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High performance thin layer chromatographic analysis of hydrolyzed tinidazole solutions I. Development and validation method[1]

Jukka-Pekka Salo*, Hannele Salomies

Pharmaceutical Chemistry Division, Department of Pharmacy, University of Helsinki, P.O. Box 56, Helsinki, FIN-00014, Finland

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Abstract

A stability-indicating high performance thin layer chromatography method for analyzing hydrolyzed tinidazole solutions using silica gel plates was developed and validated. The mobile phase used was methanol–diethyl ether–chloroform (1:9:3, v/v/v) allowing small changes in its composition. Detection was at 314 nm. R_f values being 0.1–0.4, baseline resolution was achieved for tinidazole and the hydrolysis products. The analytes were stable on the sorbent and could be precisely and accurately measured in the range 20–170 ng per band.

Keywords: Method development; Thin layer chromatography; Tinidazole; Validation

1. Introduction

Tinidazole (**2**), an N^1-substituted 2-methyl-5-nitroimidazole, is used against trichomoniasis, giardiasis, and amoebiasis. It is also effective against anaerobic infections and can be used prophylactically as well. It is susceptible to both photolysis and hydrolysis, which is especially problematic in infusion solutions. The hydrolysis products of tinidazole (**2**) have previously been identified as the 4-nitroisomer (**1**) and 2-methyl-5-nitroimidazole (**3**) [1] (Fig. 1), the latter being one of the starting materials for the synthesis of **2** [2]. **1** and **3** are also the only specifically named structurally related impurities limited by the requirements of the monograph of **2** in the European Pharmacopoeia [3]. However, metronidazole, another N^1-substituted 2-methyl-5-nitroimidazole, has been reported [4] to yield ammonia, acetic acid, and an amine with an available hydrogen on heating in 0.5 M NaOH.

A plethora of analytical methods have been described for 5-nitroimidazoles [5–7]. However, only a few densitometric thin layer chromatography (TLC) methods for tinidazole using silica sorbent have been reported [8–10]. The earlier methods [8,9] apparently made use of conventional TLC plates while the newest work [10] with metronidazole as the internal standard uses a high performance thin layer chromatography (HPTLC) method.

* Corresponding author.

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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. The validation of planar chromatographic methods has been addressed in some recent papers [11–19]. Different aspects of validation were emphasised. The work of Szepesi [11,12] covers stability problems and ruggedness thoroughly while other workers [13–16] have approached the matter in the form of a case study. The validation of a method using self-made layers has also been described [17].

The present paper describes the development and validation of an HPTLC method for the quantitation of **2** in hydrolyzed solutions. The work was mainly done according to Ferenczi-Fodor et al. [13] because of the clarity of their presentation; some ruggedness testing was carried out as described by Szepesi [12]. The method is shown to be stability-indicating and valid for its intended purpose.

2. Experimental

2.1. Chemicals and reagents

Tinidazole (**2**) was kindly supplied by Orion Pharmaceutica (Espoo, Finland). The commercially available 2-methyl-5-nitroimidazole (**3**) was from Aldrich-Chemie (Steinheim, Germany). The 4-nitroisomer of tinidazole (**1**) was prepared by the current authors as described in the literature [20]. The identity and purity of the substances had been verified by TLC, UV, IR, as well as by ^{1}H and ^{13}C NMR spectrometry. All other chemicals and reagents were of analytical grade.

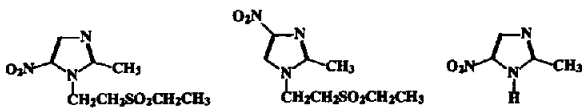

Fig. 1. Structures of tinidazole (**2**), its 4-nitroisomer (**1**), and 2-methyl-5-nitroimidazole (**3**) numbered in ascending order of R_f values.

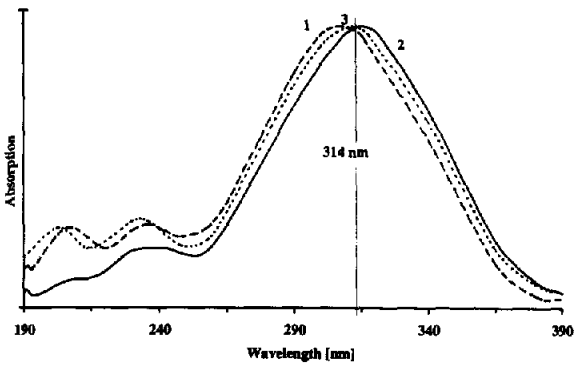

Fig. 2. UV absorption spectra **1**–**3** on silica gel sorbent.

