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MODERN CHROMATOGRAPHIC PROCEDURES IN SYSTEMATIC TOXICOLOGICAL ANALYSIS

ROKUS A. DE ZEEUW

Department of Analytical Chemistry and Toxicology, University Centre for Pharmacy, NL-9713 AW Groningen (The Netherlands)

SUMMARY

The fact that the toxicologist in systematic toxicological analysis never knows what he is looking at but has to take into account a vast number of toxicologically relevant substances makes this field a very difficult, yet challenging task. Because of the strong qualitative emphasis gas and thin-layer chromatography are at present the techniques of choice, and can be used with other relevant techniques such as spray reactions on the plate, UV spectrophotometry and mass spectrometry. However, as a single chromatographic technique will never provide unequivocal identification, the techniques have to be used side by side, so that the final identification matches the results from all the techniques applied. This approach requires that the advantages and disadvantages of each technique be well known so that a combination of techniques can be chosen that provides the optimum identification power. After the unknown substance(s) have been analysed by a number of techniques and their particular behaviour in these techniques has been established, these findings are then matched against a data bank containing the behaviour of reference substances. This data bank should be as large as possible. Moreover, the search process used with the data bank must take into account the identification power of each individual technique, otherwise a well balanced "yes–no" decision about the presence or absence of a given substance is impossible.

INTRODUCTION

Systematic toxicological analysis (STA) involves the logical chemical analytical search for a harmful substance whose presence is unsuspected and whose identity is unknown. It is an essential part of all toxicological cases and, even though it may appear relatively simple and straightforward, the analyst involved in STA faces a complex and difficult task, namely how to make sure that all substances with toxic or abuse potential are detected and, once detected, how to identify it properly against a background of thousands of others. In this content it can be noted, for example, that in 40 000 intoxication cases investigated by the Poison Centre in Munich, more than 8000 different substances were involved [1].

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Hence, when assessing the potential and pitfalls of chromatographic techniques, this strongly qualitative emphasis should be borne in mind. Further, it should be realised that in the daily practice of toxicological analysis a great number and variety of compounds are encountered, that a fast and efficient round-the-clock service must be provided and that an array of instruments and techniques must be available on a stand-by basis. This explains the need for relatively simple, straightforward and flexible equipment and procedures and the fact that the toxicological analyst can hardly afford to specialize too much in a given technique or methodology. On the other hand, as most of the work involves biological samples, other complicating factors include the occurrence of metabolites and/or endogenous components from the biological matrix, and intoxications with more than one drug (poly-intoxications) are very common nowadays.

SCREENING BY CHROMATOGRAPHIC TECHNIQUES IN SYSTEMATIC TOXICOLOGICAL ANALYSIS

Chromatographic techniques are, of course, the workhorses for screening in STA because of their separating ability and detection sensitivity. Until now, gas chromatography (GC) and thin-layer chromatography (TLC) have been the most frequently used techniques and, as a single chromatographic system will obviously not be able to provide an unequivocal identification, it is necessary to use a combination of two or more systems side-by-side. In order to compensate for variations in the experimental conditions, retention in GC is usually expressed as Kováts retention indices (RI) [2], whereas in TLC R_F values are usually corrected by means of reference substances that are run concurrently with the unknown [3]. Identification is then attempted by trying to match the retention value of the unknown(s) with that of known substances, available as reference values in a data base.

Although this approach seems straightforward, two basic problems arise: how to select the most suitable chromatographic technique or system for STA, either alone or in combination with others; and how to establish unequivocally the presence or absence of a given compound against the background of a great many others. Suitable mathematical approaches for addressing these problems are the discriminating power (DP) concept [4–6] and the identification power/mean list length (IP/MLL) concept [7,8], which have shown the following features to be the most important for a good chromatographic system in STA: an even distribution of the substances of interest across the entire length of the chromatogram; a high inter-laboratory reproducibility of the retention parameters; and, if more than one system is used, a low correlation of chromatographic properties between systems.

Gas and thin-layer chromatography

System evaluations have so far been carried out mainly for GC and TLC, because these techniques have been extensively studied and no further major developments in stationary and mobile phases are to be expected. For GC, a methylsilicone column (SE-30 or OV-1) appears to be the best column for screening

in STA. However, even though GC lends itself well to both screening and identification, SE-30 or OV-1 is also the only recommended system, because other systems, such as OV-17, Carbowax or DEGS, are all highly correlated with the SE-30 or OV-1 system, so that very little information is gained by applying a second GC system [9,10].

