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Drug screening in biological fluids
The need for a systematic approach

Rokus A. de Zeeuw

Department of Analytical Chemistry and Toxicology, University Centre for Pharmacy, Deusinglaan 1, 9713 AV Groningen, Netherlands

Abstract

In this paper the key steps towards drug screening in biological fluids are considered: (i) sample work up–isolation–concentration; (ii) differentiation–detection; (iii) identification. For (i) solid-phase extraction has very good potential; for (ii) thin-layer chromatography, gas chromatography and high-performance liquid chromatography, in combination with their respective detection modes, each have their particular advantages and disadvantages; for (iii) extensive computerized data bases are essential. It is emphasized that each step must be approached and carried out in a systematic way, not only to make sure that all substances of interest can be detected, but also for their correct identification. To this end all analytical procedures and techniques used require extensive validation of their detection and identification properties.

Keywords: Drug screening

1. Introduction

One of the principal tasks in toxicological analysis is to detect whether a potentially harmful substance is present and to identify the substance(s) correctly, regardless whether this is for clinical, forensic, environmental, workplace, drug abuse or doping control purposes. It is obvious that the analytical toxicologists face a formidable task: in our society, we are surrounded by a large number of chemicals, drugs, pesticides, household products, doping agents, etc., that may be hazardous to man and the environment. The analyst must be able to detect such compounds, identify them, differentiate between closely resembling ones and quantitate them within a reasonable amount of time. Moreover, the materials the toxicologist has to work with are usually available in limited amounts and often contain a complex biomatrix in which the toxicologically relevant substances are present in trace amounts. Finally, establishing the presence of a harmful substance is not the only task in toxicological analysis: at the same time it is equally important to establish the absence of other toxicologically relevant substances within reasonable limits. The latter aspect is often underestimated.

Thus, it will be clear that this type of analysis requires a very concise, well planned approach, also called systematic toxicological analysis (STA). It can be defined as the logical chemical-analytical search for potentially harmful substances, whose presence are uncertain and their identity unknown. In this paper the principles of STA and the present state of the art will be reviewed. Since organic substances with molecular weights in the order of 100–400 are most often encountered, the primary focus will be on the qualitative analysis of these substances.

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2. Key steps

The three key steps in STA and the potential approaches involved in these steps are as follows:

(A) Sample work up–isolation–concentration

- Hydrolysis, digestion, removal of matrix compounds
- Liquid–liquid extraction (LLE)
- Solid-phase extraction (SPE)
- Supercritical fluid extraction (SFE)
- Immunoaffinity chromatography (IAC)

(B) Differentiation–detection

- Immunoassays (IA), receptor assays (RA)
- Thin-layer chromatography (TLC), with various colour reactions
- Gas chromatography (GC), with one or more of the following detectors: flame ionization detection (FID), nitrogen–phosphorous detection (NPD), electron capture detection (ECD), and mass spectrometry (MS)
- High-performance liquid chromatography (HPLC), with one or more of the following detectors: ultraviolet spectrometry (UV), diode array detection (DAD), electrochemical detection (rather confusingly, also abbreviated as ECD, see above), and mass spectrometry (MS)

(C) Identification

- Comparison of found data with (computerized) data bases of reference substances

When applying these steps, the following prerequisites are to be kept in mind:

1. Retain all toxicologically relevant substances, yet remove as much of the non-relevant substances and interferences (matrix).
2. Obtain maximum differentiation in a minimum amount of time. Detect with optimum universality and sensitivity, yet also try to differentiate in the detection phase.
3. Maintain comprehensive and updated data bases for all relevant techniques and all relevant substances. The latter should also include data on metabolites, endogenous interferences, omnipresent contaminants, etc.

3. Sample work up

When dealing with biofluids such as urine, whole blood plasma or serum, hydrolysis of the specimens by means of enzyme preparations such as glucuronidase, sulphatase or mixtures thereof are often applied to split conjugated metabolites. Hydrolysis by means of strong acids or bases cannot be recommended because it may result in the decomposition of many substances of interest. Protein removal by means of the latter agents or by other protein precipitants should also be avoided since it may result in the occlusion of relevant substances.

Given the low concentration in which harmful substances are present in biofluids, and the fact that the latter contain many interfering compounds, STA always requires an extraction–concentration. The crucial point is that this extraction step must be able to extract a very wide variety of substances, ranging from very lipophilic to moderately polar, and exhibiting acidic, neutral, basic or zwitterion properties. In addition, the extraction method must be rapid, reproducible and give good recoveries and clean extracts.