2.2. Chromatography

10×20 cm^2 silica gel HPTLC plates (Merck Art. 5642, Darmstadt, Germany) were cleaned twice by mobile phase development before use. 1 μl of aqueous samples and 1–8 μl of the standard solution in ethanol (containing approximately 20–170 ng of each compound) were sprayed with a Linomat IV (Camag, Muttenz, Switzerland) at a speed of 15 μl s^{-1} as 5 mm bands 10 mm from the lower and vertical edges of the plate with 5 mm spaces between them. Methanol (M)–diethyl ether (D)–chloroform (C) (1:9:3, v/v/v) was used as the final mobile phase, the plates were developed at room temperature in an unsaturated horizontal chamber (Camag), after which they were allowed to dry in a fume cupboard before scanning.

2.3. Apparatus

A Camag TLC Scanner II controlled by the Cats 3.14 program was used to scan the plates at 314 nm (D_2 lamp). The scanning speed was 4.0 mm s^{-1}, and the monochromator bandwidth was set at 10 nm. The slit width and length were 0.2 mm and 2 mm respectively. The scanner was operated in absorption and reflection modes with sensitivity/span optimisation. Each track was scanned only once. For integration, the data selection and filter factors were 1 and 3 respectively. Baseline correction was used.

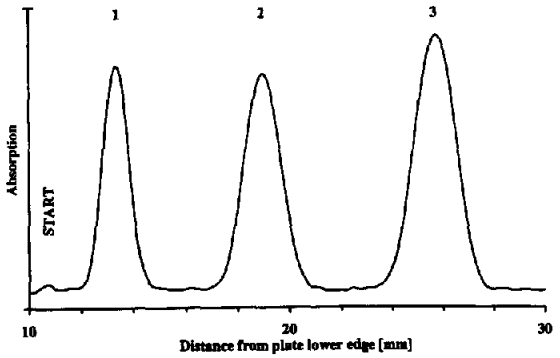

Fig. 3. Densitogram of 1–3 separated by M–D–C (1:9:3, v/v/v).

3. Results and discussion

3.1. Method development

Since compounds on a TLC plate are in contact not only with the plate surface but with atmospheric gases as well, they can undergo oxidative degradation catalyzed by the free silanol groups on the sorbent. In this respect, bonded phases with fewer available catalytic sites are often better [21]. As no problems of this kind had been reported earlier [8–10], silica gel sorbent was chosen as the standard material. The absorption maxima of 1–3 on the plate are at 306, 316, and 312 nm respectively (Fig. 2). Since 2 and 3 are the major components of hydrolyzed solutions in kinetic studies [22], 314 nm was chosen as the analytical wavelength.

Table 1
System suitability data; R_f = retention factor, $T_{0.05}$ = tailing factor at 5% peak height, R_s = resolution

Substance	R_f	$T_{0.05}$	R_s
1	0.096	0.83	2.0
2	0.24	1.0	1.9
3	0.40	0.87	
Acceptance criteria	0.1–0.9	0.9–1.1	≥1.0

None of the earlier methods [8–10] could fully resolve the three compounds (1–3). By trial and error, it was found that M–D–C (1:9:3, v/v/v) provided baseline resolution of 1–3 avoiding higher R_f values (Fig. 3) where a rise in the baseline occurred as described in the literature [21]. According to Snyder [23], M has the largest solvent strength value (5.1) of the three (4.1 and 2.8 for C and D respectively), which is reflected in the strong dependence of the R_f values on the amount of M in the mobile phase (Fig. 4a). Closer studies revealed that D is the major factor in creating the separation, and is enhanced by the addition of C, which also inhibits peak broadening.

It was decided to use peak areas for quantitation since non-linear calibration graphs could be allowed. Furthermore, peak area measurements offer higher precision and smaller sensitivity to R_f changes and baseline fluctuation in comparison with the use of peak heights [11].

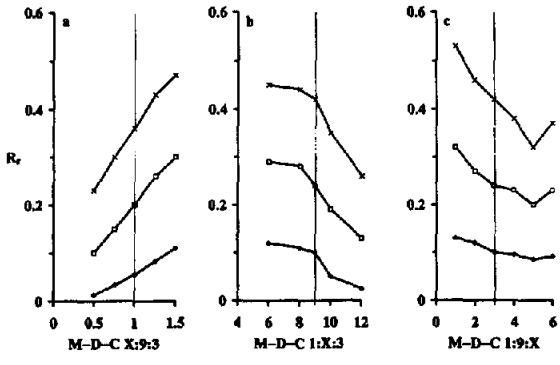

Fig. 4. The effect of the mobile phase components on the R_f values of 1 (◇), 2 (□), and 3 (×). The vertical lines represent the chosen composition.