It should be noted that the vast number of toxicologically relevant substances that have to be taken into account make it impossible for an individual analyst or institution to set up and maintain its own database of reference retention values. Instead, one has to rely on databases built up with contributions from different laboratories. For GC on SE-30 such a database now contains ca. 1600 substances [10]. The inter-laboratory standard deviation of RI measurements is of the order of 15–20 RI units [11,12], so that a "search" window of 50–60 RI units should be taken into account when comparing the RI of an unknown compound with those in the database. As RIs are temperature dependent (with the exception, by definition, of those of *n*-alkanes), their documentation would ideally require information about the temperature at which they were measured. However, the temperature dependence can be ignored by using a search window of 50–60 RI units. This also applies to RI values determined under temperature-programmed conditions [10]. Under the latter circumstances, one should realise that there is an almost linear relationship between the carbon number of the *n*-alkanes and retention time [not log (retention time) as in the isothermal mode]. This linear relationship can be used for the calculation of the RI values [13].

TLC has gained popularity for STA owing to its simplicity, universality, speed and low cost. Although its separating power is lower than that of GC, the ease of applying general or selective spray reagents enhances its identification potential. Moreover, TLC provides much better opportunities for finding two or more systems with little intercorrelation. Thus, the application of a variety of TLC systems usually yields a considerable gain in information, despite the relatively low reproducibility of the technique. This has led to the recommendation of ten standardized TLC systems for STA and a database of ca. 1100 substances [14]. These systems are given in Table I. Four systems (1–4a) were chosen for the analysis of acidic and neutral drugs (as these two classes are often extracted together in the work-up procedure prior to chromatography), whereas systems 4b–10 are suitable for basic drugs. It can be seen that systems 4a and 4b use the same solvent but different reference compounds, so that acidic, neutral and basic drugs can be run in the same tank but on separate plates. The same applies for systems 3 and 9.

Table I indicates that for general screening the best system for acidic and neutral drugs is ethyl acetate (system 2), and that for basic drugs is methanol (system 5) (the best systems are those with the highest DP or the lowest IP). This is particularly caused by the fact that single solvents provide better reproducibility than multi-component solvents, which results in smaller error windows (or search windows) for the former.

When more than one TLC system is to be used, the correlation between the systems must be taken into account. Table II gives the correlation coefficients for R_F values for pairs of TLC systems. It can be seen that for acidic and neutral

TABLE I

STANDARDIZED TLC SYSTEMS

System	Solvent[a]	Adsorbent	Reference compounds[b]	hR_F	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	Sulphathiazole	20	5	0.88	10
			Phenacetin	38			
			Salicylamide	55			
			Secobarbital	68			
3	Chloroform–methanol (90:10)	Silica	Hydrochlorothiazide	11	8	0.78	17
			Sulphafurazole	33			
			Phenacetin	52			
			Prazepam	72			
4a	Ethyl acetate–methanol–conc. ammonia (85·10:5)	Silica	Sulphadimidine	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[f]	Atropine	18	7	0.77	18
			Codeine	33			
			Chlorprothixene	56			
			Diazepam	75			
8	Cyclohexane–toluene–diethylamine (75:15:10)	Silica[f]	Codeine	6	7	0.75	19
			Desipramine	20			
			Prazepam	36			
			Trimipramine	62			
9	Chloroform–methanol (90:10)	Silica[f]	Desipramine	11	11	0.76	18
			Physostigmine	36			
			Trimipramine	54			
			Lidocaine	71			
10	Acetone	Silica[f]	Amitriptyline	15	9	0.74	20
			Procaine	30			
			Papaverine	47			
			Cinnarizine	65			

[a]Eluent composition v/v; 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 drug.
[c]The error window for each system is based on multiplying by three the inter-laboratory standard deviation of measurement of hR_F values.
[d]Discriminating power calculated using the error window.
[e]Identification power calculated using the error window and expressed as mean list length.
[f]Silica impregnated with 0.1 mol/l KOH and dried.

