Hydrolysis of tinidazole

Analytical methods and degradation kinetics

by

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Academic dissertation

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Contents

List of original publications

This dissertation is based on the following publications referred to in the text by their Roman numerals (**I**−**V**). Some unpublished results are also included.

I H. Salomies and J.−P. Salo, An HPLC study of tinidazole hydrolysis. *Chromatographia* **36** (1993) 79−82

II J.−P. Salo and H. Salomies, High performance thin layer chromatographic analysis of hydrolyzed tinidazole solutions I. Development and validation method. *J. Pharm. Biomed. Anal.* **14** (1996) 1261−1266

III J.−P. Salo and H. Salomies, High performance thin layer chromatographic analysis of hydrolyzed tinidazole solutions II. Hydrolysis kinetics of tinidazole. *J. Pharm. Biomed. Anal.* **14** (1996) 1267−1270

IV J.−P. K. Salo and H. Salomies, Two stability−indicating UV spectrophotometric methods for the analysis of hydrolyzed tinidazole. *J. Pharm. Biomed. Anal.* (2003) *in press*

V J.−P. K. Salo, J. Yli−Kauhaluoma and H. Salomies, On the hydrolytic behavior of tinidazole, metronidazole, and ornidazole. *J. Pharm. Sci.* (2003) *in press*

Abbreviations

CLC	column liquid chromatography
CN	cyano
HIV	human immunodeficiency virus
HPLC	high−performance liquid chromatography
HPTLC	high−performance thin layer chromatography
i.d.	internal diameter
ICH	International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use
Im	any 5−nitroimidazole
IR	infrared
NADH	reduced nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
ODS	octadecylsilane
r	correlation coefficient
R_F	response factor
RP	reversed−phase
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
SS_{RES}	residual sum of squares
TLC	thin layer chromatography
UV	ultraviolet
Vis	visible

Abstract

Tinidazole, chemically 1-[2-(ethylsulfonyl)ethyl]−2-methyl−5-nitroimidazole, as well as metronidazole and ornidazole, are 5-nitroimidazole drugs which are commonly used against amoebiasis, giardiasis, trichomoniasis, and anaerobic bacterial infections in humans. All 5-nitroimidazoles are synthetic drugs.

Stability studies on drugs are important not only for the correct preparation and storage of drug compounds and preparations, but they also provide useful information on degradation products and pathways, as well as valuable material for the development and validation of analytical methods. Spectrophotometric, chromatographic, voltammetric, and titrimetric methods of analysis have been widely applied to the quantitative determination of 5-nitroimidazoles.

A high−performance liquid chromatographic (HPLC), a high−performance thin layer chromatographic (HPTLC), and two ultraviolet (UV) spectrophotometric methods were developed and used for the quantitation of tinidazole in hydrolyzed aqueous solutions. The HPTLC and UV spectrophotometric methods were validated and shown to be stability−indicating. The UV methods were sensitive to the reaction pH, whereas the chromatographic methods could be used for analyzing tinidazole solutions hydrolyzed at pH 1−12. The feasibility of systematic wavelength searching in developing multi−wavelength spectrophotometric methods was demonstrated using the precision and accuracy of validation samples as selection criteria.

The hydrolysis of tinidazole followed apparent first−order kinetics in most of the conditions studied. It was hydrolytically most stable at pH 4.0−5.0. It was demonstrated that at least one of the reacting partners contributing to the transition state had a charge of 0 in the major degradation pathway at various pH values. The dominant reactions were tinidazole + OH^- at pH ≥ 7 and tinidazole + H_2O at a pH of around 4.5. In more acidic conditions, the reactions tinidazole + H^+ and/or [tinidazole+H]$^+$ + H_2O took place, but the existence of [tinidazole+H]$^+$ + H^+ could not be excluded nor corroborated. A mechanism for the alkaline hydrolysis was suggested, and it involved a proton transfer from tinidazole to the hydroxide ion and the formation of ethyl vinyl sulfone. The reaction may occur in either a step−wise or a

concerted manner. The reaction was qualitatively verified by computational methods, but the activation energy of the hydrolysis reaction, which was determined to be *ca.* 120−130 kJ/mol, could not be reproduced. The two known hydrolysis products were detected at pH 6−12, but they were absent at pH ≤ 5. No other 5−nitroimidazole products were detected in hydrolyzed tinidazole solutions at pH 1−12.

1. Introduction

Three 5−nitroimidazole drugs, namely metronidazole, ornidazole, and tinidzole, have been available in Finland on prescription (Figure 1.1). At present, only metronidazole preparations are on the market [1]; ornidazole and tinidazole were withdrawn in 1993 [2] and 1995 [3], respectively. However, all three drugs, as well as other 5−nitroimidazoles, are currently used as human drugs in several European and non−European countries; some 5−nitroimidazoles have also found potential use in veterinary practice [4]. However, these 5−nitroimidazoles belong to a list [5] of compounds for which no maximum levels in foodstuffs of animal origin can be fixed.

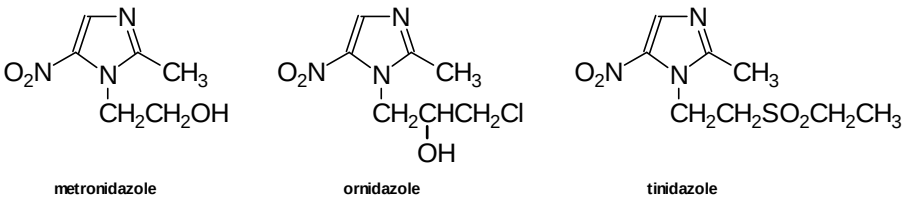


Figure 1.1: The structures of metronidazole, ornidazole, and tinidazole.

Medicinal interest in nitroimidazoles arose from the observation that azomycin, later [6] identified as 2−nitroimidazole, possessed antimicrobial activity [7]. Azomycin originates from a soil sample [7], while the 5−nitroimidazoles are synthetic drugs. The first 5−nitroimidazole drug to come on the market was metronidazole [8]; since then, several others have been synthesized and used as drugs, including ornidazole [9] and tinidazole [10]. The tautomeric 2-methyl−4(5)-nitroimidazole is the common starting material for their synthesis [9−11]. 5-nitroimidazoles are mainly used against amoebiasis, giardiasis, trichomoniasis, and anaerobic bacterial infections [4,12]. 4−nitroimidazoles are much less active than the corresponding 5-nitro isomers [11]. Also, metronidazole and tinidazole have been used in the eradication of *Helicobacter pylori* [4]. Very recently, several diarylmethyl ethers of metronidazole were shown to possess submicromolar activity against HIV-1 [13]. The structure−activity relationships of hundreds of 5-nitroimidazoles have been studied [14−19], and comprehensive reviews [12,20] on their chemistry and pharmacology exist.

Ebel *et al.* [21] published a review article on the stability−indicating assays for metronidazole. Their list included ultraviolet/visible (UV/Vis) spectrometry, high−performance thin layer chromatography (HPTLC), high−performance liquid chromatography (HPLC), and voltammetry; titrimetry, the pharmacopoeial [22] method of choice, is not suited for the purpose since interference by the degradation products is to be expected [21]. All techniques are applicable for most, if not all, 5-nitroimidazoles. However, the list is not exhaustive, and *e.g.* gas chromatography, which has been used for the quantitative analysis of 5-nitroimidazoles [23], provides comparable capabilities. It is the nitro group that is responsible for electron capture in the electrochemical methods of analysis [24] and, in conjugation with the imidazole moiety, it lies in the heart of the UV spectrophotometric methods. The latter property especially constitutes a convenient method of detection in HPTLC and HPLC.