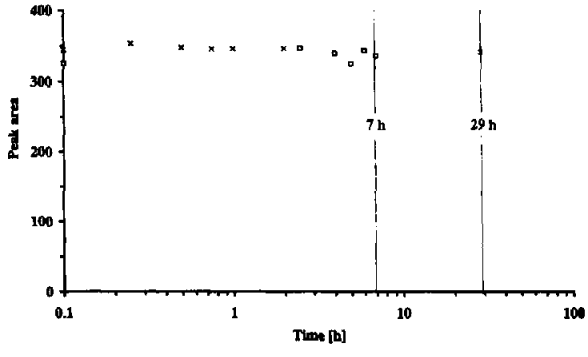

Fig. 5. The stability of 2 in sample solution and on the plate before development (□) and after development (×); the bands contained approximately 75 ng of 2. Similar results were obtained for 1 and 3.

Table 2
Repeatability of sample application ($n = 7$) at different levels of analyte expressed as relative standard deviation (RSD) (%) of peak areas

Substance	10 ng	20 ng	75 ng	430 ng	1100 ng
1	12	2.3	1.4	0.63	1.7
2	14	2.1	1.1	1.1	0.84
3	12	2.5	1.3	0.72	1.0

3.2. Validation

Although some of the system suitability data fail to comply with the acceptance criteria (Table 1), visual estimation of the densitogram (Fig. 3) confirms that the peaks are not deformed. The method is stability-indicating since baseline separation is achieved for **2** and its degradation products (**1** and **3**). Any putative degradation product involving ring-opening of the imidazole, as in the case with metronidazole [4], would not be expected to absorb at the detection wavelength and would probably have a different polarity and therefore an R_f value different from those of **1–3**.

It is good practice to study the effect of the mobile phase composition and the operating temperature on the R_f values and resolution before further experiments since the results dictate how strictly the system should be controlled in the course of validation. It can be seen that the acceptable errors (± 2–10% depending on the volume) in measuring the mobile phase components have only a small effect on the R_f values (Fig. 4); the resolution did not deteriorate either. After the mobile phase has been mixed, the volatile nature of D could cause problems leading to a change in the mobile phase composition. The experience of the present authors is that neither the R_s nor the R_f values are significantly altered even if the mobile phase has been stored in a closed vessel for a few hours before use: the resolutions typically vary from 90% to 100% and the retention factors from 95% to 105% of the tabulated values (Table 1). Therefore, the method is rugged in this respect and no special measures are needed to ensure the integrity of the system at room temperature (in this laboratory 20–26°C).

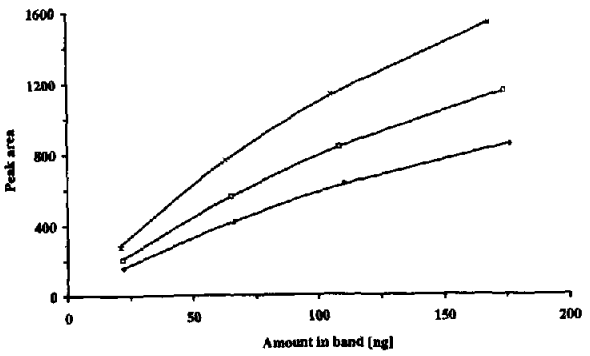

Fig. 6. Typical M–M 1 calibration graph of **1** (◇; $a_1 = 2249.59$, $a_2 = 288.6$), **2** (□; $a_1 = 3159.63$, $a_2 = 303.2$), and **3** (×; $a_1 = 3949.78$, $a_2 = 263.9$) with sdv < 2 fitted to the original calibration points.

The stability of compounds during plate development is best verified by two-dimensional chromatography using the same mobile phase in both directions and 10×10 cm^2 plates. There were only three spots (**1–3**) on the densitogram, situated on the diagonal of the plate, indicating that there was no detectable decomposition at this stage. Authentic hydrolyzed samples were stable for at least 7 h at room temperature when kept in the dark or when applied on the plate. **1–3** can be reliably measured even 29 h after the development of the plate provided it is kept in the dark (Fig. 5). The good stability of **1–3** in the samples and on the sorbent allows the simultaneous analysis of samples gathered during a working day, an advantage in kinetic studies.

