Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 523–536
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Two stability-indicating UV spectrophotometric methods for the analysis of hydrolyzed tinidazole

Jukka-Pekka K. Salo *, Hannele Salomies

Division of Pharmaceutical Chemistry, Department of Pharmacy, P.O. Box 56 (Viikinkaari 5 E), FIN-00014 University of Helsinki, Helsinki, Finland

Received 15 May 2002; received in revised form 12 August 2002; accepted 19 October 2002

Abstract

Two UV spectrophotometric methods have been validated for the analysis of hydrolyzed tinidazole solutions. The pH of the samples must be 5.00–7.00 for both methods. The multiwavelength method may be used for samples degraded at pH 6–12 if the amount of conserved 5-nitroimidazole species is at least 93 mol.% of the original; the amounts of tinidazole and its two known impurities may be determined simultaneously. The accuracy was within 100 ± 8% and the repeatability of measurement was ⩽4% at 0.03–0.33 mM, which was the measuring and calibration range. The LOD and LOQ were determined to be 0.003–0.012 mM and 0.0274 mM, respectively. The single-wavelength method may be used for samples degraded at pH 1–5, at least until approximately 50–60% of the original tinidazole has decomposed; only the amount of tinidazole can be determined. The solutions were measured at 318 nm, and quadratic fitting was used for the calibration. The mean accuracies varied between 98.0 and 101.8% and all RSDs were ⩽1.7% for the precision at 0.041–0.15 mM, which was the measuring range. The calibration range was 0.049–0.12 mM. The LOD and LOQ were 0.25 and 0.75 μM, respectively. The two methods were shown to produce statistically different results by cross-validation. At pH 10.0, tinidazole was decomposed approximately 30 times faster than its 4-nitro isomer; 2-methyl-4(5)-nitroimidazole was stable during the study.

Keywords: Hydrolysis; Multiwavelength techniques; Tinidazole; UV spectrophotometry; Validation

1. Introduction

Used against trichomoniasis, giardiasis, and amoebiasis, tinidazole (**1**) (1-[2-(ethylsulfonyl)-ethyl]-2-methyl-5-nitroimidazole) is susceptible to both hydrolysis and photolysis. It is known to hydrolytically decompose in alkaline conditions to the 4-nitro isomer (**2**) and 2-methyl-4(5)-nitroimidazole (**3**) [1] (Fig. 1); the amount of 5-nitroimidazole species is conserved in the reaction [2]. These decomposition products are also the only specifically named structurally related impurities (B and A, respectively) limited by the monograph of **1** in the European Pharmacopoeia [3]. Several analytical methods have been described for 5-nitroimidazoles in the literature [4–6], including

* Corresponding author. Tel.: +358-9-19159196; fax: +358-9-19159556.
E-mail address: jpsalo@cc.helsinki.fi (J.-P.K. Salo).

0731-7085/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0731-7085(02)00667-2

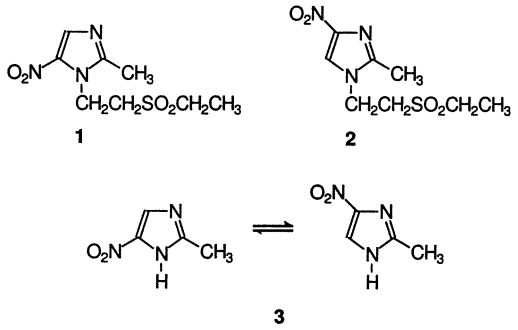

Fig. 1. The structures of tinidazole (**1**) and its 4-nitro isomer (**2**) as well as the tautomeric structures of 2-methyl-4(5)-nitroimidazole (**3**).

HPLC [7–9] and HPTLC [10–12] for the analysis of **1**. Spectrophotometric methods for the analysis of **1** in pure form or in formulations include both UV [13–15] and visible (Vis) [4,16,17] spectrometry; all methods rely on single-wavelength measurements.

There is obviously a lack of a multiwavelength UV(–Vis) spectrophotometric method for the analysis of (hydrolyzed) samples of **1**. Although HPLC [7] and HPTLC [2,11] are well suited for the purpose, the use of UV(–Vis) spectrophotometry could possibly provide even faster and simpler analyses, providing that there exist only quite small amounts of spectrally interfering compounds. The most obvious wavelength range around the maxima at 309–318 nm (Fig. 2d) is in practice useless for the purpose (unpublished results). However, there exists a 6-wavelength combination between 240 and 290 nm yielding acceptable accuracy and repeatability of measurement for quantitative work (unpublished results). It is the purpose of the present work to show by validation that a solid analytical method may be based on this wavelength combination for analyzing tinidazole solutions hydrolyzed at pH 6–12. Further, another UV spectrophotometric, single-wavelength method has been validated for the analysis of tinidazole solutions hydrolyzed at pH 1–5, and the two methods have been cross-validated for unhydrolyzed samples of **1**. Also, their suitability for analyzing samples of varying ionic strength is discussed.

2. Experimental

2.1. Materials

Tinidazole (**1**) was kindly supplied by Orion Pharmaceutica (Espoo, Finland). The commercially available 2-methyl-4(5)-nitroimidazole (**3**) was from Aldrich-Chemie (Steinheim, Germany). 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. The 4-nitro isomer of **1** (**2**) was prepared by the current authors as described in Ref. [18]; its identity and purity were verified by HPTLC as well as with UV and IR spectrometry by comparing with a batch of known [7,11] identity. All other chemicals and reagents were of analytical grade.

2.2. Apparatus

The UV-spectrophotometric determinations were carried out using a Unicam UV2-300 spectrophotometer (Unicam Ltd, Cambridge, UK). pH measurements were performed at ambient temperature with a Radiometer PHM83 Autocal pH meter (Radiometer A/S, Copenhagen, Denmark) equipped with a standard glass–calomel combination electrode or with a WTW inoLab pH Level 1 equipped with a WTW pH-Electrode SenTix 81 (WTW, Weilheim, Germany); commercial pH standard buffers (pH 1.09, 1.679, 4.005, 7.000, and 10.00) were used as appropriate. All results were calculated within the Dataplot [19] program package run under RED HAT LINUX 6.2 (Red Hat Inc., Durham, NC).