TABLE II

CORRELATION COEFFICIENTS OF R_F DATA FOR PAIRS OF TLC SOLVENT SYSTEMS 1–10

	1	2	3	4	5	6	7	8	9	10
1	-									
2	0.820	-								
3	0.890	0.748	-							
4	0.530	0.464	0.593	-						
5				0.460	-					
6				0.436	0.614	-				
7				0.700	0.745	0.552	-			
8				0.593	−0.128	−0.045	0.228	-		
9				0.723	0.748	0.472	0.728	0.342	-	
10				0.710	0.750	0.655	0.771	0.206	0.820	-

drugs systems 2 and 4 are to be preferred; for basic drugs systems 5 and 8 provide the best combination, closely followed by systems 6 and 8.

As indicated, one of the drawbacks of TLC is its low inter-laboratory reproducibility. This may become even worse when dealing with extracts from biological samples, especially with autopsy blood and liver [15]. The negative effects of the biological matrix can be counteracted by using drugs extracted from appropriate biological fluids or tissues as correction standards [16]. On the other hand, the identification potential of TLC can be enhanced by using one or more selective spray reagents.

Capillary gas chromatography

Because of its much higher separation efficiency, capillary gas chromatography (CGC) would seem to be a particularly valuable technique for STA. However, a few problems, inherent in the character of toxicological analysis, rapidly became apparent. First, in the early days of CGC when column technology was still cumbersome, RI values varied from column to column and from brand to brand. Moreover, differences in RI values were observed between packed columns and capillary columns with comparable stationary phases. This is exemplified in Table III [17]. A second problem appeared to be the load capacity in CGC. In STA the concentration of the drugs to be encountered is usually unknown and may vary over several orders of magnitude. This may easily result in overloading of the system and, subsequently, in RIs that become concentration dependent [18]. Splitless injection is not an acceptable alternative to this problem because it may easily result in substances present in low concentrations remaining undetected.

Fortunately, the situation has improved in recent years, owing to better reproducibilities in manufacturing procedures and to the introduction of wide-bore columns with an inner diameter around 0.5 mm. The latter allow on-column injection, usually provide long column lifetimes, show good reproducibility and have adequate load capacities. Notwithstanding these advantages, the inter-laboratory reproducibility that can be obtained with these columns still requires a search

TABLE III

COMPARISON OF RETENTION INDICES OF MISCELLANEOUS DRUGS ON CP-SIL 5 CAPILLARY COLUMNS AND SE-30 OR OV-1 PACKED COLUMNS [17]

Compound	CP-Sil 5 columns[a]			Packed columns [10]
	Glass WB	FS NB	FS WB	
Antazoline	2295	2299		2350
Caffeine	1780	1796		1810
Codeine	2376	2384		2385
Gallamine		2603		2700
Hexachlorophene			2860	2795
Hydroxyzine	2867	2890	2934	2850
Isoniazide		1447	1447	1630
Isopropamide		1998	2037	2060
Methadone	2150	2150	2182	2150
Methaqualone	2142	2150	2181	2115
Naphazoline	1993	1996	2044	2065
Nicotinyl alcohol	1092	1100		1150
Phenazone			1875	1830
Phencyclidine			1932	1904
Strychnine	3115	3136	>3200	3115
Theophylline	1947	1932		2105
Yohimbine	3168	3210		3290

[a]Chrompack, Middelburg, The Netherlands. NB = narrow-bore; WB = wide-bore; FS = fused-silica.

window of 50–60 RI units for SE-30 or OV-1 stationary phases [19], which is comparable to that for packed columns. This holds for temperature-programmed runs, but it should be noted that this is a mandatory requirement in screening procedures.

Recent investigations have shown, however, that under these conditions it is much more advantageous to use a mixture of drugs with known RIs instead of *n*-alkanes for the calculation of the RIs of the unknowns, resulting in a search window of only 25 RI units [20]. This is due to the fact that the retention behaviour of drugs is less dependent on temperature than that of *n*-alkanes and other alkane homologues that have been proposed for RI calculation, such as diisopropylamines or trialkylamines [21]. This is demonstrated in Fig. 1. The reference mixture of drugs for the calculation of RI consisted of amphetamine (1110), ephedrine (1365), benzocaine (1545), methylphenidate (1725), diphenhydramine (1860), tripelenamine (1980), methaqualone (2140), trimipramine (2215), codeine (2375), desmethyldiazepam (2485), prazepam (2645), paraverine (2820), haloperidol (2945) and strychnine (3120); the RIs in parentheses were taken from ref. 10.

