Thin-layer chromatography under tropical conditions: impact of high temperatures and high humidities on screening systems for analytical toxicology

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Abstract

The impact of high temperatures (33–38°C) and high relative humidities (80–100%) on the applicability of TLC systems for drug identification was studied during a six month climatologic cycle in Jakarta, Indonesia. In general, the R_F values as observed on the plates were substantially affected in comparison to values obtained at moderate climates: most substances gave higher R_F values under the tropical conditions, although exceptions may occur as well. The deviations tended to increase with increasing humidities and could amount easily to 20–30 R_F units. On the other hand, some TLC systems were more affected than others. Tropical conditions also had a negative effect on the reproducibility of the R_F values. However, when an R_F correction procedure was applied, using reference mixtures of standard drugs on each plate, accuracies as well as reproducibilities of the resulting R_F^c values were drastically improved and data thus corrected were found to be compatible with existing TLC data bases developed under moderate climatic conditions. These results are in line with earlier studies carried out in a relatively dry tropical climate. In the latter the observed R_F values tended to be lower than the ones published in the literature, but the R_F correction procedure was able to correct for this phenomenon.

1. Introduction

Because of its simplicity, speed and low costs, thin-layer chromatography (TLC) appears to be a suitable and versatile technique for qualitative and (semi)quantitative analyses in situations where financial constraints exist, such as in developing countries. The majority of these countries are in tropical areas, characterized by high temperatures (usually above 25°C) and

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humidities that may range from rather low (<30% relative humidity) to very high (above 90% relative humidity). Yet, virtually all of the commonly used TLC procedures have been or are being developed in the Western world under moderate climatic conditions. This brings the paradoxical situation that, although it is known that TLC —as an open technique— can be affected by factors such as temperature and humidity [1], little or no information exists as to how these procedures behave under tropical conditions: do they still provide adequate separation efficiencies and are the resulting R_F values

comparable to those listed as reference values, yet obtained in moderate climates?

In a previous paper [2] we described the influence of high temperatures (up to 39°C) and prevailing relative humidities (RH) of 20 to 70% (dry to moderately humid) as they occur in a semi-desert climate in Burkina Faso, West Africa. In general, the R_F values observed on the plates were found to be substantially affected as compared with values obtained at temperate climates. Most substances showed lower R_F values with lower humidities. The largest deviations were seen at the lowest humidities and were occasionally in the order of 30 R_F units.

We now report on a comparable study, done under hot and humid conditions (temperatures between 33 and 38°C and RHs between 80 and 100%), encountered during a 6-month climatological cycle in Jakarta, Indonesia, under routine laboratory conditions in non-climatized rooms. As we were primarily interested in TLC systems for drug screening in analytical toxicology, we examined a number of established screening systems with regard to the reproducibility and accuracy of the R_F values as observed on the plate and after applying a R_F correction procedure [3,4]. Accuracy was assessed by comparing the R_F values and corrected R_F values (R_F^c) under tropical conditions with the R_F data bases generated under moderate climatic conditions [3,4]. The present "on site" investigational set-up was preferred over generating tropical conditions in climatized rooms, since the latter are too constant and do not accommodate for changes during the day (tropical rainstorms), draught, open doors and windows, etc.

2. Experimental

2.1. Selection of test drugs

Two groups were selected from the WHO list of essential drugs, in order to reflect their toxicological relevance. Also, care was taken to include various relevant pharmacological and chemical classes of drugs:

Acidic and Neutral Drugs (A/N drugs)

Aminophenazone	Paracetamol
Benzocaine	Pentobarbital
Caffeine	Phenacetine
Chlordiazepoxide	Phenobarbital
Diazepam	Phensuximide
Dichlorophen	Phenylbutazone
Diflunisal	Phenytoin
Gluthetimide	Piroxicam
Guaifenesine	Prazepam
Ibomal	Salicylamide
Ibuprofen	Salicylic acid
Indometacin	Secobarbital
Lorazepam	Temazepam
Meprobamate	Theophylline
Methyprylone	Tolbutamide
Naproxen	Triazolam
Oxazepam	