2.3. UV spectra and measurements

2.3.1. pH dependence

The spectra of 0.1758 mM **1**, 0.2030 mM **2**, and 0.2250 mM (however, 0.1131 mM at pH 10–12) **3** were recorded between 220 and 400 nm (or 220–460 nm for **3** at pH 8–12) (Fig. 2) in 0.1 M phosphate buffers, whose pH was adjusted within $\pm$0.01 units of the claimed value, at 1.0 nm intervals using a spectral bandwidth of 1.0 nm and a scan speed of 30 nm min^{-1} against 0.1 M

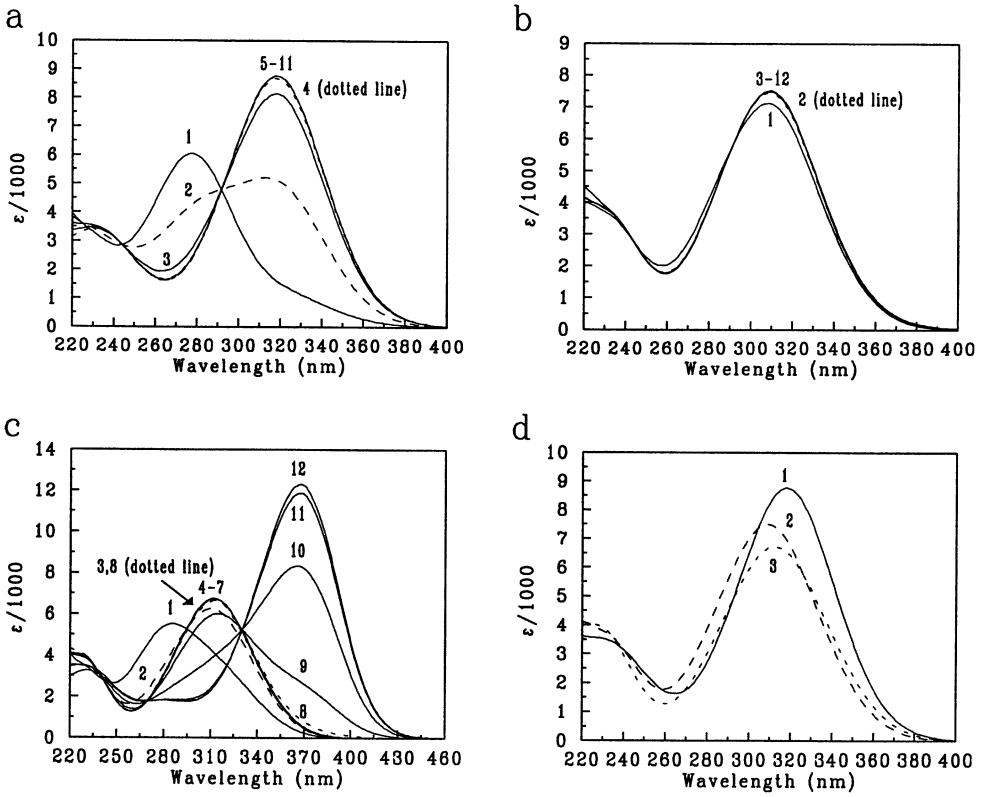

Fig. 2. The spectra at various pH values (given in panes a–c) of **1** (a); **2** (b) and **3** (c) as well as the spectra of the un-ionized species (d).

phosphate buffer (pH 6). The isosbestic points were determined visually from enlarged spectra.

2.3.2. Ionic strength

The spectra (not shown) of 0.1762 mM **1**, 0.2038 mM **2**, and 0.2251 mM **3** in 0.1 M phosphate buffer (pH 6.0) were recorded in the above mentioned conditions. The ionic strengths adjusted with sodium chloride to approximately 0.1, 0.25, 0.4, and 0.5, four solutions of each compound were studied.

2.3.3. Other compounds

The spectra (not shown) of freshly prepared 14.67 and 0.1408 mM sodium nitrite, 6.667 mM *o*-phosphoric acid, 6.667 mM citric acid, and 11.45 mM *o*-boric acid in water were determined in the above mentioned conditions except for the scan speed which was controlled by the intelliscan functionality. The spectrum of ethyl vinyl sulfone was taken from the Ref. [20].

2.3.4. Multiwavelength method

The spectra of the standards and the samples were recorded between 240 and 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^{-1}. For the quantitations, the absorbances at 6 wavelengths, namely 240, 242, 246, 248, 254 and 270 nm, were used (unpublished results). Made from stock solutions by diluting with 0.1 M phosphate buffer (pH 6), 9 freshly prepared standards (Table 1) were analyzed with the samples; an appropriate amount of the citrate–phosphate–borate buffer [21] was added to the standards before dilution. The analytical results were calculated by solving the matrix equation $A = KC$, first for K in the calibration step and then for C.

Table 1
The concentrations (mM) of **1**–**3** in the standard solutions

Compound	S1	S2	S3	S4	S5	S6	S7	S8	S9
1	0.329	0	0	0.0301	0.0602	0.209	0.0451	0.151	0.106
2	0	0.330	0	0.210	0.0302	0.0599	0.106	0.0448	0.151
3	0	0	0.330	0.0599	0.211	0.0301	0.151	0.105	0.0450
Σ	0.329	0.330	0.330	0.300	0.301	0.299	0.302	0.300	0.302

The condition number of the design matrix is 1.4.

2.3.5. Single-wavelength method

The absorbances of the standards and the samples were measured at 318 nm against a reagent blank using a spectral bandwidth of 1.5 nm and an integration time of 1 s. Made by diluting 240–600 μl (60 μl increments) of a 5 mM stock solution of **1** in water to 25.0 ml with a 0.1 M phosphate buffer (pH 6), 7 freshly prepared standards were analyzed with the samples. A second-order polynomial, $A = a_0 + a_1c + a_2c^2$, was used for the calibration.

2.4. Validation of the multiwavelength method

In general, the ICH Guidelines [22] were followed. Specifically, the following methods were used.

2.4.1. Accuracy and repeatability of measurement

A set of 24 validation samples in 0.1 M phosphate buffer (pH 6) (unpublished results) was analyzed using the present method. The nominal tinidazole concentration in unhydrolyzed samples being 0.3 mM, each of the three 5-nitroimidazoles was studied within the concentration range of 0.003–0.33 mM. Each sample was analyzed 7 times by changing the solution in the cuvette between measurements (repeatability of measurement), and the accuracy was calculated as the mean accuracy ($N = 7$). Both values were calculated for each compound at each concentration studied (Fig. 3).

2.4.2. Repeatability of the method

Seven samples were prepared by diluting 3.0 ml of a stock solution, containing approximately 1.67 mM of each 5-nitroimidazole, to 50.0 ml with 0.1 M phosphate buffer (pH 6) ([Im] = 0.1 mM for each of **1**–**3**); an appropriate amount of the citrate–phosphate–borate buffer was added to the samples.

2.4.3. Intermediate precision

On separate days during a period of 12 days, 8 samples were prepared, as described in Section 2.4.3.

2.5. Validation of the single-wavelength method

The validation scheme aims to follow the ICH Guidelines [22].