With this much smaller inter-laboratory search window of 25 RI units for wide-bore CGC, it now becomes worthwile to see if the use of a second CGC system would provide an adequate gain in information. Evaluations of various systems with regard to their identification power and their correlation with the above methylsilicone system are in progress.

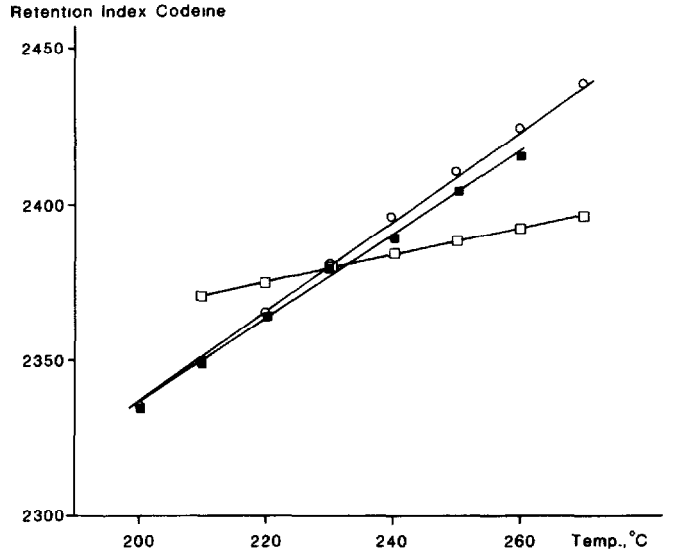

Fig. 1. Influence of temperature on the retention index of codeine using different reference substances for the calculation. Capillary GC on a 0.53 mm wide-bore methylsilicone fused-silica column. On-column injection and temperature programmed run according to ref. 21. ○ = *n*-Alkanes; ■ = diisopropylamines; □ = drugs.

High-performance thin-layer chromatography

Although increased separation power and shorter development times are key factors for STA, high-performance thin-layer chromatography (HPTLC) has not found widespread acceptance in this area. This appears to be due to the limited load capacity of HPTLC, such that "dirty extracts", which are fairly common in STA, can overload the plates or clog up the sample applicator. Hence, as in CGC, the advantages of HPTLC are overshadowed by the inherent characteristics of toxicological analysis.

On the other hand, shorter development times in classical TLC can be simply obtained by using a development distance of 7–8 cm, rather than the customary, yet arbitrary, 10 cm. This will result in a gain in development time of 30–50%, with negligible effects on the R_F values, resolution or reproducibility. Further, shorter development times result in less diffusion of the spots, so that a better sensitivity is obtained [22,23].

High-performance liquid chromatography

In view of its good separation power, flexibility and general applicability, including thermolabile and relatively non-volatile compounds, HPLC seems to be a very attractive technique for STA. Moreover, HPLC offers a wide variety of separation modes and possibilities of varying the stationary and mobile phases. However, notwithstanding extensive research, HPLC for screening purposes is still very limited, mainly because until now it has been impossible to obtain stationary phase materials of sufficient reproducibility. Similar types of packing

TABLE IV

RETENTION BEHAVIOUR OF A REFERENCE MIXTURE OF DRUGS ON C_{18}-TYPE REVERSED-PHASE HPLC COLUMNS FROM DIFFERENT BATCHES (BATCH-TO-BATCH) AND FROM DIFFERENT MANUFACTURERS (TYPE-TO-TYPE)

Adapted from ref. 24.

Type	Batch	Relative retention time		
		Diphenhydramine	MPPH[a]	Diazepam
RP-18; type 1	A	3.30	1.00	1.76
	B	4.94	1.00	1.71
	C	8.03	1.00	2.20
	D	1.51	1.00	1.75
RP-18; type 1	E	2.43	1.00	1.70
(cartridge)	F	5.58	1.00	1.81
RP-18; type 2	G	0.51	1.00	1.54
	H	0.48	1.00	1.48
	J	0.51	1.00	1.60
	K	0.45	1.00	1.60
	L	0.41	1.00	1.42
RP-18; type 3	M	0.39	1.00	1.41
	N	0.41	1.00	1.42
	O	0.36	1.00	1.39
	P	-	1.00	1.72
RP-18; type 4	Q	0.47	1.00	1.27
	R	0.63	1.00	1.38

[a]MPPH = 5-(*p*-methylphenyl)-5-phenylhydantoin.

material from different manufacturers can differ markedly in separation performance and even batch-to-batch differences in the same brand may occur.