Traditionally, LLE has been used routinely in most toxicology laboratories. However, in recent years, SPE has gained popularity for the isolation of drugs from biosamples. As compared to LLE, SPE offers several advantages, such as cleaner extracts, speed and reproducibility, ease of operation and automation, and avoidance of emulsion formation [1,2]. Numerous publications on SPE for biological fluids have been reported, but most methods deal with the extraction of single drugs or groups of related drugs, and only a few have been geared towards drug screening [3–6]. The results obtained underline how difficult it is to find a single SPE material capable of efficiently extracting drugs from various classes.

Therefore, research in our laboratory has focused on the use of multimodal SPE columns, i.e. columns that exhibit more than one retention mechanism, and this has proven to be a good strategy. We initially selected a Bond Elut Certify column (Varian, Harbor City, CA), but the approach proved to be equally well applicable to similar columns from other manufacturers. The type of mixed mode columns exhibit hydrophobic interactions and cation-exchange interactions. In addition, we utilized a pH shift during the extraction to differentiate between acidic and neutral drugs on the one hand and basic drugs on the other. Fig. 1 gives a schematic overview of the SPE procedure for plasma, serum or urine [7]. Acidic and neutral drugs are eluted in fraction A, whereas basic drugs and remaining neutral drugs are eluted in fraction B. Apart from GC, fractions may also be analysed by TLC and/or HPLC. The extracts obtained were found to be quite clean and recoveries were usually between 80 and 100% with R.S.D. values of less than 10%. It should be noted that drugs with strong plasma protein binding, such as antidepressants and phenolthiazines also gave recoveries of nearly 100%, suggesting that their binding to proteins is disrupted in favour of a stronger binding to the cation-exchange groups of the sorbent. Sonication of the diluted specimen prior to application to the column will enhance this disruption. After suitable sample pretreatment, the same approach also worked well for the analysis of whole blood and for tissues [8,9]. In addition, the methods can be fully automated [10] and, contrary to earlier reports, various batches of column material showed excellent lot-to-lot reproducibilities [11].

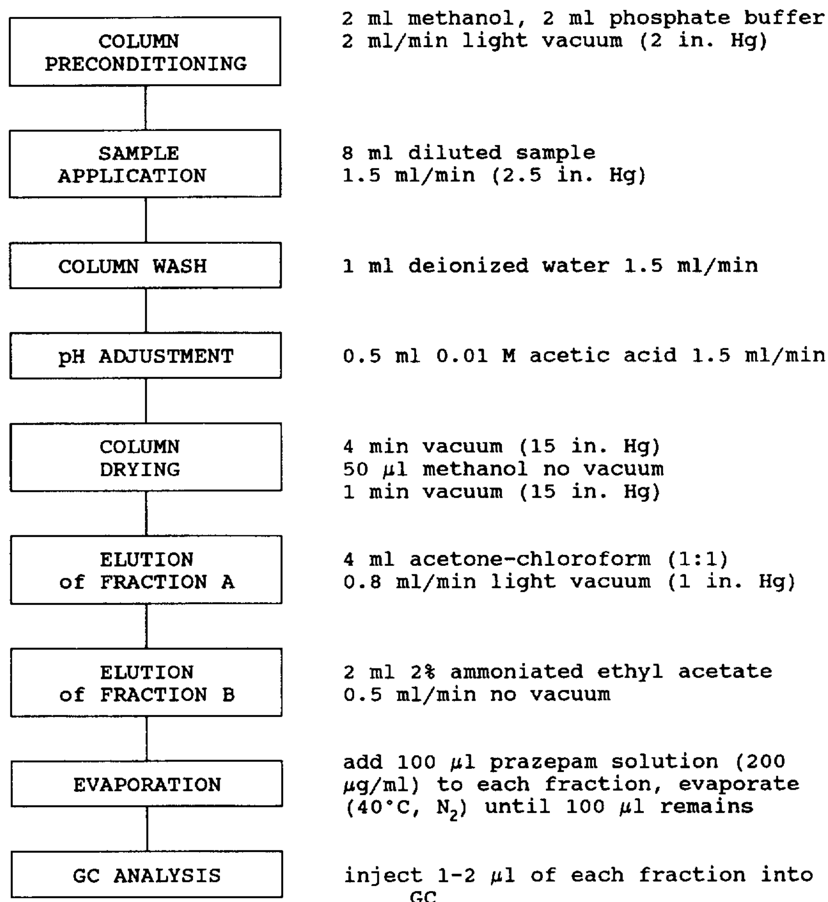

Fig. 1. Solid-phase extraction scheme on Bond Elut Certify columns for broad spectrum drug screening in plasma, serum or urine.