The validation of an analytical method is an integral part of method development since its purpose is to demonstrate that the method is appropriate for the intended application. Since 1996, an ICH guideline final draft [25] has existed for the methodology with an associated text [26]; all ICH guidelines concerning analytical methods, and quality in general, are accessible on the ICH Quality Topics homepage (*http://www.ich.org/ich5q.html*). Being guidelines, the ICH documents cannot go into details, and the analyst may still need to resort to the scientific literature in order to find the proper procedures for individual validation tests. (HP)TLC is an off−line technique that serves as an excellent example of an analytical method that imposes additional requirements on the validation scheme: the analytes on the plate are vulnerable to oxidative degradation catalyzed by the free silanol groups and light [27] both before, during, and after the chromatographic separation. A less than exhaustive list of publications on the validation of HPTLC methods is shortly reviewed in paper **II**.

The objective of this study was to develop HPLC, HPTLC, and UV spectrophotometric methods for investigating the hydrolytic stability of tinidazole over a wide pH range. The alkaline hydrolysis mechanism of tinidazole was also studied.

2. Review of the literature

2.1. Stability of 5−nitroimidazoles

"Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures" [28]. Being sufficient for a registration application in the European Union, Japan, and the United States, the stability testing of new drug substances and products is covered in two ICH guidelines [28,29]. Stress testing should establish the effect on stability of temperature, humidity, oxidation, and photolysis; hydrolytic stability over a wide range of pH values should also be studied for substances in solution or suspension [28]. The general testing conditions are listed in Table 2.1. Photostability testing is described in another guideline [29].

Table 2.1: General conditions for stability testing of new drug substances [28].

Study	***Storage condition***	***Minimum time period covered by data at submission of registration application***
Long term	25±2 °C and 60±5 % relative humidity	12 months
Intermediate	30±2 °C and 60±5 % relative humidity	6 months
Accelerated	40±2 °C and 75±5 % relative humidity	6 months

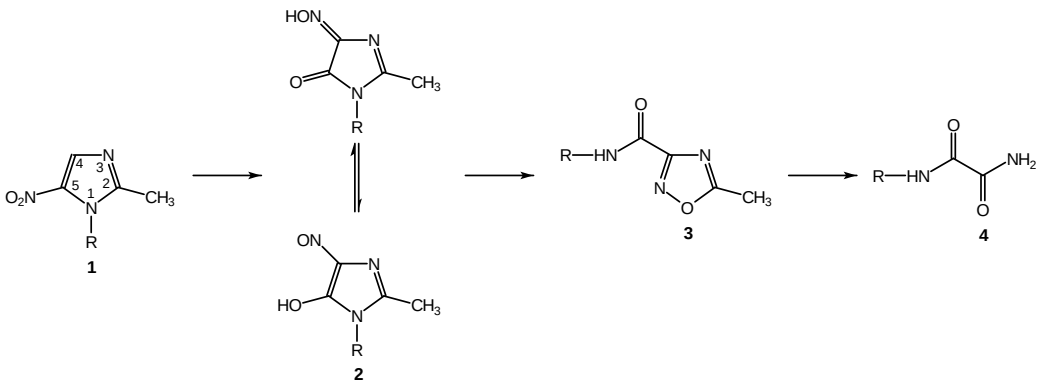
2.1.1. Photolysis

The photolytic disintegration of 5−nitroimidazoles is a versatile reaction. The initial formation of a nitro radical anion has been proposed [30] as one reaction pathway; this has later [31] been shown to be incorrect for metronidazole. The radical anion would then decompose to yield nitrite or the corresponding amine by multistep reduction [32]; other reports [33,34] proposing different mechanisms also exist. By far the most complete reaction sequence described involves several rearrangements of the 5−nitroimidazole structure. This sequence (Scheme 2.1) has been verified [33,35,36] for the compounds in Table 2.2; the side chain on the imidazole N^1 has to be a group other than hydrogen for the reaction to take place [35]. The reaction proceeds via the very labile tautomeric 2−imidazoline−4,5−dione−4−oxime (**2**) to the corresponding 1,2,4−oxadiazole−3−carboxamide (**3**) [33,35,36]. A more detailed reaction mechanism has been presented as well [33,35]. The oxadiazole is the main photolysis product for the compounds in Table 2.2 [33,35]. It has also been reported [33] to be the end product of

the reaction, but other workers [35,36] have shown that the compounds decompose further, yielding an oxamide (**4**). The degradation kinetics of several 4− and 5−nitroimidazoles, including metronidazole, ornidazole, and tinidazole, has been found to be of the first order both in solution [37] and in the solid state [38]; in solution, metronidazole was by far the most photolabile 5−nitroimidazole [37].

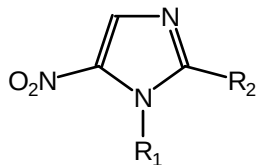
Table 2.2: 5−Nitroimidazole drugs (**1** in Scheme 2.1) known to decompose photolytically according to Scheme 2.1.

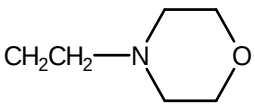
R	***Generic name***	***Reference***
CH_3	Dimetridazole	[35]
CH_2CH_2OH	Metronidazole	[33,35]
$CH_2CH(OH)CH_2Cl$	Ornidazole	[35]
$CH_2CH(OH)CH_3$	Secnidazole	[35]
$CH_2CH_2SO_2CH_2CH_3$	Tinidazole	[36]
$CH_2CH(O{-}COCH_3)CH_3$	(Secnidazole, acetate ester)	[35]



Scheme 2.1

Table 2.3: Liberation of nitrite ions used for the quantitative analysis of 5−nitroimidazole drugs.



Compound	***R_1***	***R_2***	***Hydrolytic conditions***	***Reference***
Dimetridazole	CH_3	CH_3	*e.g.* 2 h in 0.2 M NaOH under copper catalysis at 100 °C	[40]
Ipronidazole	CH_3	$CH(CH_3)_2$	2 h in 0.1 N NaOH at 100 °C	[39]
			e.g. 2 h in 0.2 M NaOH under copper catalysis at 100 °C	[40]
Metronidazole	CH_2CH_2OH	CH_3	15 min in 1 N NaOH at 100 °C	[42]
Nimorazole		H	15 min in 2.5 N NaOH at 100 °C	[43]
Ornidazole	$CH_2CH(OH)CH_2Cl$	CH_3	1 h UV irradiation in 0.1 N NaOH	[44]
			3 min in 1 N NaOH at 100 °C	[43]
Ronidazole	CH_3	$CH_2O{-}CONH_2$	1 h in 1.67 % NaOH under copper catalysis at 80 °C	[45]
			e.g. 2 h in 0.2 M NaOH under copper catalysis at 100 °C	[40]
Secnidazole	$CH_2CH(OH)CH_3$	CH_3	10 min in 0.5 M NaOH at 100 °C	[46]

2.1.2. Chemical hydrolysis

According to the approach used, the literature on the hydrolysis reactions can be divided into two groups: the formation of nitrite ions, and the degradation of the parent compound (with possible analysis of the emerging products).