An example from the work of Daldrup and Kardel [24] is given in Table IV. Not only were there considerable changes in relative retention times, but even changes in elution order were observed. Although this work was done over 4 years ago and despite extensive efforts to improve this situation both in industry and in academia, the lack of reproducible column material is still with us today [25]. This situation severely hampers investigations in other areas of qualitative HPLC such as the evaluation of suitable detection systems (e.g., diode-array detectors) and an acceptable method for expressing retention in a standardized form. The use of relative retention times has the same drawbacks as in GC, whereas *n*-alkanes are incompatible with UV detection. Recently nitroalkanes were suggested for this purpose [26] and an extensive evaluation of their properties is to be expected soon.

SUBSTANCE IDENTIFICATION BY MEANS OF A DATABASE

Comparison of the retention values found, i.e., RI values for GC and corrected R_F values (R_F^c values) for TLC, with those of known compounds provides the basis for identification. This can be combined with other parameters such as

spray reactions on the plate, UV and mass spectra and differential responses on flame ionization and nitrogen–phosphorus detectors in GC. Hence, a collection of these parameters that have been measured with reference substances under well defined and reliable conditions is of paramount importance. This database should contain data on as many relavant substances as possible and not be limited to the parent drugs but also include metabolites, precursors, endogenous compounds and ubiquitous substances such as plasticizers, antioxidants and PCBs. To set up such a data bank and keep it up-to-date is a virtually impossible task for an individual laboratory, also in view of the ongoing introduction of new substances. Fortunately, joint efforts to overcome this problem by the STA Committee of the International Association of Forensic Toxicologists (TIAFT) and the Senate Commission for Clinical–Toxicological Analysis of the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) has led to the compilation of two databases for inter-laboratory use, one for GC on OV-1 or SE-30 for ca. 1600 substances [10] and one for TLC for ca. 1100 substances in ten systems [11]. A computerized data bank, containing the above GC and TLC databases and UV data, is also available [27] and will soon be expanded to include other parameters, such as spray reactions on the plate, mass spectrometric data and HPLC data as they become available.

The following examples will show how this data bank can be used in practice. A case sample seized by the police was analysed for the presence of controlled substances by TLC using methanol (system 5, Table I). The unknown revealed one spot, for which a R_F^c value of 10 was calculated. With this parameter a computerized search was started in the database containing reference data for ca. 700 relevant substances which resulted in printout of possible candidates with their relative probabilities and similarity indices. A similarity index of 100 means a full match between all parameters found and listed.

PRINTOUT 1

Value(s) found:
System 5: $R_F^c = 10$

Substance	Relative probability	Similarity index
Ephedrine	2.0%	100.0
Dextrorphan	2.0%	100.0
Dextromethorphan	2.0%	100.0
Bufotenine	2.0%	100.0
Bromodimethoxyamphetamine	2.0%	100.0
Pholedrine	1.9%	93.4
Phentermine	1.9%	93.4
Nortriptyline	1.9%	93.4
Metopon	1.9%	93.4
Methylenedioxyamphetamine/mda	1.9%	93.4

Methoxyamphetamine/4-	1.9%	93.4
Methamphetamine	1.9%	93.4
Mesoridazine	1.9%	93.4
Levorphanol	1.9%	93.4
Hydrocodone	1.9%	93.4
Hexobendine	1.9%	93.4
Disopyramide	1.9%	93.4
Dimetindene	1.9%	93.4
Dimethoxy-4-methylamphetamine/2-5	1.9%	93.4
Dihydrocodeine	1.9%	93.4
Deoxyephedrine/[+/−]-	1.9%	93.4
Amphetamine	1.9%	93.4

One can easily deduce that trying to identify a substance by using a single analytical technique is impossible. It should also be noted that in this instance the error window had been reduced to 1 in order to keep the length of the list within accceptable limits for this paper, otherwise a list of ca. 100 candidates would have resulted.