Thus, the developed concept of using hydrophobic and cation-exchange interactions makes SPE well suited for systematic analysis. Some rather polar acidic and basic substances, such as salicylic acid and benzoylecgonine, may give low recoveries but recent investigations have shown that this can be circumvented by slight adaptations of the procedure [12]. The use of anion-exchange interactions, which appeared another variable option for screening, especially with regard to retaining acidic substances, was found to be less suitable due to interference by matrix components [12].

The new trend of miniaturizing SPE column procedures into disc procedures is certainly of interest for STA. Disc filtration may result in shorter procedures and smaller amounts of solvents to be used. It remains to be seen, however, whether the discs provide sufficient sample load capacity and sufficient analyte retention, particularly when dealing with multi-component intoxications representing different substance classes.

4. Differentiation–detection

A variety of analytical techniques are available for the differentiation and detection of a relevant substance in a sample. After sample work up has been carried out, chromatographic and spectroscopic systems then come into focus. Other frequently applied techniques are immunoassays and receptor assays. They are geared to give a yes/no answer with respect to substances belonging to a certain class and can often be carried out directly in the biological fluid. They will not be discussed further in this article.

With regard to the actual analysis, one may choose from a series of analytical techniques such as TLC, GC and HPLC. In addition, within a technique, one has the option of selecting a large variety of developing systems (i.e. the combination of stationary phase and mobile phase). Furthermore, the chromatographic techniques may be combined with appropriate detection modes. In order to obtain maximum benefit from these techniques and systems, it is important that one selects the best in the various categories and that they be designated as recommended. The following criteria are crucial in the selection of analytical methods most suitable for identification [15].

1. The substances measured in the analytical systems should cover the total range and be evenly distributed over that range (e.g. for TLC the R_F range 0–100).
2. The standard deviations of measurement should be as small as possible and the values (e.g. R_F-values) must be standardized in such a way that good reproducibility is obtained on an inter-laboratory scale.
3. When more than one system or technique is used, there should be low correlation between these systems or techniques.

In order to establish the extent to which an analytical method fulfils the above criteria in an objective way, a number of mathematical approaches have been developed such as mean list length (MLL) [13,14], discriminating power [15,16] and information content [17]. Since MLL can also be used to identify unknown substances, we have a preference for this technique [18].

In the MLL approach, it is established for a given analytical parameter in a given system [e.g. a retention index (RI) in a GC system] how many substances from a large population would qualify if an unknown substance with that RI is encountered. The number of qualifying substances is called the list length. This can be done for all RI values in that GC system and the list lengths thus obtained can be averaged to give the MLL. The shorter the MLL, the better that system is for STA. This can be done for individual systems as well as for combinations of systems, including detection modes. The ultimate is reached when an MLL of 1 is found, which means that all substances from the large population can be identified. As unequivocal identification can never be achieved by applying a single analytical system, it is essential that two or more systems are used in combination. It will also be clear that the number of systems required for unequivocal identification will increase when the number of substances in the population increases. On the other hand, when an MLL of 1.00 is found, i.e. only one candidate for the unknown substance under investigation, all other

substances in the population are automatically excluded. This reflects the ultimate aim in STA: to exclude all substances except one.

In recent years, extensive evaluations of TLC and GC systems have been carried out on an interlaboratory basis, which also provided extensive data bases [19,20]. This resulted in the recommendation of a number of TLC systems for acidic and neutral drugs and for basic and neutral drugs. To compensate for variations in the experimental conditions, the R_F values observed are to be corrected by means of a set of reference substances that are co-chromatographed on the same plate. For GC a dimethylsilicone column, preferably a capillary one and in a temperature-programmed run, is best suited for STA. Retention is expressed as Kovats RIs. Due to the fact that almost all GC systems are highly correlated with the dimethylsilicone system, no other GC system can be recommended. Evaluations of HPLC systems have long been hampered by lack of reproducible behaviour between different batches. However, this situation has improved substantially in the early nineties and a first HPLC screening system has been recommended [21,22]. It uses reversed-phase C_{18} or C_8 columns and gradient elution with a triethylammonium phosphate buffer and acetonitrile. Retention is expressed as RIs derived from a mixture of homologous nitroalkanes. As in GC, mixtures of reference drugs can be used to correct for variations in experimental conditions, especially in interlaboratory comparisons [23].