Basic and Neutral Drugs (B/N drugs)

Amitriptyline	Oxycodone
Amphetamine	Papaverine
Atropine	Pentazocine
Codeine	Pethidine
Desipramine	Pheniramine
Diphenhydramine	Procainamide
Dipyridamole	Procaine
Emetine	Promethazine
Ephedrine	Propranolol
Haloperidol	Pseudoephedrine
Hydrocodone	Quinine
Hydoxizine	Timolol
Lidocaine	Trazodone
Methamphetamine	Trifluperidol
Morphine	Trimipramine
Orphenadrine	

The above subdivision is based on the fact that specimens in analytical toxicology are usually extracted first at an acidic pH to isolate A/N drugs, then followed by extraction under alkaline conditions to isolate B/N drugs.

The test substances were of pharmacopoeial quality, with the basic substances usually present as their salt. Solutions were made in ethyl acetate in concentrations of 2 mg/ml. One or 2 μl was spotted, either by means of a Nanomat II automatic applicator (Camag, Muttenz, Switzerland, or by hand with glass capillaries. Solvents were of analytical grade (Merck, Darmstadt, Germany).

2.2. TLC systems

According to the recommendations of TIAFT/ DFG [3,4], systems 1–4A were used to chromatograph A/N drugs and systems 4B–10 for B/N drugs. These systems are described in Table 1, together with the reference mixtures to be used with each system, the error windows and the respective discrimination powers [5] and identification powers [6] for the systems.The systems were run on Silica gel 60 F254 with fluorescence indicator, 20×10 cm (Merck), for systems 7–10 impregnated with KOH [3,4]. Paper-lined, saturated tanks (Camag) were used (presaturation time 30 min), except for systems 5 and 6 which were run in unsaturated tanks. Samples were spotted 2 cm from the bottom of the plate and at least 2 cm from the side edges. The running distance was 7 cm over the starting points [7]. The error window for a given system equals three times the interlaboratory standard deviation for that system.

Detection was done under UV light of 254 nm and by means of location reactions [8]. For each drug, R_F values were determined in 10 independent runs, spread over a period of 6 months (January through June).

2.3. R_F correction procedure

On each plate mixtures of four reference substances were spotted as described in Table 1, and the R_F values observed were compared with their corresponding values in a general data base, determined under moderate climatic conditions [4]. This allowed the construction of a six point correction graph, including the starting point (0,0) and the solvent front (100,100). The observed R_F values for the test drugs on the same plate were then corrected by means of the graph or by calculation [4]. Fig. 1 depicts typical correction graphs for systems 1, 5 and 9, respectively.

Mixtures of the four reference substances in ethyl acetate contained approximately 2 mg/ml of each substance and were stored in the refrigerator. One or two μl was spotted.

2.4. Climatic conditions

The temperature and relative humidity of each experiment was recorded when the plate was put in the tank for development. Temperatures varied from 26 to 38°C and RHs from 80 to 100%. The majority of the experiments was carried out between 33 and 38°C and 85 to 95% RH.

2.5. Evaluations

Reproducibility was assessed as follows. For each substance in a given system the individual standard deviations around the mean (S.D.) were calculated. Then, these S.D. values were averaged over all substances investigated in that system to give $\overline{S.D.}$ This was done for uncorrected R_F values as well as for corrected ones. The number of observations per substance was at least 10.