Lau *et al.* [39] reported in 1969 that N^1−substituted 5−nitroimidazoles yield quantitative amounts of nitrite ions when reacted in 0.1 N NaOH at 100 °C for 2 hours; however, the reaction seemed to be complete in 1 hour (Figure 2 in *Ref.* [39]). They also demonstrated the applicability of the reaction for the quantitative analysis of ipronidazole: the nitrite ion so formed reacts with sulfanilamide and an aromatic amine to produce a colored diazo compound which can then be quantitated spectrophotometrically. It has since been shown [40] that even N^1−unsubstituted 5−nitroimidazoles can be similarly decomposed in 1.5 M NaOH under prolonged (up to 5 h) heating at 100 °C and copper catalysis; the alkali concentration can be used to distinguish the unsubstituted compounds from the substituted ones. This reaction has been used for the quantitative analysis of several 5−nitroimidazole drugs (Table 2.3); the diazotization reagents and conditions, as well as the analytical wavelengths (typically 535-550 nm), usually vary. A possible reaction mechanism for the nitrite formation, at least for 5-nitro−2-styrylimidazoles at pH ≥ 13, has been presented [41]. Theuer [34] demonstrated the formation of ppm−level nitrite during the autoclavation of metronidazole.

The hydrolysis of metronidazole was a first−order reaction in both acidic and alkaline conditions [47,48], and it exhibited maximum stability at pH 4−6 [34,47,48]. The alkaline hydrolysis of metronidazole yielded almost quantitative amounts of ammonia and acetic acid [49], as well as an amine with at least one attached hydrogen [47,49]; an amine has also been shown [47] in acid hydrolyzed solutions. A kinetic salt effect was detected at pH 3.1, while it was less pronounced at pH 7.4 [48]. The defects in the mathematical treatment of the kinetic data [48] have been pointed out and corrected [50]. The activation energy of hydrolysis was 26.6 kcal/mol at pH 8.0 [47] and 15.35 kcal/mol at pH 3.1 [48]. No general acid/base catalysis could be detected [48].

As both reactions involve well−known organic chemistry, the pseudo first−order alkaline hydrolysis (pH 6−10) of ornidazole has been shown to produce an epoxide from the side−chain chlorohydrin, and subsequently the corresponding diol [51,52] (Fig. 1 in paper **V**), the

latter reaction being of pseudo first−order, as well [51]. On the other hand, ornidazole has also been reported [52] to lose the nitroimidazole structure in strongly acidic conditions (≥ 0.1 M HCl). 2−methyl−4(5)−nitroimidazole and the 4−nitro isomer of ornidazole, as well as the previously mentioned epoxide and diol, were already known to the scientific community as possible impurities of ornidazole [53]. The most recent study [52] is based on the ICH guideline [28] for stability testing.

Tinidazole is known to decompose hydrolytically in alkaline conditions to its 4−nitro isomer and 2−methyl−4(5)−nitroimidazole, the latter being the almost exclusive product in 0.1 M NaOH [36]. These decomposition products are also the only specifically named structurally related impurities (*B* and *A*, respectively) limited by the monograph of tinidazole in the European Pharmacopoeia [54]. In the presence of a catalytic amount of base, however, tinidazole has been reported [55] to isomerize almost quantitatively to the 4−nitro isomer; the corresponding sulfoxides and sulfides do not react. It was suggested [55] that the process takes place via *N*−dealkylation followed by *N*−realkylation on the other nitrogen, involving the formation of ethyl vinyl sulfone. Indeed, treating 2−methyl−4(5)−nitroimidazole with the sulfone gave the 4−nitro isomer exclusively [55] and in a high yield [56]. Even before an insight into the reaction was gained, hydrolysis to 2−methyl−4(5)−nitroimidazole of tinidazole in strongly alkaline conditions was used for quantifying the drug. A pale yellow color is produced, thereby facilitating spectrophotometric determinations at 360−370 nm [57−59]; since then, this reaction has been erroneously claimed to be previously unknown [60]. In a recent study [61], tinidazole was reported to exhibit constant hydrolytic stability over the pH range 3.00−6.50; the activation energy at pH 5.25 can be calculated to be 133 kJ/mol.

Dimetridazole was not stable when boiled in a mildly alkaline solution, and no decomposition was observed in a slightly acidic solution. No attempt was made to identify the decomposition product(s) [62].

2.2. Quantitative analysis of tinidazole

The analytical literature on tinidazole comprises several hundred scientific papers. Therefore, the following review does not aim to be comprehensive; instead, it is a representative selection covering three analytical techniques, namely HPLC, (HP)TLC, and

UV spectrometry. However, it should be borne in mind that other analytical techniques, like gas chromatography [23], polarography [63], and titrimetry [22], are also applicable to the quantitative analysis of tinidazole although they have not been used as frequently in the literature.

2.2.1. High−performance liquid chromatography

HPLC, which is probably the most frequently used analytical method for the analysis of tinidazole, has been used to analyze the drug substance in simple formulations [64,65], as well as in biological samples [64,66−74]. Several methods for stability studies have been published, too [61,75−79] (Table 2.4). Tinidazole and metronidazole have been frequently used as internal standard for each other [64,68,71,72,74−78].

2.2.2. High−performance thin layer chromatography

Only a few densitometric thin layer chromatographic (TLC) methods for tinidazole using silica sorbent have been reported [65,73,81−84] (Table 2.5). The earlier methods [81−83] apparently made use of conventional TLC plates, while the most recent reports [65,73,84] with metronidazole as the internal standard are true HPTLC methods. Methods exist for the analysis of tinidazole in both dosage forms [65,84] and biological samples [73,81−83]. The earlier method [84] designed for analyzing tinidazole in dosage forms was found to be comparable with polarography. A study [65] of tinidazole in a combined dosage form (with furazolidone in suspension) contained no comparison of the HPTLC and HPLC methods developed. For serum samples, HPTLC was found to produce comparable results with those given by HPLC [73]. An outdated, laborious, and error−prone method involving scraping and extraction of the "spots" after chromatography is given for reference [85] only.

Table 2.4: Determination of tinidazole by HPLC.

Mobile phase	*Column*	*Detection*	*Sample matrix*	*Reference*
Acetate buffer (0.05 M, pH 4.7) containing 22 % acetonitrile	C_{18} (30 × 0.4 cm)	UV (313 nm)	Plasma Tablet	[64]
Water—acetonitrile—triethylamine 80:20:0.1 (V/V) (pH 3.0 with phosphoric acid)	C_{18}	UV (335 nm)	Suspension	[65]
Phosphate buffer (0.01 M, pH 5.5) with 15 % acetonitrile	ODS (5 µm; 0.15 m × 4.6 mm i.d.)	UV (320 nm)	Feces Plasma	[66]
Methanol—KH_2PO_4 (0.005 M, pH 4) 20:80 (V/V)	C_{18} (30 cm × 4 mm i.d.)	UV (320 nm)	Serum Urine	[67]
35 % Acetonitrile in acetate buffer (0.02 M, pH 4.0)	C_{18} (10 µm; 30 × 3.9 mm)	UV (313 nm)	Serum Various tissues	[68,80]
(No data available.)	ODS	UV	Breast milk Serum	[69]
Various compositions (methanol/acetonitrile—phosphate buffer)	C_{18} (10 µm)	UV (280 nm)	Gingival crevicular fluid Gingival tissue Serum	[70]
Methanol—water 23:77 (V/V)	C_{18} (5 µm; 4.6 mm i.d. × 250 mm)	UV (310 nm)	Saliva	[71]
SDS (0.05 M)—propan−1−ol 94:6 (V/V)	CN (3.9 × 300 mm)	UV (320 nm)	Serum	[72]
Acetate buffer (0.05 M, pH 4.7) containing 22 % (V/V) acetonitrile	C_{18}			
Acetate buffer (0.05 M, pH 4.7) containing 22 % acetonitrile	C_{18} (0.39 × 30 cm)	UV (313 nm)	Serum	[73]
KH_2PO_4 (0.002 M, pH 4.8)—methanol—acetonitrile 85:7.5:7.5 (V/V)	C_{18} (5 µm; 25 cm × 4.6 mm i.d.)	UV (320 nm)	Serum	[74]
Methanol—water—acetic acid 20:80:0.1	ODS (4.6 mm × 20 cm)	UV (317 nm)	Injection	[75][a]
Methanol—water—acetic acid 20:80:0.5	ODS	UV (316 nm)	Solution	[61][a]
Methanol—water 25:75	ODS (5 mm × 150 mm)	UV (318 nm)	Injection	[76][a]
Methanol—water 20:80	CLC−ODS (150 × 6.0 mm)	UV (310 nm)	Injection	[77][a]
Methanol—water—acetic acid 20:80:0.1	ODS (4.6 × 250 mm)	UV (310 nm)	Collutorium	[78][a]
Acetonitrile—KH_2PO_4 (0.05 M) 25:75	(4.6 mm × 150 mm)	UV (317 nm)	Injection	[79][a]

[a] stability study

Table 2.5: Determination of tinidazole by thin layer chromatography with densitometric quantitation.

