatographic conditions as in Fig. 2.

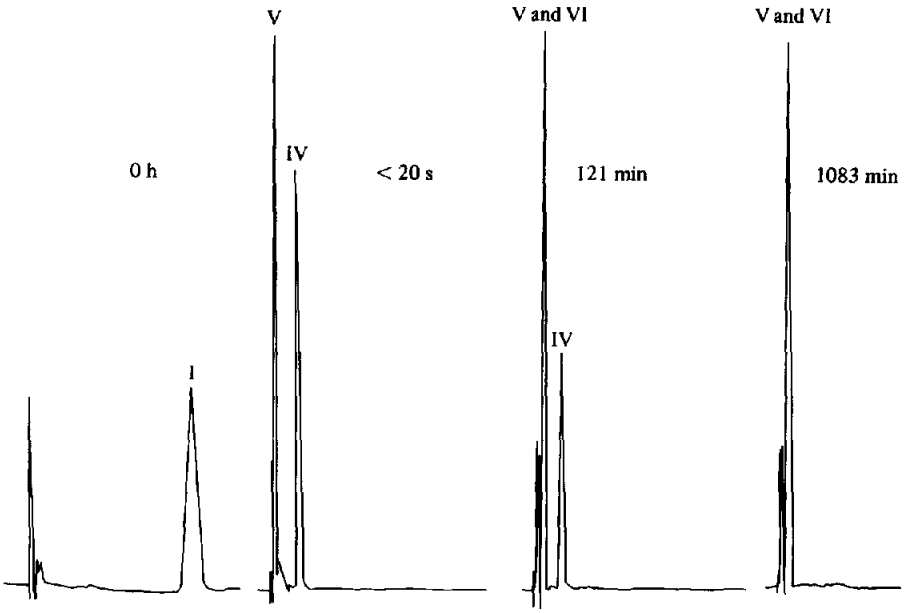


Figure 5
Chromatograms showing the rapid loss of fetindomide (I), appearance of mitindomide (IV) and phenylalanine (V), and the subsequent degradation of mitindomide and the appearance of its degradation products (VI) in phosphate buffer (pH 10.5, μ = 0.5, 25 ± 0.1°C). Chromatographic conditions as in Fig. 2.

converted to mitindomide within 20 s at pH 10.5 and that the mitindomide itself degrades further with an approximate half life of about 4 h which is consistent with the previous study of Vishnuvajjala and Cradock [2].

During the kinetic experiments, peaks II and III attributed to the isomers of *N*-phenylalanyloxymethylmitindomide were observed. The peak area ratios of these two isomers remained constant and close to unity throughout the course of the kinetic experiments. Since authentic samples of II and III were not available, it was not possible to calibrate the system for these two components. However, their response factors could be determined indirectly from the known concentrations of I and IV and their molar response factors by the following approach. The appropriate mass balance equation is:

$$[I]_0 = [I] + [II] + [III] + [IV] \tag{3}$$

and

$$[II] + [III] = [I]_0 - [I] - [IV]. \tag{4}$$

The relationships between molar response factor (RF), peak area (A) and the concentrations of II and III are given by equations (5) and (6), respectively:

$$[II] = A(II)/RF(II), \tag{5}$$

$$[III] = A(III)/RF(III). \tag{6}$$

Assuming $RF(II) = RF(III)$, which is not unreasonable for isomeric compounds, it follows that

$$[II] + [III] = [A(II) + A(III)]/RF(II), \tag{7}$$

$$= [A(II) + A(III)]/RF(III). \tag{8}$$

Substitution of equations (7) or (8) into equation (4) gives, after rearrangement, equation (9):

$$RF(II) = RF(III) = \{A(II) + A(III)\}/\{[I]_0 - [I] - [IV]\}. \tag{9}$$

The response factors of II and III were calculated from their peaks areas and the concentrations of I and IV at various times during the degradation of fetindomide in 0.1 M HCl [equation (9)]. The response factors for II and III were found to be constant over the first 184 h of the experiments and the mean values (±SD) for two determinations (separate kinetic experiments) were 234.9 ± 17.9 μm^{-1} ($n = 5$) and 237.9 ± 15.6 μm^{-1} ($n = 5$). It is interesting to note that the response factor for the two isomers of *N*-phenylalanyloxymethylmitindomide is greater than half that of fetindomide itself [$RF(I) = 304.5\ \mu m^{-1}$], suggesting that the absorbance of light by II and III does not arise solely from the phenyl groups and that there is some contribution from the imide groups. Absorbance of light by the imide groups would explain the relatively high and somewhat unexpected response factor of 245.0 μm^{-1} for mitindomide itself at 254 nm.

Having obtained the response factors for II and III it was then possible to convert the peak areas of II and III into concentrations [equations (5) and (6)]. Figure 4 shows the change in concentration of I, II, III and IV in a solution originally containing 0.58 mM (400 μg/ml) fetindomide in 0.1 mM HCl at 25 ± 0.1°C. It can be seen that the loss of fetindomide was first order (Fig. 4) and the appearances of II, III and IV were consistent with Scheme 1. A lag of about 23 h was seen before mitindomide itself was detected at a concentration of about 300 nM. Mass balance calculations [equation (3)] were also performed and Fig. 4 shows that all the species (I–V) were accounted for at all time periods up to 260 h.

Acknowledgements: This work was supported by the National Cancer Institute Contract NO1-CM1-67912.

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[*First received for review 2 February 1987; revised manuscript received 6 April 1987*]