Accuracy was also assessed per system before and after correction of the R_F values. First, for each substance, the mean deviation (M.D.) between the observed R_F value and the one available in the literature was calculated:

$$\text{M.D.} = \frac{\sum (R_{F,\text{observed}} - R_{F,\text{literature}})}{n}$$

in which n represents the number of observations (at least 10) per substance. Then, these M.D.s were averaged over all substances investigated in that system to give the averaged mean deviation from the literature R_F values:

$$\overline{\text{M.D.}} = \frac{\sum M.D.}{m}$$

in which m represents the number of substances investigated. In addition, the mean *absolute* deviation (M.A.D.) from the literature was calculated in a similar way:

$$\text{M.A.D.} = \frac{\sum |R_{F,\text{observed}} - R_{F,\text{literature}}|}{n}$$

and for the averaged mean deviation from the literature:

$$\overline{\text{M.A.D.}} = \frac{\sum M.A.D.}{m}$$

Table 1
Details on the TLC systems

Solvent [a]		Adsorbent compound	Reference [b]	hR_F^c	Error window [c]	DP [d]	IP [e]
1	Chloroform–acetone (80:20)	Silica	Paracetamol	15	7	0.83	14
			Clonazepam	35			
			Secobarbital	55			
			Methylphenobarbital	70			
2	Ethyl acetate	Silica	Sulfathiazole	20	8	0.88	10
			Phenacetin	38			
			Salicylamide	55			
			Secobarbital	68			
3	Chloroform–methanol (90:10)	Silica	Hydrochlorothiazide	11	8	0.78	17
			Sulfafurazole	33			
			Phenacetin	52			
			Prazepam	72			
4a	Ethyl acetate–methanol–conc. ammonia (85 + 10 + 5)	Silica	Sulfadimidine	13	11	0.76	19
			Hydrochlorothiazide	34			
			Temazepam	63			
			Prazepam	81			
4b	Ethyl acetate–methanol–conc. ammonia (85:10:5)	Silica	Morphine	20	10	0.71	21
			Codeine	35			
			Hydroxyzine	53			
			Trimipramine	80			
5	Methanol	Silica	Codeine	20	8	0.83	17
			Trimipramine	36			
			Hydroxyzine	56			
			Diazepam	82			
6	Methanol–n-butanol (60:40); 0.1 mol/l NaBr	Silica	Codeine	22	9	0.78	19
			Diphenhydramine	48			
			Quinine	65			
			Diazepam	85			
7	Methanol–conc. ammonia (100:1.5)	Silica impregnated with 0.1 mol/l KOH and dried	Atropine	18	9	0.77	18
			Codeine	33			
			Chlorprothixene	56			
			Diazepam	75			
8	Cyclohexane–toluene–diethylamine (75:15:10)	Silica impregnated with 0.1 mol/l KOH and dried	Codeine	6	8	0.75	19
			Desipramine	20			
			Prazepam	36			
			Trimipramine	62			
9	Chloroform–methanol (90:10)	Silica impregnated with 0.1 mol/l KOH and dried	Desipramine	11	11	0.76	18
			Physotigmine	36			
			Trimipramine	54			
			Lidocaine	71			
10	Acetone	Silica impregnated with 0.1 mol/l KOH and dried	Amitriptyline	15	9	0.74	20
			Procaine	30			
			Papaverine	47			
			Cinnarizine	65			

[a] Eluent composition: volume:volume; saturated systems are used except for systems 5 and 6 which are used with unsaturated solvent tanks. System 4 is split: 4a for acidic and neutral substances and 4b for basic and neutral substances.
[b] Solutions of the four reference compounds at a concentration of approximately 2 mg/ml of each substance.
[c] The error window for each system is based on multiplying by three the interlaboratory standard deviation of measurement of hR_F values.
[d] DP = Discriminating power calculated using the error window in the fifth column.
[e] IP = Identification power calculated using the error window in the fifth column and expressed as mean list length.
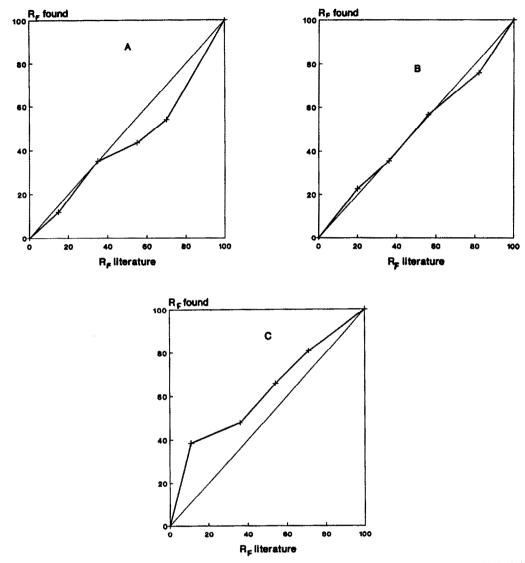
Fig. 1. Typical R_F correction graphs for individual TLC systems. Temperatures 33–38°C, relative humidities >90%. (A) System 1, reference substances ($R_{F,\text{literature}}$ in brackets): paracetamol (15), clonazepam (35), secobarbital (55), methylphenobarbital (70). (B) System 5, reference substances: codeine (20), trimipramine (36) hydroxyzine (56), diazepam (82). (C) System 9, reference substances: desipramine (11), physostigmine (36), trimipramine (54), lidocaine (71).