Mobile phase	*Sorbent*	*Detection*	*Sample matrix*	*Reference*
Chloroform—acetic acid 9:1	Silica gel F_{254}	Quenching of plate fluorescence	Serum	[81−83]
Dichloromethane—acetone—diethyl ether 2+2+1 (V/V)	HPTLC silica gel GF_{254}	Densitometry (311 nm)	Infusion solution Pure substance Tablet	[84]
Chloroform—acetonitrile—acetic acid 60+40+2	HPTLC silica gel 60	Densitometry (320 nm)	Serum	[73]
Chloroform—methanol—ammonia 9:1:0.1 (V/V)	HPTLC silica gel $60F_{254}$	Densitometry (335 nm)	Suspension	[65]

Table 2.6: Determination of tinidazole by UV spectrophotometry.

Analytical wavelength (solvent)	*Sample matrix*	*Reference*
315 nm (chloroform) 310 nm (alcohol, methanol) 322 nm (benzene) 277 nm (0.1 N HCl)	Bulk drug Tablet	[86]
316 nm (ethanol)	Bulk drug Tablet	[87]
310 nm (ethanol)	TLC scrape−off	[85]
317±2 nm (water)	Medicinal films	[88]
317 nm	Buccal tablet Saliva	[89]

2.2.3. UV spectrophotometry

Tinidazole has been determined spectrophotometrically as such [86,87], in drug preparations [86−89], and in biological samples [89] (Table 2.6). An error−prone method using measurements on *n* wavelengths for the analysis of *n* components has been used for tinidazole in a combined dosage form [90]; the interactions between L−amino acids and tinidazole (and metronidazole) have been studied as well [91]. Sometimes the other components do not absorb at the absorption maximum of the component of interest, which can then be determined by a direct single−wavelength measurement, as was described for tinidazole (311 nm in methanol) in another combined dosage form [92]. It has been shown [93] that tinidazole (and metronidazole) sometimes interferes with clinical measurements involving NADH at 340 nm. However, few methods based on a true multi−wavelength analysis have been published for tinidazole [94].

2.3. Multi−wavelength methods in spectrophotometry

(True) multi−wavelength methods are a more reliable method for the analysis of multi−component samples. For these methods (*cf. e.g.* [95−97]), the familiar Lambert−Beer law is written in matrix form, *i.e.* $\boldsymbol{A} = \boldsymbol{KC}$, where $\boldsymbol{A}$ is the matrix of sample absorbances, $\boldsymbol{C}$ the matrix of sample concentrations, and $\boldsymbol{K}$ the matrix of proportionality constants $k = \varepsilon b$. The matrix equation is solved for $\boldsymbol{K}$ in the calibration step, and later for $\boldsymbol{C}$ in the analysis step. The least squares solutions of the equation are $\boldsymbol{K} = \boldsymbol{AC}^{\boldsymbol{T}}(\boldsymbol{CC}^{\boldsymbol{T}})^{-1}$ and $\boldsymbol{C} = (\boldsymbol{K}^{\boldsymbol{T}}\boldsymbol{K})^{-1}\boldsymbol{K}^{\boldsymbol{T}}\boldsymbol{A}$, respectively. For $\boldsymbol{CC}^{\boldsymbol{T}}$ and $\boldsymbol{K}^{\boldsymbol{T}}\boldsymbol{K}$ to be invertible, the columns of $\boldsymbol{C}$ and $\boldsymbol{K}$, respectively, have to be linearly independent [98].

Sternberg *et al.* [95] demonstrated in 1960 that, in their specific application, the best results were achieved when the analytical wavelengths were evenly spaced. Since then, many strategies have been studied for the selection of the optimal set of analytical wavelengths in spectroscopy. The approaches can be grouped broadly into two classes. In one class, the analytical results between several wavelength sets are compared with the aid of standard statistical parameters, while the wavelength sets are formed *e.g.* by even spacing or by choosing the maxima, minima and crossover points in the analyte spectra [95,96,99]. In the other class, the wavelength candidates are chosen from a large number of (evenly spaced)

wavelengths by awarding a figure of merit to each, hence ordering the wavelengths from "best" to "worst". Starting with a minimum number of (best) wavelengths, the search for the optimal wavelength combination then continues by adding the wavelength next in order to the previous set [100−103]; finally, each combination has to be evaluated in some way. However, the need for wavelength ranking arises from a time when computational efforts, especially inverting a larger matrix, were non−trivial.

2.4. Mathematical treatment of kinetic data

van der Houwen *et al.* [50] demonstrated in 1997 that the interpretation of pH degradation profiles is, in many cases, flawed in the scientific literature. Making some assumptions that are not too limiting [104], the observed reaction rate constant k_{obs} of a substance can be expressed as a function of its n acidity constants K, the hydrogen ion concentration, and the "macro reaction constants" M (combining kinetically indistinguishable reactions):

$$k_{obs} = \frac{\sum_{i=1}^{n+3} \frac{M_i}{[H^+]^{i-2}}}{\sum_{i=0}^{n} \frac{\prod_{j=0}^{i} K_j}{[H^+]^i}} \tag{2.1}$$

For a substance with one ionizable group, such as tinidazole, the above equation reduces to [104]

$$k_{obs} = \frac{M_1 \times [H^+]^2 + M_2 \times [H^+] + M_3 + M_4 \times [H^+]^{-1}}{[H^+] + K_a} \tag{2.2}$$

with

$$M_1 = k_{0,H} \tag{2.3}$$

$$M_2 = k_{1,H}\times K_a + k'_{0,S}\times[H_2O] = k_{1,H}\times K_a + k_{0,S} \tag{2.4}$$

$$M_3 = k'_{1,S}\times K_a\times[H_2O] + k_{0,OH}\times K_w = k_{1,S}\times K_a + k_{0,OH}\times K_w \tag{2.5}$$

$$M_4 = k_{1,OH}\times K_a\times K_w \tag{2.6}$$

(The individual reaction rate constants take the form $k_{n,N}$, where n = 0,1 states the number of dissociated protons in the acid, whose K_a appears in the equations, and N = H,S,OH denotes the other reacting species, H^+, H_2O (or solvent), and OH^-, respectively.) A similar approach has been adopted in the presence of ligands [105] and buffer catalysis [106]. A statistically robust method for handling replicate cases in determining the pH degradation profile has been described recently [107−109].

3. Aims of the study

The primary aim of the study was to investigate the hydrolysis kinetics of tinidazole using different analytical techniques.

The specific aims of the research were

1. to develop HPTLC and UV spectrophotometric methods for the analysis of hydrolyzed tinidazole (**II**, **IV**)
2. to conduct a preliminary study on the alkaline hydrolysis of tinidazole by HPLC (**I**), and to elucidate the hydrolysis kinetics of tinidazole over a wide pH range (**III**, **V**)
3. to study the hydrolysis mechanism(s) of tinidazole in relation to its kinetic behavior (**V**)

4. Materials and methods

Only the major experimental features are described in this section. More detailed descriptions can be found in the original publications **I**−**V**.