2.5.1. Linearity

A 7-point calibration graph in the range of 0.049–0.12 mM tinidazole (**1**) was constructed, with the lowest, middle, and highest points measured in triplicate; the every-day working graphs were constructed without the replications. Analysis of variance (ANOVA) was used to analyze the replication case.

2.5.2. Accuracy and precision

Accuracy was assessed as the mean percent recovery ($N = 7$); the relative standard deviation (in %) of the recoveries was taken as the appropriate type of precision. Repeatability of measurement was studied at five concentrations by measuring seven aliquots of the same solution: 83.3% of the lowest calibration point; lowest, middle, highest calibration points; and 120% of the highest calibration point.

For the remaining tests, five stock solutions were prepared, from which the samples were diluted as described for 0.6 mM tinidazole (**1**)

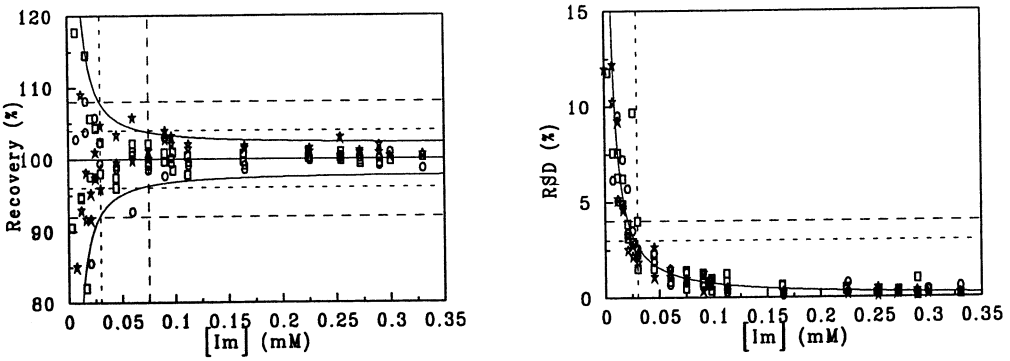

Fig. 3. Accuracies and repeatabilities of measurement for the multiwavelength method: **1** (★); **2** (○) and **3** (□). For the lines and curves, see Section 3. [Im] = concentration of a 5-nitroimidazole (mM).

solutions below. The final sample concentrations were identical to the ones for the repeatability of measurement.

Repeatability of the method was studied by preparing 7 separate samples, each of which was analyzed once. Intermediate precision was studied by preparing the samples on 7 separate days. The short-time repeatability of the spectrophotometer was confirmed by calculating the pooled standard deviation of absorbances for the ANOVA replication case (no associated accuracy).

2.5.3. LOD and LOQ

Detection ($q = 3.3$) and quantitation limits ($q = 10$) were calculated as $q\sigma/S$, where σ was estimated by the standard deviation of the absorbances of 30 blank samples, S was $\varepsilon_{318} \approx 8889$ ($n = 20$) for **1** (Table 2).

2.6. Cross-validation

Five solutions of 0.6 mM tinidazole (**1**) in 0.1 M phosphate buffer (pH 2.00) with varying ionic strengths (0.30–0.90) were prepared at ambient temperature. Two aliquots of each solution were taken (one aliquot for each analytical method), prepared like authentic samples (cf. below) and analyzed. The procedure was repeated after 7 days. A 3-way ANOVA was performed to discover the effects on the analytical result of the chosen analytical method (2 levels), analysis date (2), and the ionic strength of the tinidazole (**1**) solution

Table 2
UV data for the evaluation of spectral interferences

Structure	Medium	λ_{max} (nm)	ε_{max} (1 mol^{-1} cm^{-1})	Reference
Tinidazole (**1**)	0.1 M phosphate buffer (pH 7)	318	8889 ± 74[a]	Present work
Sodium nitrite	Water	355	23[b]	Present work
Non-conjugated nitro group	Not available	275	200	[35]
Nitrobenzene[c]	Not available	270	800	[35]
Non-conjugated sulfonyl group	Not available	< 180	Not available	[35]
Ethyl vinyl sulfone[d]	Ethanol	208–212	280	[20]
C=C–C–C=O[e]	Not available	290	110	[35]

[a] Mean ± S.D. ($N = 20$).
[b] $\varepsilon_{240} \approx 358$, $\varepsilon_{318} \approx 10$.
[c] Representative of a highly conjugated species.
[d] Representative of a conjugated sulfonyl compound, $\varepsilon_{240} \approx 3$.
[e] Approximation to the 5-keto tautomer of the proposed [30] 5-hydroxyimidazole structure.

(5). Thus, the significance of the factors were estimated by the F test.

2.7. Sample preparation for 0.6 mM solutions of *1*

2.7.1. Multiwavelength method

10.0 ml of the (hydrolyzed) solution was added to 5 ml of 0.1 M phosphate buffer (pH appropriate to ensure a final pH of 6.0 ± 0.7) in an ice-water bath. After cooling to ambient temperature, the mixture was diluted to 20.0 ml with the same buffer.

2.7.2. Single-wavelength method

Five milliliter of the (hydrolyzed) solution was added to 10 ml of 0.1 M phosphate buffer (pH appropriate to ensure a final pH of 6.0 ± 0.7) in an ice-water bath. After cooling to ambient temperature, the mixture was diluted to 25.0 ml with the same buffer.

2.8. Hydrolysis at pH 10

Five millimoler solutions of each 5-nitroimidazole were prepared in a citrate–phosphate–borate buffer (pH 10.0) [21] by dissolving the solid in 50.0 ml of the buffer solution at 80 ± 0.5 °C in a 100 ml volumetric flask submerged in a water bath kept at the same temperature. 3.0 ml aliquots of the hydrolyzed solution were drawn at intervals (seven aliquots in total), added to 25 ml of 0.1 M phosphate buffer (pH 5.8) in an ice-water bath and, after cooling to ambient temperature, diluted to 50.0 ml with the same buffer (final pH 5.9). Additionally, the effect on the hydrolysis of 5 mM **1** of 0.5 mM **2** and **3**, respectively, was studied.

2.9. Kinetic results

Data points with calculated concentrations below the respective quantitation limit, if any, were discarded from the kinetic considerations. The decision between zero- and first-order kinetics was made on the basis of the correlation coefficient, the residual standard deviation, the magnitude of the residuals, and the visually evaluated distribution of the residuals.