M.A.D. is the parameter of choice to assess accuracy because it considers deviations from the literature irrespective of sign. With M.D., deviations will level out if some substances run higher and with others running lower than their literature values. As a result, M.D. may be close to zero, even though the deviations can be substantial.

3. Results and discussion

In general, all systems could be used under the tropical conditions encountered, i.e. there was reasonable separation and the separation sequences were the same as under temperate conditions. However, at very high humidities (RH close to 100%) the silica on the plates

appeared to adsorb so much water vapor that the separation power was lost, with the spots running near to or with the solvent front. Such results were not further evaluated.

An other important observation was made when it was tried to avoid the rather unpleasant odor of the diethylamine in system 8 by carrying out the developments with that system in the fume hood. The air currents in the hood caused such a cooling of the walls of the tanks that solvent vapors condensed on the inside walls, thus causing highly irregular solvent flows and substance movements on the plate. Similar observations were made when some control experiments were done at temperatures around 25°C in an air conditioned room: the air currents were again so strong that solvent vapors condensed on the inside walls of the tanks, ruining the standard separation patterns.

Spotting was affected under very high humidities in that the organic spotting solution had to be applied rather slowly and preferably intermittently, to keep the spots small enough. The latter was occasionally a problem in the automatic spotting procedure with the Nanomat because it did not allow intermittent spotting. Therefore, spotting was done by hand when RHs exceeded 95%.

Our earlier observation [2], that at higher temperatures (>32°C) the ammonia lost gas bubbles when the bottle was opened, was also noted in the present study. Since this may lead to unacceptably high losses of ammonia when using bottles of 1 l that are opened frequently, ammonia was stored in bottles of 100 ml.

When the *observed, uncorrected* R_F values were considered, it became clear that the tropical conditions, and the high humidities in particular, could cause drastic changes as compared to the R_F data in the literature [4]. Conceivably, when exposed to the high ambient humidity, the silica on the plate will adsorb a substantial amount of surface water, which will make the stationary phase more polar and reduce the chances for solute interaction [9]. As a result, one may expect higher R_F values with increasing RHs, with the deviations becoming larger when the developing solvent becomes less polar. In-

deed, the largest deviations were seen with the rather non-polar system 9. This is demonstrated in the correction graph for this system in Fig. 1 and in the M.D. values in Table 2: the uncorrected R_F values show a mean deviation of 27 R_F units. Yet, systems that employ more polar solvents are much less affected when high amounts of water vapour are being adsorbed. This is reflected for example by systems 5 and 7, in which the impact of extra water against the large amounts of methanol is relatively small (See Fig. 1B and Table 2). However, exceptions to the above general rule apparently exist, as demonstrated by system 1. In the latter, most substances were found to give lower R_F values with increasing humidities. This can be seen in Fig. 1A and in an M.D. for uncorrected R_F values of -5.1 in Table 2. The reasons for this behavior of system 1 remains as yet unknown.