4.1. Materials

Tinidazole was kindly supplied by Orion Pharmaceutica (Espoo, Finland). The commercially available 2−methyl−4(5)−nitroimidazole was from Aldrich−Chemie (Steinheim, Germany). The 4−nitro isomer of tinidazole was prepared as described in the literature [55]. The identity and purity of these compounds had been verified by TLC, UV, IR, as well as by ^{1}H and ^{13}C NMR spectrometry. For studies **IV** and **V**, another batch of the 4−nitro isomer was prepared [55], and its identity and purity were verified by HPTLC, as well as with UV and IR spectrometry by comparing it with the previous batch. Metronidazole, the internal standard for HPLC (**I**), was obtained from the Farmos Group (Oulu, Finland). The methanol used in study **I** was of HPLC grade; all other chemicals and reagents were of analytical grade. In study **V**, sodium salts were used for the phosphate buffers; the ionic strengths were adjusted with sodium chloride.

4.2. High−performance liquid chromatography (I)

The HPLC system consisted of an LKB 2150 pump, LKB 2151 variable−wavelength monitor (LKB, Bromma, Sweden), D−2000 chromato−integrator (Merck−Hitachi, Tokyo, Japan), and a 20 µl loop injector. The compounds were separated on a HP 79915MO−174 RP−8 column (200 mm × 4.6 mm i.d., 10 µm particle size). The mobile phase was an isocratic mixture of acetonitrile—phosphate buffer (50 mM, pH 3) 18:82 (V/V), and the flow rate was 1.0 ml/min. The analytical wavelength was 318 nm.

Samples from the 5 mM tinidazole solutions were prepared by mixing 1.0 ml of the hydrolyzed solution with a sufficient volume of a neutralizing solution (0.1 M HCl) and then diluting to 25.0 ml with water; metronidazole was added as internal standard (final concentration 16 µg/ml). Peak area ratios (analyte to internal standard) were used for the quantitations.

4.3. High−performance thin layer chromatography (II, III)

Samples were sprayed with a Linomat IV (Camag, Muttenz, Switzerland) at a speed of 15 s/µl as 5 mm bands on silica gel HPTLC plates (Merck, Darmstadt, Germany). Methanol—diethyl ether—chloroform 1:9:3 (V/V) was used as the final mobile phase; the plates were developed at room temperature in an unsaturated horizontal chamber (Camag). The plates were scanned at 314 nm (D_2 lamp) with a Camag TLC Scanner II controlled by the Cats 3.14 program. The scanning speed was 4.0 mm/s, and the monochromator bandwidth was set at 10 nm. The slit width and length were 0.2 mm and 2 mm, respectively. Each track was scanned once, and baseline correction was used (**II**).

Samples from the 5 mM tinidazole solutions were prepared by mixing 1.0 ml of the hydrolyzed solution with a sufficient volume of a neutralizing solution (0.1 M HCl or water) and then diluting to 10.0 ml with water. Samples were applied on the HPTLC plate in duplicate (**III**). Quantitations were based on peak areas and the Michaelis−Menten 1 equation (**II**).

4.4. UV spectrophotometry

The UV spectrophotometric determinations (**IV**, **V**) were carried out using a Unicam UV2-300 spectrophotometer (Unicam Ltd., Cambridge, UK). The reliability of the quantitative methods was confirmed based on the ICH guideline [25], and the specific methodology is described in detail in paper **IV**.

*4.4.1. Single−wavelength method (**IV**)*

The absorbances of the solutions (pH 6.0±0.7) were measured at 318 nm against a reagent blank using a spectral bandwidth of 1.5 nm and an integration time of 1 s. Samples from the 0.6 mM tinidazole solutions were prepared by mixing 5.0 ml of the hydrolyzed solution with 10 ml of a 0.1 M phosphate buffer and diluting to 25.0 ml with the same buffer. A second−order polynomial was used for the calibration.

Table 4.1: Design matrix for the validation samples S1−S24 (1 = tinidazole, 2 = the 4-nitro isomer of tinidazole, 3 = 2-methyl−4(5)-nitroimidazole).

	Concentration (mM)																							
	S1	***S2***	***S3***	***S4***	***S5***	***S6***	***S7***	***S8***	***S9***	***S10***	***S11***	***S12***	***S13***	***S14***	***S15***	***S16***	***S17***	***S18***	***S19***	***S20***	***S21***	***S22***	***S23***	***S24***
1	0.003	0.007	0.012	0.017	0.021	0.025	0.030	0.045	0.060	0.075	0.091	0.097	0.113	0.165	0.225	0.254	0.272	0.290	0.300	0.000	0.000	0.330	0.000	0.000
2	0.007	0.290	0.017	0.272	0.025	0.254	0.045	0.226	0.075	0.165	0.113	0.091	0.097	0.060	0.030	0.021	0.012	0.003	0.000	0.300	0.000	0.000	0.330	0.000
3	0.290	0.003	0.272	0.012	0.254	0.021	0.224	0.030	0.164	0.060	0.098	0.112	0.090	0.075	0.045	0.026	0.016	0.007	0.000	0.000	0.300	0.000	0.000	0.330
sum	0.301	0.300	0.301	0.300	0.300	0.300	0.299	0.301	0.299	0.300	0.301	0.299	0.299	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.330	0.330	0.330

4.4.2. Selection of analytical wavelengths for the multi−wavelength method

A set of 24 validation samples was designed (Table 4.1). The samples were prepared in a 0.1 M phosphate buffer (pH 6). The spectra of the samples were recorded at 240−290 nm at 2.0 nm intervals, *i.e.* at 26 wavelengths, against a reagent blank using a spectral bandwidth of 1.5 nm and a scan speed of 30 nm/min. Each sample was analyzed 7 times by changing the solution in the cuvette between measurements (*repeatability of measurement*). Analyzed on separate days, two sets of samples S1−S18 were prepared; one set of samples (S19−S24) was analyzed on a third day. All calculations were performed within the Dataplot [110] program suite.

Preselection of wavelengths was performed by analyzing all combinations of 4−6 wavelengths (310,960 wavelength sets). With the nominal tinidazole concentration in unhydrolyzed samples being 0.3 mM, wavelength combinations with all mean accuracies (n = 7) 100±10 % and all repeatabilities ≤ 4 %, for analyte concentrations [Im] ≥ 0.03 mM (≥ 10 % of the original amount of tinidazole in real samples), were considered acceptable.

Taking into account the results of the preselection step, a systematic search of selected combinations of 4−20 wavelengths was conducted in the final step (786,069 wavelength sets). Combinations with all mean accuracies 100±4 %, when [Im] ≥ 0.075 mM, and all repeatabilities ≤ 4 %, when [Im] ≥ 0.03 mM, were selected for visual inspection, and the combination providing the (visually) best accuracies and repeatabilities was chosen for quantitative work.

*4.4.3. Multi−wavelength method (**IV**)*

The spectra of the solutions (pH 6.0±0.7) were recorded at 240−290 nm at 2.0 nm intervals against a reagent blank using a spectral bandwidth of 1.5 nm and a scan speed of 30 nm/min. For the quantitations, the absorbances at 6 wavelengths (240, 242, 246, 248, 254 and 270 nm) were used. The results were calculated using matrix algebra.