3. Results and discussion

3.1. UV spectra

3.1.1. pH dependence

Tinidazole (**1**) has a pK_a value of 1.82 [23]; a computational [24] estimate was 2.34 ± 0.35. The protonation of the aromatic imidazole ring strongly reduced the absorbance at $\lambda_{max} = 318$ nm (Fig. 2a), which is due to the conjugated nitroimidazole structure; the observed maxima were in accordance with previous results [25]. The isosbestic points were found at 244 nm and 291–292 nm. Between 220 and 400 nm, the spectrum of **1** remained unchanged at pH 5–7. As compared to pH 5–7, changes were seen at pH 4 (throughout the studied wavelength range) and pH 11 (below 240 nm); furthermore, basic pH enhances the decomposition of tinidazole [1,2,7,15]. Spectral changes and stability considered, pH 5.00–7.00 provides a safe range for the UV-spectrophotometric determination of **1**.

No pK_a data has been found in the literature for the 4-nitro isomer of tinidazole (**2**), but a value of -0.53 has been reported [26] for 1-methyl-4-nitroimidazole (2.13 for 1-methyl-5-nitroimidazole [26]); a computational [24] estimate for **2** was 0.28 ± 0.38. The lower pK_a value was reflected in the fact that the spectrum of **2** at pH 1 was altered to a comparable extent as the spectrum of **1** at pH 3 (Fig. 2a–b) when compared to the spectra of the un-ionized species. The spectra at pH 4–11 were identical, and $\lambda_{max} = 309$ nm; a value of 307 nm has been reported previously [25]. The isosbestic points were at 243–244 nm and 291 nm, practically at the same wavelengths as for **1**. **2** was much more stable (Section 3.6) in basic conditions than **1**. Nevertheless, pH 4.00–7.00 would be the optimal sample pH for **2**.

2-Methyl-4(5)-nitroimidazole (**3**, below denoted by BH) is an ampholyte. Of several (different) pK_a values reported in the literature, 1.1 ± 0.1 and 9.7 ± 0.1 [27] for the HBH^+/BH and BH/B^- equilibria, respectively, are quoted here since they coincide well with the computational [24] values of 0.79 ± 0.11 and 9.66 ± 0.50, respectively. The pure spectrum of the uncharged species was seen at pH 4–7 with $\lambda_{max} = 312$ nm (Fig. 2c); a

value of 310 nm has been reported previously [25], The isosbestic point for the BH/B^- equilibrium was at 330 nm, and for the HBH^+/BH equilibrium at 241 nm and 294–295 nm. The absorption maximum of the negatively charged species was at 367 nm, previously reported values are 361 ± 2 nm [14] (although the measurements took place at 368 ± 2 nm!), 363 nm [25], and 368 nm [15]; there is appreciable absorption well above 400 nm causing a yellow discoloration for alkaline solutions containing **3**.

1, therefore, imposes the strictest demands on the pH of any hydrolyzed solution whose constitution should be analyzed UV spectrophotometrically. Purely spectral properties lead to the demand that the pH of such solutions be 5.00–7.00; it is luckily for the analyst also a pH range of good analyte stability [2].

3.1.2. Ionic strength

The spectra of **1** were visually identical between 220 and 270 nm. At longer wavelengths, the spectrum at the largest ionic strength was below the others until the absorption maximum, and above the others at longer wavelengths still. At 318 ± 3 nm, the absorbances of the solutions were unaltered; nor was the absorption maximum shifted at the studied ionic strengths. In the spectra of **2**, there were clear changes even between 240 and 270 nm. Below the unshifted minimum at 259 nm, the spectrum at the smallest ionic strength was the weakest, and above the minimum it was the strongest. At 309 nm and beyond, the spectra at the larger ionic strengths were clearly stronger. The changes between 240 and 270 nm in the spectra of **3** are more subtle than those for **2**. Below the minimum at 259–260 nm, the spectrum at the largest ionic strength was the strongest, and above the minimum it was the weakest. At 312 ± 3 nm, the absorbances of the solutions were unaltered. At 320 nm and beyond, the spectrum at the largest ionic strength was clearly the strongest. Based on these findings, some problems are expected to emerge with the quantitation of all three analytes (**1**–**3**) at higher ionic strengths. It is easy to verify from $C = (K^T K)^{-1} K^T A$ (the least-squares solution to $A = KC$) that, if the spectrum of even one compound is altered, the calculated concentrations of all compounds will be different; it must be remembered that K is not affected by the ionic strength of the samples.

3.2. Specificity

It has been shown [2] that only traces of the products of alkaline hydrolysis are present in acid-hydrolyzed (pH 1–7, 80 ± 0.5 °C) solutions of 1, whereas considerable amounts of the products are formed in basic solutions (pH 8–12, 60–80 °C) [2,7]; in fact, the total amount (in moles) of the 5-nitroimidazole species seems to be conserved at pH 8–12 [2].

A re-analysis of this raw data (not shown here) lets us further identify between two groups of pH values at pH 1–7. Based on both the calculated detection limits of the HPTLC method used [11] and the *de facto* absence of detected peaks, **2** and **3** were absent in solutions hydrolyzed at pH $\leqslant 5$. On the other hand, at pH 6–7 these products were formed, since they could be detected, although their absolute amounts remained small owing to short hydrolysis times. According to the results of the HPLC study [7] (not shown here), the total amount of 5-nitroimidazole species was conserved at pH 8–12, whereas the HPTLC study [2] showed some loss of total 5-nitroimidazoles in most cases at pH 6–12. However, the loss was always quite limited within the studied time spans. It should be further noted that apart from **1**–**3** no other 5-nitroimidazole species have been detected in hydrolyzed solutions of **1** at pH 1–13 [1,2,7,11].

3.2.1. Multiwavelength method

It is therefore concluded that the overwhelming majority of UV active species absorbing above 240 nm at pH 6–12 are the three 5-nitroimidazoles discussed here. The issue is very important since any unidentified UV active compounds in the samples would definitely cause interference in calculating the results. It is well documented [28,29] that in extreme basic conditions ($\geqslant 0.1$ M NaOH) *N*-substituted 5-nitroimidazoles can be degraded to produce equimolar amounts of nitrite; on the other hand, the other degradation products have not been identified. It has been proposed [30] that in some cases it might be the corresponding 5-

hydroxyimidazole. (To be exact, the tautomeric 5-keto compound is the stable tautomer in water [31,32]). It is then a question of whether the nitrite ion and/or the 5-keto compound are able to interfere with determinations between 240 and 270 nm. For the nitrite ion, $\varepsilon_{240} \approx 358$, which means that it contributes less than 1% to the total absorbance at 240 nm until 7.7 mol.% of the original 5-nitroimidazoles have decomposed (given equimolar production of nitrite). On longer wavelengths, the effect is weaker still. A similar calculation for the hypothetical 5-keto compound (Table 2) shows a conservative lower limit of 29 mol.% of the total 5-nitroimidazoles decomposed. Ethyl vinyl sulfone has been proposed [18] to exist in hydrolyzed solutions of **1** (the other decomposition product when **1** is degraded to **3**). It is by no means capable of interfering with the present multiwavelength method since it has a molar absorptivity of $\varepsilon_{240} \approx 3$ [20]. Of the solvents and buffer species discussed here, only citric acid is able to absorb above 240 nm at the concentrations used. Since $\varepsilon_{240} \approx 14$ for citrate, it causes an absorbance of 0.006 at the concentration present in the samples (0.4 mM), and it may be compensated for by the addition of an equivalent amount of citrate in the blank and standard solutions. In interpreting the situation, one should remember that strong interference by the nitrite and/or a nitro compound is to be expected in acid-hydrolyzed solutions (Fig. 4), and the multiwavelength method should only be used for the analysis of solutions stored at pH 6–12 if the amount of conserved 5-nitroimidazole species is at least 93 mol.% of the original.