To assess the potentials of combinations of chromatographic systems and their respective detection modes, the MLL concept proved very valuable [24]. Evaluations were made with a test set of 99 basic and neutral drugs, representing various classes and structures of toxicologically relevant drugs. These substances were evaluated by TLC, GC and HPLC as follows.

For TLC, three different systems were used and corrected R_F values were measured. Then, on each plate, 4 colour reactions were carried out in sequence and after each step the colour was noted and encoded by means of a colour wheel [25]. The colour reactions were taken from the Toxi-Lab Drug Compendium [26].

MLL systems

- TLC 1, ethylacetate–methanol–25% ammonia (85:10:5) on silica, S.D. 3.8
- TLC 2, methanol on silica, S.D. 2.8
- TLC 3, cyclohexane–toluene–diethylamine (75:15:10) on silica, S.D. 3.0

Colour reactions

- CR 1, expose to formaldehyde vapor, then dip in concentrated sulphuric acid with 0.1% ammonium vanadate and observe the colour
- CR 2, dip in water (exothermic reaction) and observe the colour
- CR 3, observe the fluorescence under UV light of 366 nm
- CR 4, dip in modified Dragendorff reagent and observe the colour

The GC system used a capillary fused-silica dimethylsilicone column (HP-1, 12.5 m×0.53 mm, film thickness 0.88 μm) with a temperature programmed run from 120 to 300°C, with FID or NPD. Retention times were converted to retention indices according to [20]. The S.D. was 25 RI units. As an additional detection parameter, the molecular mass was used (M_r) as obtained from chemical ionization mass spectrometry.

The HPLC systems consisted of an RP-Select B C_8 column (125×4.6 mm I.D.) in a gradient mode, starting at 100% 0.025 triethylammonium phosphate buffer pH 3.0, to 30% acetonitrile in 70% of the above aqueous solution in 30 min. Detection was by means of a Hitachi-Merck L-3000 diode array detector. Retention times were converted to retention indices based on nitroalkanes (RI_{NO2}), according to [21]. The S.D. was 7 RI_{NO2} units. Special comparisons were made over the range of 200–360 nm.

MLL calculations were made for single systems and detection modes [13], as well as for combinations of systems and detection modes [27]. Table 1

Table 1
MLL values for single systems and single detection modes

System	MLL	Detection	MLL
TLC 1	19.60	CC 1	14.99
TLC 2	14.09	CC 2	16.36
TLC 3	22.12	CC 3	15.60
GC	7.20	M_r	1.40
HPLC	9.80	DAD	10.13

Table 2
MLL values for combinations of single systems with their appropriate detection modes

System and detection	MLL	System and detection	MLL
TLC 1–CC 1	9.46	GC–M_r	1.10
TLC 2–CC 2	7.61	HPLC–DAD	3.52
TLC 3–CC 3	9.88		

gives the MLL values for single systems and detection modes for the set of 99 test substances. Because of their limited separation efficiencies and low reproducibilities, the TLC systems clearly have the highest MLL values. The best is TLC 2 because of its good spread and lowest S.D. values. GC and HPLC provide substantially lower MLL values indicating that these are better suited for STA as single systems. For the detection modes, as expected, the identification power of M_r is by far the best, but even for a small data base of only 99 substances, the MLL does not yield unequivocal identification. On the other hand, it is interesting to know that the combination of 4 colour reactions and DAD provide MLL values of the same order as the corresponding chromatographic techniques. These examples show how MLL can be utilized to select the best systems for STA.

Table 2 gives the MLL values for combinations of single systems and their corresponding detection modes. It can now be seen that a single, rapid TLC test plus a few colour reactions provides similar IP values as a GC or HPLC retention index. The highest IP is obviously obtained by GC–M_r, but again, no unequivocal identification is obtained for all 99 substances. Thus, the widely stated belief that identification can generally be obtained by two techniques of which one should be MS appears to be far from correct. This is further demonstrated in Table 3, which lists the MLL values for combinations of two systems with their corresponding detection modes. Again, the most powerful combination of GC–M_r with HPLC–DAD does not result in the ideal MLL value of 1.00.