Samples from the 0.6 mM tinidazole solutions were prepared by mixing 10.0 ml of the hydrolyzed solution with 5 ml of a 0.1 M phosphate buffer and then diluting to 20.0 ml with the same buffer. Samples from the 5 mM tinidazole solutions (pH 10.0) were prepared by

mixing 3.0 ml of the hydrolyzed solution with 25 ml of 0.1 M phosphate buffer (pH 5.8) and then diluting to 50.0 ml with the same buffer.

4.5. Preliminary degradation studies (I, III)

In order to obtain the pH degradation profile, the hydrolysis of 5 mM tinidazole solutions, prepared in citrate−phosphate−borate/HCl buffers [111] (pH 1−12), was carried out in a thermostated water bath at 80±0.5 °C.

The activation energy for the basic hydrolysis was calculated using the Arrhenius equation. Hydrolysis of 5 mM tinidazole solutions, prepared in the citrate−phosphate−borate/HCl buffer at pH 10.0 [111], was carried out in a thermostated water bath in the temperature range 60-80 °C.

4.6. Kinetic salt effect (V)

Within the pH range of 1.00−4.70, the ionic strengths varied from approximately 0.1 to 1. Solutions of tinidazole (0.6 mM) were prepared in 0.1 M phosphate buffer by dissolving the solid in the buffer solution at room temperature. The solutions were placed in an oven kept at 80±0.5 °C, and the samples were analyzed using the single−wavelength method.

At pH values of 7.00−8.45, the ionic strengths varied from approximately 0.2 to 1. Solutions of tinidazole (0.6 mM) were prepared in 0.1 M phosphate buffer of appropriate pH (7.50-8.45) by dissolving the solid in the buffer solution at 80±0.5 °C, and the containers were submerged in a water bath kept at the same temperature. Tinidazole solutions (0.6 mM) at pH 7.00 were also prepared in 0.1 M phosphate buffer by dissolving the solid in the buffer solution at room temperature, and the solutions were placed in an oven kept at 80±0.5 °C. The samples were analyzed using the multi−wavelength method.

4.7. Molecular modeling (V)

The alkaline hydrolysis mechanism of tinidazole was studied using molecular modeling. The calculations were performed with Spartan 5.0.3 (Wavefunction Inc., Irvine, CA, USA) at the

pBP/DN**//HF/3−21G$^{(*)}$ level of calculation, run on an SGI R5000 processor (SGI, Mountain View, CA, USA).

Table 5.1: Reliability test results of the quantitative methods developed (1 = tinidazole, 2 = the 4-nitro isomer of tinidazole, 3 = 2-methyl−4(5)-nitroimidazole).

	HPLC (I)			***HPTLC (II)***			***UV (IV)***			
							Single−wavelength	***Multi−wavelength***		
	1	***2***	***3***	***1***	***2***	***3***	***1***	***1***	***2***	***3***
Specificity	Apparently stability−indicating			Stability−indicating			Stability−indicating	Stability−indicating		
Linearity	Straight line			Michaelis−Menten 1 calibration			2nd order polynomial	Established		
slope[a]	8651±24.71	6760±7.362	6180±6.347							
y−intercept (× 10^{-3})[a]	−49.93±6.023	−20.27±1.871	2.897±1.683							
r[a]	0.9999	1.000	1.000							
SS_{RES}[a]	0.4928	0.1531	0.1377							
Range[a]	µg/ml			ng on plate			49−120 µM (calibration) 41−150 µM (measurement)	30−330 µM		
	10.6−106	11.0−110	5.90−59.0	21.7−174	22.1−177	21.0−168				
Accuracy[b]				105.2 %	104.6 %	103.6 %	100±2 %	100±8 %		
Precision[c]										
Repeatability[a]	< 3 %	< 3 %	< 1 %							
Measurement of peak area				0.60 %	0.40 %	0.60 %				
Measurement							≤ 0.20 %	< 4 %		
Positioning				0.70 %	0.60 %	1.1 %				
Sample application				1.8 %	1.4 %	1.0 %				
Method				5.3 %			≤ 0.25 %	1.1 %	2.5 %	1.5 %
Intermediate precision				2.4 %	2.5 %	1.5 %	≤ 1.7 %	1.3 %	1.6 %	1.0 %
Detection limit				3 ng on plate			0.247 µM	3 µM	7 µM	12 µM
Quantitation limit				20 ng on plate			0.750 µM	27.4 µM		
Robustness				Mobile phase composition Laboratory temperature			pH of reaction mixture pH of sample/standard solutions Ionic strength of samples			
Wavelength precision[c]				1.0 %	0.93 %	0.89 %				

[a] The data for the HPLC method were not reported in paper **I**, but were later calculated from the original data.

[b] mean recovery

[c] RSD

5. Results and discussion

The main results obtained in this work are briefly described in this section. More details can be found in the original publications **I**−**V**.

5.1. High−performance liquid chromatography (I)

The hydrolysis products were easily separated from each other, as well as from tinidazole and the internal standard metronidazole; the total analysis time was 11 minutes (Figure 1 in paper **I**). Calibration graphs for tinidazole, its 4−nitro isomer, and 2−methyl−4(5)−nitroimidazole, had correlation coefficients better than 0.9999. This method was originally developed for the preliminary degradation studies, and some relevant data have been collected in Table 5.1. It is apparently stability−indicating since the reasoning given in paper **II** obviously applies to this method, too: any putative degradation product involving ring−opening of the imidazole moiety would not be expected to absorb at the detection wavelength and it would probably have a different polarity and, hence, a different retention time from the three 5-nitroimidazoles. It also follows that this method may be used for solutions hydrolyzed at pH 1−12.

5.2. High−performance thin layer chromatography (II)

This method was developed because the earlier (HP)TLC methods [81,82,84] could not fully resolve tinidazole and its degradation products. The chosen eluent, methanol—diethyl ether—chloroform 1:9:3 (V/V), provided baseline resolution that eliminated higher R_F values (Fig. 3 in paper **II**) where a rise in the baseline occurred as described in the literature [27]; the system suitability data were acceptable (Table 1 in paper **II**). Methanol affected the R_F values very strongly (Fig. 4a in paper **II**), and more detailed studies revealed that diethyl ether was the major factor in creating the separation enhanced by the addition of chloroform, which also inhibited peak broadening. The method was deemed stability−indicating, and it could be used for solutions hydrolyzed at pH 1−12.

The stability of the compounds during plate development was verified by two−dimensional chromatography using the same mobile phase in both directions; no decomposition was

detected at this stage. Authentic hydrolyzed samples were stable for at least 7 hours at room temperature when kept in the dark or when applied on the plate. The compounds could be reliably measured even 29 hours after the development of the plate, provided it was kept in the dark (Fig. 5 in paper **II**).

The reliability data for the HPTLC method are presented in Table 5.1. The calculated quantitation limit would have been less than 10 ng for each compound, but it was shown that only at 20 ng was the repeatability of sample application less than 3 % (Table 2 in paper **II**), which is the acceptance criterion [112]. The precision tests confirmed that the method, including the analytical equipment, is free of any large random errors. On the other hand, although the method passed the accuracy test, it had a slight positive bias, which was especially pronounced at the lower end of the range. Taking into account the intended use of the method, this could be overlooked because of the possible experimental errors in kinetic studies.

5.3. UV spectrophotometry

The UV spectra of tinidazole and its hydrolysis products are depicted in Figure 2 in paper **IV**. Tinidazole imposes the strictest demands on the pH of any hydrolyzed solution whose constitution should be analyzed by UV spectrophotometry. Spectral and stability considerations lead to the demand that the pH of such solutions be 5.00−7.00. The reliability data (**IV**) for both methods are presented in Table 5.1.