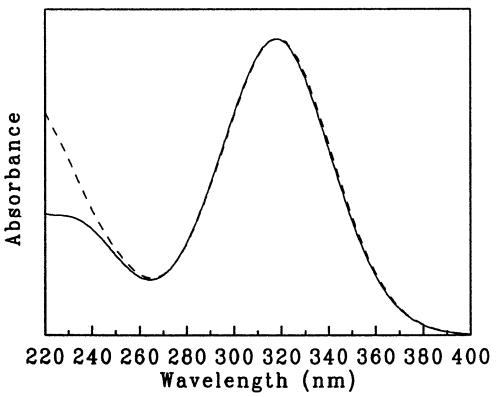

Fig. 4. Normalized spectra of intact (—) and hydrolyzed (– – –) (pH 2.00, 80 ± 0.5 °C, $\mu = 0.90$, 90 h) solutions of **1**.

3.2.2. *Single-wavelength method*

The only conceivable hydrolysis products of **1** at pH $\leqslant 5$ involving ring-opening or the loss of the nitro group, and therefore without an absorption maximum at around 318 nm, would be the nitrite ion and the resulting hydroxyimidazole [30] (or its keto tautomer), or the products would contain a conjugated or a non-conjugated nitro or sulfonyl group. Their amounts in the reaction mixture can at most be equal to the moles of tinidazole decomposed. It is clear from Table 2 that the putative sulfonyl moieties cannot compromise the measurements at 318 nm. On the other hand, if one makes the reasonable assumption that the spectrum of a non-conjugated nitro compound at $\lambda \geqslant 275$ nm may be approximated—in terms of relative absorptivities—by the spectrum of **1** at $\lambda \geqslant 318$ nm, a simple calculation reveals that this hypothetical hydrolysis product contributes less than 1% to the total absorbance at 318 nm until 70% of the original **1** has decomposed; interference by the more simple nitrite ion would be weaker still (90%). A similar approach with nitrobenzene yields a limit of 45% tinidazole decomposed. (One should bear in mind that nitrobenzene as an aromatic species overestimates the effect of the hypothetical conjugated nitro compound in this context.) The spectrum of a hydrolyzed solution of **1** at $\lambda < 260$ nm resembles that of the nitrite ion (Fig. 4), and it is quite safe to assume that a species with a conserved nitro moiety is indeed present in acid-hydrolyzed solutions of **1**. Finally, a limit of 60% tinidazole decomposed can be calculated for the predicted 5-keto tautomer. In interpreting these results, one should remember that—since the products would be formed by different pathways—1) only some of these feasible degradation products may be formed in equimolar amounts, or 2) products of several pathways may be present at a lower concentration than the equimolar amount of degraded **1**. Therefore, it is safe to claim that the single-wavelength UV-spectrophotometric method of the present work possesses the specificity required [22] of an analy-

tical method for investigations of hydrolyzed solutions of **1** at pH 1–5, at least until approximately 50–60% of the original **1** has decomposed.

3.3. Validation of the multiwavelength method

3.3.1. Accuracy

The mean accuracies for each 5-nitroimidazole at different concentration levels are shown in Fig. 3, from which it can be seen that these values were $100 \pm 8\%$ for [Im] $\geqslant 0.03$ mM, and apart from 2 values at [Im] = 0.06 mM, $100 \pm 5\%$; all mean accuracies were $100 \pm 4\%$ for [Im] $\geqslant 0.075$ mM. It may be stated that this method showed an accuracy comparable to the accuracy at low-range of the previously reported [11] HPTLC method. All the individual accuracies calculated fell between $100 \pm 10\%$ for [Im] $\geqslant 0.03$ mM and between $100 \pm 5\%$ for [Im] > 0.11 mM. These values are very adequate for kinetic purposes, and they totally lack deviations $> 10\%$ seen with the HPTLC method [11]. As Fig. 3 shows, the present method seemed to lack the positive bias seen with the HPTLC method [11], as well.

3.3.2. Precision

The repeatability of measurement was better than 3% for [Im] $\geqslant 0.03$ mM apart from one value which lied below the 4% limit (Fig. 3). Comparable repeatabilities for the HPTLC method [11] were calculated at approximately mid-range, and they varied from 0.40 to 1.8%. For the present method, the repeatability of measurement at mid-range, [Im] = 0.16–0.17 mM, was well below 1% (Fig. 3). In this respect, these two methods are similar.

The repeatability of the method (RSDs with ranges for observed individual accuracies) was 1.1% (99–102%), 2.5% (93–101%), and 1.5% (99–104%) for **1**–**3**, respectively. It can be seen that only for **2** were the results clearly worse than the corresponding value for the repeatability of measurement (Fig. 3). It is also evident that the accuracies were only slightly inferior to those in Fig. 3. Due to methodological differences, the present values cannot be compared with the HPTLC method [11].

Measuring day-to-day variability, the intermediate precision (RSDs with ranges for observed individual accuracies) was 1.3% (97–102%),1.6% (95–101%), and 1.0% (98–103%) for **1**–**3**, respectively. The results were partly better than those for the repeatability of the method, no apparent reason for this could be identified. These values were clearly better than those for the HPTLC method ('within-laboratory reproducibility') [11].

3.3.3. Linearity and range

It was hard to estimate the linearity of the method in other terms than the residuals of the 24 calibrations performed using this method. The residuals were plotted against the concentration of each 5-nitroimidazole (not shown), and the graphs were evaluated visually. Although the residuals were not entirely random, no obvious patterns could be recognized, either. On the other hand, there were calibrations whose residuals were almost randomly distributed, too. It should also be emphasized that the residuals at 0 concentration were of the same magnitude as at other concentrations. Based on this very elementary analysis, the responses of the compounds were considered linear throughout the studied range 0–0.33 mM. Since the range of the method is dictated by acceptable linearity, accuracy and precision [22], it was taken to be 0.03–0.33 mM for each of the three 5-nitroimidazoles.