The limit of detection was approximately 3 ng for each compound (**1–3**). The calculated limit of quantitation 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), which is the acceptance criterion. Michaelis–Menten regression, which is represented by a saturation curve $[y = a_0 + (a_1 x)/(a_2 + x)]$, is widely used for linearisation of HPTLC data. Michaelis–Menten regression 1 (M–M 1), which provided better fits than the second-order polynomial, forces the calibration graph through the origin ($a_0 = 0$) but it can be used if the results are not distorted (personal communication by Peter Jänchen in March 1995), which was verified in this case. Generally, Michaelis–Menten regression 2 with a non-zero

Table 3
Repeatability and reproducibility expressed as RSD (%) of the peak areas

Test	1	2	3		Acceptance criterion
Repeatability ($n = 7$)					
Measurement of peak area	0.40	0.60	0.60		$\leqslant 1$
Positioning	0.60	0.7	1.1		$\leqslant 2$
Sample application	1.4	1.8	1.0		$\leqslant 3$
Method				5.3	$\leqslant 8$
Reproducibility ($n = 5$)	2.5	2.4	1.5		$\leqslant 10$

a_0 term is preferred to M–M 1, but in this particular case the Cats program could not make the iterative process converge. A good agreement (sdv < 2) between the standards (at 20, 65, 110, and 170 ng) and M–M 1 could be achieved in the range 20–170 ng (Fig. 6); the exact ranges were 22.1–177 ng, 21.7–174 ng, and 21.0–168 ng for **1**–**3** respectively. Since non-linear calibration is used, the sensitivity of the method changes with sample concentration. In the case of M–M 1, the method becomes less sensitive as the concentration increases (Fig. 6). A 1% change in the sample amount causes a change in the peak area of approximately 0.9% at the lower end and approximately 0.6% at the higher end of the range.

Precision, a measure of random errors, can be divided into repeatability and reproducibility. Scanning the same track seven times or seven different tracks depending on the test, repeatability was studied at the 85 ng level, except for the method repeatability where a 1 μl sample volume containing approximately 10 ng of both **1** and **3** was used (totaling 2% of the amount of **2**). For method repeatability, the assayed amounts of **1** and **3** were summed and the RSD calculated for these sums of total impurities. Within-laboratory reproducibility was determined as a five-day analysis of the same sample at the 130 ng level (Table 3), the expected amount of **2** in unhydrolyzed samples. The tests confirm that the method, including the equipment, is free of any large random errors.

Accuracy gives an indication of systematic errors. Water was spiked with three different amounts of each compound (**1**–**3**) in duplicate. The measured values were compared with the known amounts, and the RSD value for the method repeatability (5.3%) served as the acceptance criterion for the mean differences calculated for each compound (Table 4). It can be argued whether the use of mean differences is actually the best way to analyze these data; this fairly simple method of analysis was chosen to enable comparison with previous work [13]. Although the method passes the test, it has a slight positive bias, which is especially pronounced at the lower end of the range. Taking into account the intended use of the method, this can be overlooked because of the possible experimental errors in kinetic studies; the use of this method for pharmaceutical applications would, however, require a closer look at the reasons underlying the problem.

To end the validation, a final ruggedness test was performed. Wavelength precision [12] was tested by selecting the same wavelength (314 nm) and scanning the same track seven times. The RSDs of peak areas were 0.93% (**1**), 1.0% (**2**), and 0.89% (**3**). These values are very close to those of the repeatability of positioning (Table 3), which inevitably has an effect on these measurements too. It can be deduced that the method is rugged in this respect as well.

4. Conclusions

The results prove the method to be valid for monitoring the hydrolysis kinetics of **2**. It has been shown to be stability-indicating. It is both precise and accurate, and **1**–**3** can be quantified over a wide range of concentrations. The compounds are stable on the sorbent over a long period of time, a prerequisite for simultaneously

Table 4
Accuracy of the method determined as the difference between spiked and measured amounts

Substance	Amount							Mean difference (%)
1	Spiked (ng)	22.08	22.08	33.12	33.12	44.16	44.16	+4.6
	Measured (ng)	23.26	24.16	33.54	34.66	45.10	46.25	
	Difference (%)	+5.34	+9.28	+1.27	+4.65	+2.13	+4.73	
2	Spiked (ng)	21.72	21.72	32.58	32.58	43.44	43.44	+5.2
	Measured (ng)	25.09	22.80	32.66	33.45	45.90	44.46	
	Difference (%)	+15.5	+4.97	+0.246	+2.67	+5.66	+2.35	
3	Spiked (ng)	21.00	21.00	31.50	31.50	42.00	42.00	+3.6
	Measured (ng)	23.24	23.48	29.99	31.47	43.44	42.35	
	Difference (%)	+10.7	+11.8	−4.79	−0.0952	+3.43	+0.833	

analyzing the samples of a kinetic study. The method is also rugged with respect to mobile phase composition, ambient temperature, and wavelength precision. Thus, it can be reliably used in the second part of this study.

Acknowledgements

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