*5.3.1. Single−wavelength method (**IV**)*

This method should only be used for analyzing reactions taking place within the pH range of 1.00−5.00, where the product profile is known to lack any strong interferants at 318 nm. The pH of the samples and standard solutions should be adjusted to 6.00±0.70 with a 0.1 M phosphate buffer of appropriate pH. The HPTLC sample stability data (**II**) apply to this method, too. Sample ionic strengths of 0.1−0.5 were well tolerated without any spectral changes for tinidazole at 318 nm. It was shown that quadratic fitting, *i.e.* a second−order polynomial, is better than a straight line for calibration.

5.3.2. Selection of analytical wavelengths for the multi−wavelength method

The preselection step revealed the following: there were about 1000 acceptable 4−6 wavelength combinations, one of the wavelengths was always 240 nm or 242 nm, and wavelengths between 258−268 nm (limits included) never occurred. The large number of acceptable combinations would have led to an overwhelming amount of results requiring some form of screening, *e.g.* visual inspection. On the other hand, the total run time would have grown prohibitively long since the analysis of all 4−26 wavelength combinations (approximately 67.1 million) would have taken almost 9 years! It was also noticed that the accuracy for some compounds in some of the samples was very sensitive to the choice of wavelengths (Figure 5.1). Since the applied accuracy criteria were rather loose, it was decided to choose a tighter accuracy range for the final selection step that would bring these values as near 100 % as possible. To shorten the analysis time in the final step, the wavelengths of 258-268 nm (limits included) were omitted and at least one of 240 nm and 242 nm had to be included in acceptable combinations.

In the final step, only 13 wavelength combinations had to be evaluated visually. All acceptable combinations had 4−7 wavelengths; no 8−wavelength or higher combinations passed the criteria. All the acceptable wavelength combinations apart from the best one yielded rather similar results, especially in respect of the sensitive points, *i.e.* the ones showing potential outlier behavior. The best combination was formed by 6 wavelengths: 240, 242, 246, 248, 254, and 270 nm (Figure 5.1). This wavelength combination was used in all the multi−wavelength analyses.

*5.3.3. Multi−wavelength method (**IV**)*

This method should only be used for analyzing reactions taking place within the pH range 6.00−12.00. The requirements for sample and standard pH, as well as for the storage of samples, are identical to the single−wavelength method.

This method showed an accuracy comparable to the accuracy at low−range of the HPTLC method (**II**). All the calculated individual accuracies fell between 100±10 % for [Im] ≥ 0.03 mM. These values are very adequate for kinetic purposes, and they totally lacked deviations > 10 % seen with the HPTLC method (**II**). This method seemed to lack the positive

bias seen with the HPTLC method (**II**), as well (Figure 5.1, Table 4 in paper **IV**). The repeatability of measurement was better than 3 % for [Im] ≥ 0.03 mM apart from one value which lied below the 4 % limit (Figure 5.1). Comparable repeatabilities for the HPTLC method (**II**) were calculated at approximately mid−range. In this respect, these two methods are similar. The intermediate precision was clearly better than within−laboratory reproducibility for the HPTLC method (**II**). Due to methodological differences, the repeatabilities of the method cannot be compared.

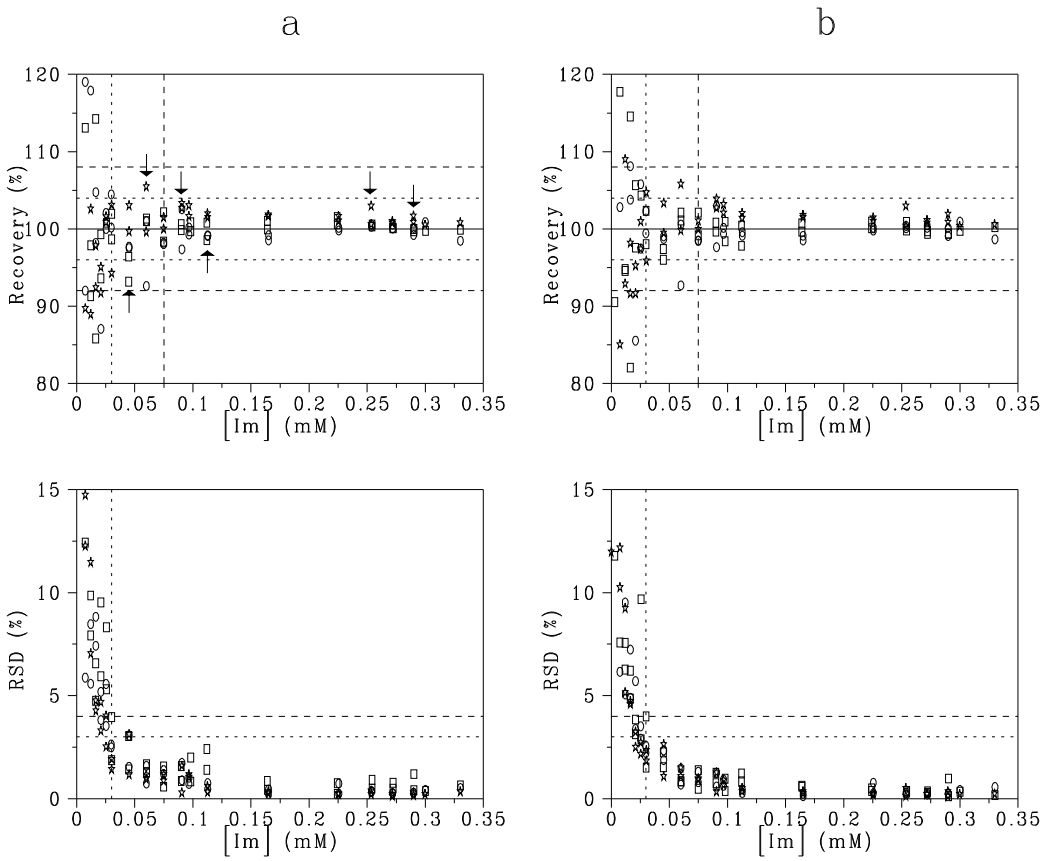


Figure 5.1: Accuracies and repeatabilities of measurement for a typical 4−7 wavelength combination in the final step (a) and the best 6−wavelength combination (b): tinidazole (☆), its 4−nitro isomer (○) and 2−methyl−4(5)−nitroimidazole (☐). Some sensitive points have been marked with an arrow.

*5.3.4. Cross−validation (**IV**)*

The single−wavelength and multi−wavelength UV spectrophotometric methods were cross−validated. Analysis of variance identified the analytical method (confidence level: 99.61 %) and the ionic strength of the tinidazole solution (96.99 %) as significant factors, whereas the analysis date was statistically non−significant (44.53 %). It was therefore concluded that these UV spectrophotometric methods are not equal; the multi−wavelength method always gave higher concentrations of tinidazole (typically by 2−3 %).

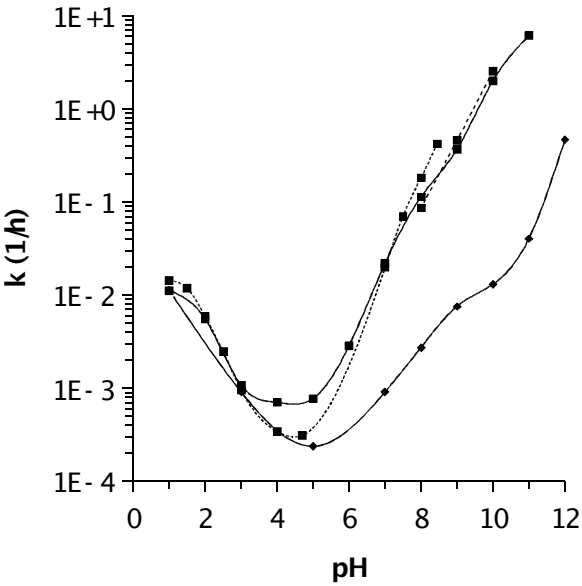


Figure 5.2: The pH degradation profiles of tinidazole (■; −−− paper **I**, — paper **III**, ··· paper **V**) and metronidazole (♦) [47].