3.3.4. Detection and quantitation limits

A set of standard samples at low concentrations (around the LOD and LOQ, respectively) is required for the determination of the detection and quantitation limits. Since the validation samples used for determining the accuracy and the precision covered concentrations down to [Im] = 0.003 mM (actually, [Im] = 0 mM was represented for each 5-nitroimidazole, too), the same set was used for this task, as well. It is evident from Fig. 3 that the repeatabilities depended on [Im] in a hyperbolic manner. An iterative fit for $\mathrm{RSD}(\%) = p_0 + p_1/[\mathrm{Im}]^{p_2}$ yielded the constants (with approximated standard deviations) $p_0 = 0.08180$ (0.8818), $p_1 = 0.04991$ (0.04698), and $p_2 = 1.131$ (0.1583) with a residual standard deviation of 5.889 (df = 109) and a replication standard deviation of 4.888 (df = 52). The lack-of-fit F ratio was 1.863 (98.78%). Although p_0 and p_1 were

statistically equal to 0 at the 95% level of confidence, it is evident from Fig. 3 that this model describes the data points reasonably well even if there was a statistically significant lack of fit. According to this model, the 3% repeatability cut-off point is at 0.0274 mM.

The pattern for the mean accuracies was not as clear cut. However, one can see that the observations can easily be grouped in terms of [Im]. Furthermore, one would expect from Fig. 3 that the extreme values, i.e. the minima and maxima in each group, respectively, would approximate a hyperbola each. The extrema were treated as one set of values by reflecting the maxima in respect of the line $y = 100$. An iterative fit for $\text{Accuracy}_{\text{min}}(\%) = p_3 + p_4/[\text{Im}]^{p_5}$ yielded the constants (with approximated standard deviations) $p_3 = 97.91$ (4.032), $p_4 = -0.04923$ (0.08418), and $p_5 = 1.355$ (0.2861) for the lower band, and therefore $p_3' = 102.1$, $p_4' = 0.04923$, and $p_5' = 1.355$ for the upper band, with a residual standard deviation of 18.49 (df = 37) and a replication standard deviation of 8.399 (df = 4). The lack-of-fit F ratio was 5.311 (94.28%) meaning that the chosen model correctly describes the dependence on [Im] of the extremum values although p_4 was statistically equal to 0 at the 95% level of confidence. According to this model, some accuracy cut-off points are at 0.0671 mM (100 ± 4%), 0.0292 mM (100 ± 8%), and 0.0236 mM (100 ± 10%).

The limit of quantitation was taken as the minimum [Im] for which both the calculated recovery was within 100 ± 10% and the calculated repeatability $\leqslant 3\%$. Then, the LOQ was 0.0274 mM for each of **1**–**3**. It is easy to check visually (Fig. 3) that at concentrations above this value, the observed accuracies are indeed 100 ± 5% apart from two observations (which lie within 100 ± 10%) and the observed repeatabilities are $< 3\%$ with one exception (which is below the 4% limit). This is essentially the same value as was deduced when defining the range.

For the limit of detection, the calculated concentrations were plotted (not shown) against the known concentrations for each 5-nitroimidazole separately, and the detection limits were evaluated visually as the lowest concentrations at which all calculated values were positive. The approximate LODs were 0.003, 0.007 and 0.012 mM for **1**–**3**, respectively.

3.3.5. Robustness

Robustness 'should show the reliability of an analysis with respect to deliberate variations in method parameters' [22]. pH of the reaction mixtures as well as of the sample and standard solutions is a key element in devising a spectrophotometric method for the determination of an ionizable analyte. Some aspects have already been discussed under Section 3.2. To provide adequate safety margins, the pH of the samples and standard solutions should be adjusted between 5.30 and 6.70 with a 0.1 M phosphate buffer of appropriate pH. Another issue is the ionic strength of the samples since it was shown to influence some analyte spectra and it is known [33] to affect the pK_a values. Considering the ionic strengths of 0.1–0.5 in real samples, the latter effect is not an issue if it is ensured that the sample pH at high ionic strengths stays below 6.70. Lastly, it has been previously shown [11] that hydrolyzed samples (of comparable composition with the present study) are stable for at least 7 h at room temperature if kept in the dark. Therefore, all samples gathered during a working day can be analyzed at the end of the day.

3.4. Validation of the single-wavelength method

3.4.1. Linearity

According to a lack-of-fit F test ($\alpha = 0.05$), the straight line, $A = k_0 + k_1 c$ (for the replication case: $A = 8.941$ (±0.02734) $c + 0.002733$ (±0.002448); $r^2 = 0.9999$ ($r = 0.9999$); $\text{SS}_{\text{RES}} = 0.00007821$), was not an adequate model to describe the relationship between the measured absorbance, A, and the concentration of **1**, c, in the sample, whereas the parabola, $A = a_0 + a_1 c + a_2 c^2$ (for the replication case: $A = -3.707$ (±0.5953) $c^2 + 9.574$ (±0.1024) $c - 0.02155$ (±0.004068); $r^2 = 1.000$ ($r = 1.000$); $\text{SS}_{\text{RES}} = 0.00001603$), was. The question about the correct fitting strategy was therefore further studied under sections 3.4.2 and 3.4.3. The working range covered absorbances of about 0.43–1.1, which should be well within the linear range of the detector. Although the residuals were very small

(usually < 0.005 absorbance units) even in the case of a linear fit, the use of quadratic fitting reduced them still and removed their paraboloid distribution. On the other hand, one should bear in mind that it may have been explaining only noise.

3.4.2. Accuracy

The accuracies in the repeatability of measurement test (Table 3) were generally worse using the quadratic fit. However, in both cases the mean recoveries were 99–102%. The real value of the quadratic fit is seen in Tables 4 and 5. Although the quadratic fit provided slightly worse recoveries in the middle of the studied range, the recovery values were better at both lower and higher concentrations. A substantial enhancement in the quality of the results was seen at both extremes, which lie well outside the calibration range. This, especially the ability to reliably reach concentrations below the calibration curve, is an added value to an analytical method used for kinetic studies since it allows some flexibility in designing the experiment in respect of sampling intervals and total duration. Using quadratic fitting, the accuracies in both the repeatability of the method and the intermediate precision tests fell into the range of 98–100% over the entire studied range.

3.4.3. Precision

The good short-time stability of the spectrophotometer was reflected by the pooled standard deviation of 0.0009655 determined for the ANOVA replication case. When going from linear to quadratic fit, no dramatic changes were seen in the relative standard deviations (Tables 3–5) used to describe the different aspects of precision. In some cases the values were smaller, in others they were greater; in a few cases they remained (practically) the same. The overall tendency was that worst values were seen at the lowest concentration tested ($A \approx 0.355$) although they cannot be regarded as useless; the worst value seen was the decent 1.7% for intermediate precision at 0.04 mM using quadratic calibration.