When, in addition to methanol, a second solvent system was used, the number of candidates that matched both results could be substantially reduced. In this instance system 8 (cyclohexane–toluene–diethylamine) was selected because of its low correlation with system 5. The search for substances that match both the R_F^c values of 10 in system 5 and 14 in system 8 and applying the relevant error windows as given in Table I now yields a list of seven candidates (see printout 2). Four of them have relatively high probabilities and similarity indices. The use of a third system, system 6 (the ion-pair system methanol–butanol and sodium bromide), gave a list of four candidates with amphetamine as the highest probability (printout 3). It may also be noted that maproteline and protriptyline are now on the list, although they were absent in printout 2. This is because the search system was set at a cut-off level of 95% cumulative probability.

PRINTOUT 2

Value(s) found:
System 5: $R_F^c = 10$
System 8: $R_F^c = 14$

Substance	Relative probability	Similarity index
Levorphanol	19.2%	92.2
Amphetamine	19.2%	92.2
Pipazethate	15.6%	83.2
Dextrorphan	9.6%	65.4
Methylenedioxyamphetamine/mda	9.0%	63.2
Diethyltryptamine/NN-	6.9%	55.1

PRINTOUT 3

Value(s) found:
System 5: $R_F^c = 10$
System 8: $R_F^c = 14$
System 6: $R_F^c = 73$

Substance	Relative probability	Similarity index
Amphetamine	58.7%	87.9
Methylenedioxyamphetamine/mda	20.9%	62.3
Maproteline	9.8%	48.5
Protriptyline	5.1%	38.8

It is our experience that the combination of three TLC systems usually provides the maximum amount of information and that using more systems is not very effective. Additional identification parameters, either to confirm the identity if only one candidate is listed or to reduce further the number of candidates to one, are then obtained by other techniques, such as colour or spray reactions, UV spectrophotometry or GC. In our case a UV spectrum was recorded in a methanol extract and the maximum of 262 nm, added to the TLC information obtained with systems 5 and 8, then resulted in printout 4, listing amphetamine as the single candidate. This could be confirmed by a ninhydrin spray reaction on the plate.

PRINTOUT 4

Value(s) found:
System 5: $R_F^c = 10$
System 8: $R_F^c = 14$
UV: 262.0

Substance	Relative probability	Similarity index
Amphetamine	92.5%	80.2

The following example describes a case in which two spots were found with both systems 5 and 8. In system 5 the R_F^c values were 28 and 10 whereas in system 8 they were 15 and 8. Now, a problem arises in that one does not know a priori which spot in system 5 corresponds to that in system 8 and vice versa. This has been solved by the program taking into account all possible combinations (preferentially called "configurations") and printing them out separately. In configuration A it is assumed that unknown substance I produced the R_F^c values 28 and 15 and that substance II produced the R_F^c values 10 and 8 in systems 5 and 8, respectively. The resulting printout 5 lists diamorphine (heroin) as best candidate for substance I and dihydrocodeine for substance II. For the other configuration B, the results are given in printout 6. Spray reactions with Marquis reagent then revealed that only the spots with R_F^c 28 in system 5 and R_F^c 15 in system 8

gave a positive reaction in the form of a purple colour. This indicated that configuration B was false and that in configuration A the spot with the Marquis reaction would be diamorphine as the other listed compounds would not react. In order to identify the second spot in this mixture, the TLC behaviour in system 6 was also introduced in the search system, with printout 7 giving the result for the appropriate configuration. Strychnine is now listed as the best candidate for substance II whereas the presence of diamorphine is further confirmed. Additional information from other techniques provided full confirmation for both substances.