5.4. pH degradation profile (I, III, V)

The hydrolysis of tinidazole followed apparent first−order kinetics in almost all of the studied cases (**I**, **III**−**V**); for exceptions, *cf.* Table 1 in paper **III**. It can be clearly seen that, at 80 °C, tinidazole is hydrolytically most stable at pH 4.0−5.0 (Figure 5.2). However, tinidazole has been reported to exhibit constant hydrolytic stability at 70 °C over the pH range 3.00−6.50

[61]. For comparison, metronidazole has maximum stability around pH 5.0 [34,47]. Despite slightly different reaction conditions [47], metronidazole clearly seems to be more stable in alkaline solutions than tinidazole. One possible reason for the difference was proposed (**III**) to be the intrinsic stability of metronidazole. It was considered (**III**) plausible that tinidazole would decompose by a different mechanism in basic and acidic conditions, while metronidazole would be degraded by a single mechanism, which could be identical to that of tinidazole in acidic conditions. This postulation was considered to be substantiated by the different product profiles of acid− and base−catalyzed hydrolysis of tinidazole. However, it was found (**III**) that the determined pH profiles (**I**, **III**) suffered from a lack of constant ionic strength, thereby warranting a study of the kinetic salt effect in order to gain an insight into the reaction mechanisms in question.

5.5. Activation energy (I, III)

The activation energy for the basic hydrolysis of tinidazole at pH 10.0 was calculated to be 130.9 kJ/mol (**I**) and 122 kJ/mol (**III**) (31.3 kcal/mol and 29.2 kcal/mol, respectively). These values are in good agreement with each other, as well as with a recent study [61] that reported a value of 133 kJ/mol at pH 5.25. For the hydrolysis of metronidazole, the activation energy at pH 8.0 is 26.6 kcal/mol [47].

5.6. Reaction mechanisms (V)

It was shown that, in all the studied cases, within the pH range of 1.00−8.45 at least one of the reacting partners contributing to the transition state in the hydrolysis of tinidazole had a charge of 0 in the major degradation pathway. In alkaline conditions (pH ≥ 7) tinidazole is mainly decomposed through reaction between the uncharged tinidazole and the hydroxide ion. In mildly acidic conditions (around pH 4.5), the course of the reaction is governed by solvent−assisted degradation of the nitroimidazole moiety in the uncharged tinidazole. In more acidic conditions, the nitroimidazole structure is also decomposed, but it was not possible to elucidate whether it is tinidazole in the charged or uncharged form that reacts, or both.

The mechanism in Scheme 1 in paper **V** was proposed for the alkaline hydrolysis of tinidazole (Table 3 in paper **V**). A stable encounter pair between the hydrated hydroxide ion (4 H_2O)

[113] and tinidazole was present in the gas phase in a micro−hydrated environment. The transition state described a proton transfer to the hydroxide ion. The deprotonated tinidazole was then able to decompose to ethyl vinyl sulfone and the deprotonated 2-methyl−4(5)-nitroimidazole through another transition state. The activation energy of the hydrolysis reaction could not be reproduced at the calculation level used. Although the scheme involves a cascade of reactions, it cannot be excluded that the proton transfer and formation of ethyl vinyl sulfone may take place simultaneously.

5.7. Hydrolysis products (I−V)

The formation of 2−methyl−4(5)−nitroimidazole was not of the first−order, apparently because of the small amounts of the 4−nitro isomer formed at the same time (**I**, **IV**, **V**); this was corroborated by the HPTLC study (**III**). Quantitation of the 4−nitro isomer in hydrolysis solutions was usually not possible because of the very small amounts formed (**I**, **IV**, **V**). These results were characteristic of the pH range 6.0−12.0 (**I**, **III**−**V**). Addition of the hydrolysis products did not significantly affect the hydrolysis rate of tinidazole (**IV**). At pH ≤ 5, the 4-nitro isomer and 2−methyl−4(5)−nitroimidazole were absent in hydrolyzed tinidazole solutions (**III**, **IV**). It should be further noted that, apart from tinidazole and the two hydrolysis products, no other 5−nitroimidazole species have been detected in hydrolyzed tinidazole solutions within the pH range of 1−13 [36 and present studies **I**−**III**].

The hydrolysis of the 4−nitro isomer and 2−methyl−4(5)−nitroimidazole were studied (**IV**) at pH 10.0 and 80 °C. The decomposition rate of the 4−nitro isomer was approximately 30 times slower than that of tinidazole; the reaction yielded an almost quantitative amount of 2-methyl−4(5)-nitroimidazole and only a very small amount of tinidazole. 2-methyl−4(5)-nitroimidazole did not decompose at all under the test conditions.

6. Conclusions

An HPTLC method and two UV spectrophotometric methods were developed for the analysis of hydrolyzed tinidazole. The HPTLC method was shown to be stability indicating and robust. Tinidazole and its two hydrolysis products were stable enough before, during, and after chromatography to enable analyses to be carried out easily. The method was found to be free of any large random errors. It had a slight positive bias, which could be overlooked because of the possible experimental errors in the kinetic studies. Thus, the HPTLC method could be used reliably for monitoring the hydrolysis kinetics of tinidazole.

The UV spectrophotometric methods were stability−indicating and robust. The single−wavelength method is suited for analyzing reactions taking place within the pH range of 1−5, whereas the multi−wavelength method can be used for tinidazole solutions hydrolyzed at pH 6−12. The multi−wavelength method gave validation parameters comparable to the HPTLC method, but it lacked the positive bias. Both methods could be reliably used for kinetic purposes, although cross−validation indicated that they produce statistically different results. The feasibility of systematic wavelength searching in developing multi−wavelength spectrophotometric methods was demonstrated using the precision and accuracy of validation samples as selection criteria.

The hydrolysis of tinidazole followed apparent first−order kinetics in most of the conditions studied. At 80 °C, tinidazole was hydrolytically most stable between pH 4.0−5.0, and it was clearly more labile in alkaline conditions than metronidazole, which is the most frequently used 5−nitroimidazole. Over the pH range 1.00−8.45, at least one of the reacting partners contributing to the transition state had a charge of 0 in the major degradation pathway. The dominant reactions were tinidazole + OH^- at pH ≥ 7 and tinidazole + H_2O around pH 4.5. In more acidic conditions, the reaction tinidazole + H^+ and/or $[\text{tinidazole}+H]^+ + H_2O$ took place; the existence of $[\text{tinidazole}+H]^+ + H^+$ could be neither excluded nor corroborated. The suggested alkaline hydrolysis mechanism involved a proton transfer from tinidazole to the hydroxide ion and the formation of ethyl vinyl sulfone. The reactions may occur either in a step−wise manner or simultaneously. The reaction was qualitatively verified by computational methods. The activation energy of the hydrolysis reaction, which was determined to be *ca.* 120−130 kJ/mol, could not be reproduced.

The formation of 2−methyl−4(5)−nitroimidazole over the pH range 6−12 was not of first−order, apparently because of the small amounts of the 4−nitro isomer of tinidazole formed at the same time. At pH ≤ 5, these products could not be detected. Apart from tinidazole and the two hydrolysis products, no other 5−nitroimidazole species were detected in hydrolyzed tinidazole solutions over the pH range 1−12.

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8. References

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