3.4.4. Range

Taking into account the results and discussion presented under Sections 3.4.1, 3.4.2 and 3.4.3, the following conclusions may be drawn. The chosen calibration range of 0.049–0.12 mM was justifiable, and it provided for good precision and accuracy with quadratic fitting, Furthermore, quadratic fitting allowed the expansion of the measuring range to 0.041–0.15 mM without compromising the results.

3.4.5. Detection and quantitation limits

The measured responses of the blank samples are a time series whose statistical nature may be studied using standard tools [34]. The measured series was in statistical control, i.e. there was no drift with respect to location nor variation, and the measured absorbances were random, normally distributed values; no outliers could be detected. The standard deviation of the responses, used for the calculation of the detection and quantitation limits, was 0.0006663. The slope of the calibration was estimated by the mean of slopes from 20 linear calibrations (Table 2), and it was 8889 ($1\ mol^{-1}$ for a 1 cm cell). Using these values, the calculated detection limit for **1** was 0.2474 μM (corresponding to 0.0022 absorbance units), which is a very optimistic value since the range of the original data was -0.0013 to $+0.0022$ absorbance units. The

Table 3
Accuracy as mean recovery and repeatability of measurement (linear–quadratic fit) for the single-wavelength method

Level (mM)	Recovery range (%)		Mean recovery (%)		SD (%)		RSD(%)	
0.04063	101.3–101.9	101.5–102.1	101.6	101.8	0.21	0.21	0.20	0.20
0.04876	99.5–99.7	100.0–100.2	99.5	100.1	0.066	0.064	0.067	0.064
0.08532	99.8–99.9	99.4–99.5	99.8	99.5	0.047	0.047	0.047	0.047
0.1219	100.0–100.1	100.2–100.4	100.0	100.2	0.055	0.057	0.055	0.057
0.1463	99.8–100.0	100.7–100.9	99.9	100.8	0.064	0.067	0.064	0.067

Table 4
Accuracy as mean recovery and repeatability of the method (linear–quadratic fit) for the single-wavelength method

Level (mM)	Recovery range (%)		Mean recovery (%)		S.D. (%)		RSD (%)	
0.04066	97.0–97.6	98.2–98.8	97.4	98.5	0.25	0.25	0.26	0.25
0.05083	97.5–98.1	97.9–98.5	97.7	98.1	0.25	0.24	0.25	0.25
0.08133	97.9–98.4	97.7–98.2	98.2	98.0	0.18	0.18	0.18	0.18
0.1220	98.6–98.9	98.8–99.0	98.8	99.0	0.099	0.10	0.10	0.10
0.1525	98.6–99.1	99.4–100.0	98.8	99.6	0.20	0.20	0.20	0.21

calculated quantitation limit was 0.7495 μM (or 0.0067 absorbance units). Previous experience with this particular spectrophotometer suggests that approximately threefold values for both parameters would hold better. In any case, the lower ends of the calibration and measuring ranges were at 49 and 41 μM, respectively, and therefore well above the calculated LOQ.

3.4.6. Robustness

As stated under Section 3.2, this method should only be used to analyze reactions taking place at pH 1.00–5.00, where the product profile is known to lack any strong interferants at 318 nm. The requirements for sample and standard pH as well as for the storage of samples are identical to the multiwavelength method. Sample ionic strengths of 0.1–0.5 were well tolerated without spectral changes for **1** at 318 nm.

3.5. Cross-validation

One may justifiably argue that the cross-validation scheme used is biased. In fact, the proper experimental set-up would include several different tinidazole concentrations. This would, however, mean great practical difficulties since there does not exist a common pH for hydrolyzing a tinidazole solution for both methods. Therefore, one would be compelled either to analyze pure tinidazole solutions of varying concentrations or to add some amount of the alkaline hydrolysis products to the tinidazole samples analyzed with the multiwavelength method, in order to conserve the amount of total 5-nitroimidazoles. Either method would be susceptible to criticism as being unauthentic. After consideration, it was decided to resort to the methodology described in Section 2.

ANOVA identified the analytical method (confidence level: 99.61%) and the ionic strength of the tinidazole solution (96.99%) as significant, whereas the analysis date was statistically insignificant (44.53%). The results were validated by visually inspecting the residuals plotted against the predicted values and each of the three variables; the residuals appeared to be randomly and normally distributed. It was therefore concluded that the analytical methods described here are not equal, and the use of the multiwavelength method always resulted in a higher concentration of **1** (typically by 2–3%). Since both methods were found out to be valid for the intended purpose and the spectrum of **1** was shown to remain unaltered at the wavelengths used and at the ionic strengths present in

Table 5
Accuracy as mean recovery and intermediate precision (linear–quadratic fit) for the single-wavelength method

Level (mM)	Recovery range (%)		Mean recovery (%)		S.D. (%)		RSD (%)	
0.04066	95.7–100.0	96.8–101.5	97.6	98.8	1.5	1.6	1.6	1.7
0.05083	95.6–99.7	96.0–100.0	98.1	98.6	1.5	1.5	1.5	1.5
0.08133	98.0–99.9	97.7–99.6	98.8	98.6	0.92	0.89	0.93	0.90
0.1220	98.6–100.4	98.7–100.7	99.3	99.5	0.76	0.81	0.76	0.81
0.1525	98.3–100.3	99.1–101.5	99.1	100.0	0.63	0.83	0.64	0.83

the cross-validation samples, the underlying factors for this behavior should be established. The validation results give some explanations to the problem at hand. At concentrations of approximately 0.3 mM of **1**, recoveries of slightly above 100% with a very small RSD were achieved for the multiwavelength method (Fig. 3). On the other hand, the mean recoveries reported for the single-wavelength method with quadratic fit (Tables 3–5) tended to be slightly below 100% for 0.12 mM of **1**. (The quoted sample concentrations are the result of prescribed dilution of the original 0.6 mM solution.) Another explanatory factor may have been that the batch of **1** used was only about 99.9% pure, the rest being mainly **3** (determined by HPTLC using the pharmacopoeial [3] eluent). Since the spectrum of the impurity was shown to be affected by the ionic strength at the wavelengths used, one would expect greater interferences for a method using several wavelengths rather than one.

3.6. Kinetics at pH 10

1. showed a first-order rate constant (with approximated standard deviation) of 2.413 h^{-1} (0.07082 h^{-1}) during a 30-min decomposition. The calculated amount of **2** stayed below the LOQ and many times below the LOD. The amount of **3** grew steadily yet not according to either 0 or 1. order kinetics.