PRINTOUT 5

Value(s) found substance I:
System 5: $R_F^c = 28$
System 8: $R_F^c = 15$

Substance	Relative probability	Similarity index
Diamorphine	44.9%	87.2
Cyclazocine	19.7%	57.8
Pentazocine	10.6%	42.4
Lobeline	7.3%	35.1

Value(s) found substance II:
System 5: $R_F^c = 10$
System 8: $R_F^c = 8$

Substance	Relative probability	Similarity index
Dihydrocodeine	12.2%	96.6
Disopyramide	11.1%	92.2
Trimethoxyamphetamine/2/4/6	9.9%	87.2
Strychnine	9.9%	87.2
Hexobendine	8.3%	80.0
Ephedrine	5.6%	65.4
Dextrorphan	5.6%	65.4

PRINTOUT 6

Value(s) found substance I:
System 5: $R_F^c = 10$
System 8: $R_F^c = 15$

Substance	Relative probability	Similarity index
Levorphanol	19.7%	96.6
Amphetamine	19.7%	96.6
Pipazethate	16.0%	87.2
Methylenedioxyamphetamine/mda	13.5%	80.0
Diethyltryptamine/NN-	7.0%	57.8

Value(s) found substance II:
System 5: $R_F^c = 28$
System 8: $R_F^c = 8$

Substance	Relative probability	Similarity index
Oxymorphone	21.9%	72.2
Tiotixen	20.6%	70.1
Morphine-6-acetate	15.5%	60.8
Quinidine	7.0%	40.9
Cinchonidine	6.0%	37.8
Procaine	5.2%	57.8

PRINTOUT 7

Value(s) found substance I:
System 5: $R_F^c = 28$
System 8: $R_F^c = 15$
System 6: $R_F^c = 30$

Substance	Relative probability	Similarity index
Diamorphine	93.7%	77.3

Value(s) found substance II:
System 5: $R_F^c = 10$
System 8: $R_F^c = 8$
System 6: $R_F^c = 10$

Substance	Relative probability	Similarity index
Strychnine	46.8%	89.6
Disopyramide	33.5%	80.2
Methenamine	8.7%	51.2
Hydrocodone	8.1%	50.0

It should be borne in mind, however, that even computerized search programs become difficult when larger numbers of substances are present, which is not uncommon as multi-drug intoxications or complex drug mixtures are more and more often encountered. With five substances present and using two analytical techniques there are 120 theoretical configurations, and the application of a third technique will increase this number to more than 14 000. Fortunately, in practice these numbers turn out to be much lower because an experienced analyst will be able to rule out certain configurations as being false. Nevertheless, multi-component mixtures remain extremely difficult to analyse and the computer serves an important task in pointing out the various possibilities so that one does not jump too soon to scientifically unsound conclusions.

One may ask the question of what would happen if a substance is encountered in a case sample that is not (yet) included in the database. The answer is that this substance will not be identified, in which event the computer will report "no candidates", or it will be misidentified in that the computer gives the best possible match. This is illustrated in printout 8: a sample containing methylpiperidyl benzilate (JB-336) was analysed using TLC solvent systems 4, 5 and 6, resulting in a listing of the correct candidate at 83% probability and its ethyl analogue, JB-318, as a second candidate at 13% probability and a much lower similarity index (see printout 8A). When we used an older version of the database in which JB 336 and JB 318 were not yet taken up, the listing in printout 8B was obtained with flurazepam as best candidate.

PRINTOUT 8

A
Value(s) found:
System 4: $R_F^c = 73$
System 5: $R_F^c = 58$
System 6: $R_F^c = 50$

Substance	Relative probability	Similarity index
Methylpiperidyl [JB-336]	83.1%	100.0
Ethylpiperidyl [JB-318]	12.8%	53.6

B
Value(s) found:
System 4: $R_F^c = 73$
System 5: $R_F^c = 58$
System 6: $R_F^c = 50$

Substance	Relative probability	Similarity index
Flurazepam	41.2%	27.3
Piperidolate	28.1%	24.0
Benactyzine	17.2%	20.4

The above discussions make it clear that, for a rational approach to STA, it is first necessary to evaluate carefully the various chromatographic systems and techniques with respect to their identification power and inter-laboratory reproducibility, so that the best can be selected. Thereafter, one can start to build up a data bank for these optimum systems. This data bank must be made as large as possible and be kept up-to-date. The combination of optimum systems and a comprehensive data bank then provides a powerful tool for the detection and identification of potentially harmful substances.

There is one other critical factor that remains to be emphasized, however, namely that STA almost invariably requires a sample pretreatment step to extract the substance(s) of interest from their matrix. Here too the basic problem

is to find suitable methodologies that extract compounds of all types and properties in sufficient yields without leaving too much or too many behind and on the other hand without obtaining too much interfering material in the extract [28]. However, a detailed discussion of this problem would be beyond the scope of this paper.

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