Table 2 summarizes the impact of the tropical conditions on the uncorrected R_F values (the U-columns) with regard to precision (S.D.) and accuracy (M.D. and M.A.D.). It can be seen that some systems were more affected than others. Moreover, it should be noted that Table 2 shows the averages for the sets of about 30 substances each, obtained in 10 independent experiments. For individual drugs, deviations from the literature of some 30 R_F units were not unusual. This may be seen in Fig. 1C for desipramine: listed with an R_F value of 11 in the literature, an actual R_F of 40 was found in the experiment on the plate. Thus, the above observations clearly show that uncorrected R_F values obtained under hot and humid conditions cannot be compared with reference data collected under moderate climatic conditions.

However, *the use of the R_F correction procedure* drastically improved the applicability of all the systems under tropical conditions. This is reflected in a significant reduction in S.D. (better precision), but even more so by substantial reductions in the C-columns of M.D. and M.A.D. (better accuracy). Yet, despite the correction procedure, systems 1, 3, 7, 9 and 10 remain less suitable for work under hot and humid conditions because their M.A.D. values are still larger than 5. The other systems, nos. 2

Table 2
Reproducibilities and accuracies of uncorrected (U) and corrected (C) R_F values. Temperatures 33–38°C; relative humidities 80–100%

TLC system	S.D.		M.D.		M.A.D.	
	U	C	U	C	U	C
1	1.6	1.6	−5.1	0.8	7.6	5.6
2	1.9	1.9	2.9	1.2	4.7	3.1
3	1.6	1.2	1.4	−0.4	5.9	5.2
4A	2.4	2.2	3.4	−1.1	5.2	2.9
4B	2.7	2.3	3.0	−0.8	5.4	2.9
5	2.2	1.6	−1.8	0.6	4.4	2.6
6	4.0	2.0	1.6	1.3	4.5	3.5
7	3.2	2.2	−1.6	2.2	6.0	5.0
8	2.8	1.3	5.9	0.3	6.9	2.8
9	3.8	3.4	27.2	13.6	27.2	15.2
10	4.1	2.5	7.6	3.6	12.6	7.3
Average	2.7	2.0	5.6	2.4	8.2	5.1

S.D. = averaged standard deviation of the mean per system.
M.D. = averaged mean deviation from the literature: $R_{F,found} - R_{F,literature}$ per system.
M.A.D. = averaged mean absolute deviation from the literature: $|R_{F,found} - R_{F,literature}|$ per system.

and 4A for A/N drugs and nos. 4B, 5, 6 and 8 for B/N drugs, behave well and can be recommended for screening in analytical toxicology under hot and humid conditions. Their M.A.D. values are between 2.6 and 3.5, which means that corrected R_F values obtained with these systems can be checked against the existing TLC data bases developed in moderate climates [4]. Virtually all data will then fall within the Error Windows as listed in Table 1. As for system 8, it was noted that high humidities (>90%) had an especially pronounced effect on the second substance in the reference mixture, desipramine. Listed with an R_F value of 20, it showed R_F values up to 35 at high humidities, which is very close to that of the third reference, prazepam, whose listed R_F of 36 was far less affected. We are presently considering whether desipramine in the reference mixture can be replaced by noscapine with a listed R_F value of 21 [4].

When these findings are compared with those obtained in a semi-desert climate [2], it can be noted that the same systems also did well under hot and dry conditions. Other systems that did well under hot and dry conditions, such as nos. 3 and 7, were less satisfactory under humid conditions.

Thus, R_F corrections using reference mixtures on the same plate appear to be essential for TLC work under tropical conditions, especially to correct for the impact of very dry or of very humid conditions. The idea behind the correction is that the influence of the humidity is being reflected in the behavior of the substances in the reference mixture and that this influence is being corrected for in the behavior of the unknown substance by means of the correction graph that is being made for each plate. Corrected R_F^c values obtained in this way are then compatible with existing TLC data bases generated in temperate climates.

4. Acknowledgements

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