2. exhibited a first-order rate constant of 0.08127 h^{-1} (0.006463 h^{-1}) during a 6-h decomposition. The calculated amount of **1** stayed below or just above the LOD. The amount of **3** grew steadily yet not according to either 0 or 1. order kinetics.

3. did not decompose during a 6-h test.

In all the three experiments, the total amount (in moles) of the 5-nitroimidazole species remained constant. It is evident that the formation of **2** was very slow in decomposed solutions of **1**. It is equally clear that **2** decomposed approximately 30 times more slowly than **1** in the test conditions. Several reasons can be listed for the non-equimolar formation of **3** in decomposed tinidazole and isomer solutions; at least the following reactions may exist: 1) the decomposition of **1** and/or **2** to **3**, 2) isomerization of **1** to **2** and vice versa, 3) the formation of **1** and/or **2** from **3** and ethyl vinyl sulfone [18]. The ionic strength was not an issue in these tests.

0.5 mM **2** or **3** did not significantly alter the decomposition rate of 5 mM **1**, the first-order rate constants being 2.615 h^{-1} (0.1043 h^{-1}) and 2.312 h^{-1} (0.1203 h^{-1}), respectively. Again, **1** was mainly decomposed to **3**. There were some problems with maintaining a constant pH, and it was decided to discontinue the work with the citrate-phosphate-borate buffer [21]. Furthermore, the pK_a values of *o*-phosphoric acid are temperature insensitive [33] thereby insuring that the reactions take place at the claimed pH even at elevated temperatures.

Acknowledgements

We wish to thank Ms Inkeri Huttunen for carrying out the synthesis of **2**. J.-P.K.S. wishes to acknowledge the financial support by the Academy of Finland through the Graduate School in Pharmaceutical Research (Ministry of Education, Finland).

References

[1] H. Salomies, Acta Pharm. Nord. 3 (1991) 211–214.
[2] J.-P. Salo, H. Salomies, J. Pharm. Biomed. Anal. 14 (1996) 1267–1270.
[3] European Pharmacopoeia, Council of Europe, Strasbourg, (2001) 2039–2040.
[4] R.B. Patel, A.A. Patel, T.P. Gandhi, P.R. Patel, V.C. Patel, S.C. Manakiwala, Indian Drugs 18 (1980) 76–78.
[5] D. Tonelli, E. Gattavecchia, Boll. Chim. Farm. 123 (1984) 117–123.
[6] S. Ebel, M. Ledermann, B. Mümmler, Arch. Pharm. (Weinheim, Germany) 323 (1990) 195–200.
[7] H. Salomies, J.-P. Salo, Chromatographia 36 (1993) 79–82.
[8] M. Feng, H. Cao, F. Yu, Yaowu Fenxi Zazhi 17 (1997) 247–249 through Chem. Abstr. 129, 72321 (1998).
[9] X.Z. Zhang, Y.A. Zhou, Y. Peng, H.S. Cai, Sepu 14 (1996) 127–128.
[10] H. Salomies, J. Planar Chromatogr.—Mod. TLC 5 (1992) 291–293.
[11] J.-P. Salo, H. Salomies, J. Pharm. Biomed. Anal. 14 (1996) 1261–1266.

[12] M.H. Guermouche, D. Habel, S. Guermouche, J. AOAC Int. 82 (1999) 244–247.
[13] N.M. Sanghavi, N.G. Joshi, Indian J. Pharm. Sci. 41 (1979) 144–145.
[14] R.G. Bhatkar, C.V. Nagvankar, East Pharm. 25 (1982) 117.
[15] L. López-Martínez, F.J. Luna Vázquez, P.L. López-de-Alba, Anal. Chim. Acta 340 (1997) 241–244.
[16] C.S.P. Sastry, M. Aruna, D. Vijaya, Indian J. Pharm. Sci. 49 (1987) 190–192.
[17] C.S.P. Sastry, M. Aruna, A. Rama Mohana Rao, Talanta 35 (1988) 23–26.
[18] A.K.S.B. Rao, R.S. Prasad, C.G. Rao, B.B. Singh, J. Chem. Soc., Perkin Trans. 1 7 (1989) 1352–1353.
[19] J.J. Filliben, A. Heckert, Dataplot v. 8/1999. Computing and Applied Mathematics Laboratory, National Institute of Standards and Technology, Gaithersburg, MD (1999) available from http://www.itl.nist.gov/div898/software/dataplot.html.
[20] E.A. Fehnel, M. Carmack, J. Am. Chem. Soc. 71 (1949) 231–237.
[21] K. Diem, C. Lentner (Eds.), Scientific Tables, Ciba-Geigy Limited, Basle, 1970.
[22] International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, ICH harmonised tripartite guideline. Validation of analytical procedures: methodology. (1996) available from http://www.ich.org/pdfICH/Q2B.pdf.
[23] M.C. Guerra, A.M. Barbaro, G.C. Forti, M.T. Foffani, G.L. Biagi, P.A. Borea, A. Fini, J. Chromatogr. 216 (1981) 93–102.
[24] ACD/pK_a DB v. 4.06. Advanced Chemistry Development Inc., 133 Richmond St. West, Suite 605, Toronto, ON, M5H 2L3 Canada, Toronto.
[25] K. Nagarajan, V. Sudarsanam, P.C. Parthasarathy, V.P. Arya, S.J. Shenoy, Indian J. Chem. 21B (1982) 1006–1021.
[26] A. Grimison, J.H. Ridd, B.V. Smith, J. Chem. Soc. (1960) 1352–1356.
[27] Y. Henry, A. Guissani, B. Hickel, Int. J. Radiat. Biol. 51 (1987) 797–809.
[28] E.P.K. Lau, C. Yao, M. Lewis, B.Z. Senkowski, J. Pharm. Sci. 58 (1969) 55–57.
[29] A.K. Sanyal, J. Assoc. Off. Anal. Chem. 71 (1988) 849–851.
[30] H. Theuer, Pharm. Ztg. 128 (1983) 2919–2923.
[31] R. Gompper, H. Herlinger, Chem. Ber. 89 (1956) 2816–2824.
[32] R. Gompper, H. Herlinger, Chem. Ber. 89 (1956) 2825–2833.
[33] A. Albert, E.P. Serjeant, Ionization Constants of Acids and Bases. A Laboratory Manual, Methuen and Co Ltd, London, 1962.
[34] NIST/SEMATECH Engineering Statistics Internet Handbook. National Institute of Standards and Technology and Sematech, (2002) available from http://www.nist.gov/itl/div898/handbook/index.htm.
[35] T. Hase, Tables for Organic Spectrometry, Otatieto Oy, Helsinki, 1994.