The MLL values for combinations of three separation systems and detection modes are given in Table 4. The use of all three TLC systems and the colour codes now results in an MLL value of 1.35. Combinations including GC–M_r or HPLC–DAD, or both, are even better but there is only one combination that provides the ideal MLL value of 1.00, namely TLC 2-CC plus GC–MS plus HPLC–DAD.

Hence, these MLL evaluations provide an objective assessment of the potentials of the various systems and detection modes for the identification of unknown substances, leading to some valuable and interesting conclusions. Obviously, HPLC–DAD and GC–M_r are very powerful techniques, but even for this limited test set additional information from a suitable TLC–CC system is necessary to yield unambiguous identification. This contrasts with the widely held belief in analytical toxicology that identification can be achieved by applying two techniques based on different physico-chemical principles. Clearly, the latter is a serious oversimplification, even when MS is included. Although we utilized molecular masses derived from MS, we have good reasons to believe that the same is true if MS information is used in the form of electron impact (EI) mass spectra. More detailed calculations on EI-MS are in progress.

On the other hand, for those workers who do not have access to the more powerful coupled detection modes, such as DAD and MS, it is good to see that identifications can be approached very well on the basis of TLC–CC plus GC and HPLC retention indices alone. This is demonstrated in Table 4, in

Table 3
MLL values for combinations of two systems and their appropriate detection modes

System and detection	System and detection			
	TLC 2–CC	TLC 3–CC	GC–M_r	HPLC–DAD
TLC 1–CC	2.17	2.65	1.04	1.24
TLC 2–CC	–	2.20	1.04	1.19
TLC 3–CC	–	–	1.06	1.18
GC–M_r	–	–	–	1.02

Table 4
MLL values for combinations of three systems and their appropriate detection modes

System and detection	System and detection		
	TLC 3–CC 3	GC–M_r^a	HPLC–DAD[a]
TLC 1–CC 1 plus			
TLC 2–CC 2	1.35	1.02 (1.06)	1.08 (1.11)
TLC 3–CC 3	–	1.04 (1.19)	1.12 (1.16)
GC–M_r^a	–	–	1.02 (1.06)
TLC 2–CC 2 plus			
TLC 3–CC 3	–	1.02 (1.08)	1.08 (1.10)
GC–M_r^a	–	–	1.00 (1.04)
TLC 3–CC 3 plus			
GC–M_r^a	–	–	1.02 (1.08)

[a] Values in brackets have been obtained by omitting M_r and DAD parameters.

which the MLL values in brackets were obtained by excluding M_r and DAD parameters. The best combination of TLC 2-CC plus GC and HPLC now gives an MLL value of 1.04, which is only marginally higher than 1.00 when DAD and M_r are included. The values for combinations of two TLC–CC systems with either GC or HPLC RIs are not much higher, between 1.06 and 1.19, respectively. Moreover, even those laboratories which can only afford TLC (e.g. in developing countries) should not despair. The combination of the present three TLC systems and the colour codes already has very good identification power with an MLL of 1.35.

5. Identification

After having selected the most suitable systems and detection modes for STA on the basis of their MLL values, data bases need to be established on an interlaboratory scale. As explained, these bases are to contain data on a multitude of substances of toxicological relevance. When unknown substances are encountered, their parameters are then to be compared with those in the data base to find possible matches. It will be clear that this is to be done with the aid of a bench-top computer that is directly accessible in the laboratory.

We have recently developed such a system, capable of handling TLC–CC, GC–M_r and HPLC–DAD. This so-called MTSS system is commercially available from Merck (Darmstadt, Germany) in a Windows version. It contains data for 5 TLC systems (2 for acidic and neutral drugs; 3 for basic and neutral drugs), 1 GC system and 1 HPLC system, together with data on colour reactions, M_r and DAD spectra. It comes with data for at least 900 toxicologically relevant substances. In addition, users may create data bases on their own (e.g. for a GC or HPLC system of their choice) or for other applications (e.g. for analysing pesticides in environmental toxicology). However, it should be realized that the utility of a data base is directly related to the number of entries it contains, because if a substance is not represented in the data base, it will never be found or it will be misidentified. Furthermore, a good data base will also contain data on appropriate metabolites, on ubiquitous substances like plasticizers, antioxidants, caffeine, nicotine, etc. and on coextracted substances originating from the matrix, such as cholesterol, fatty acids, etc.

The system works as follows. After having run the unknown sample in any combination of analytical systems and developing mode one chooses, the parameters found are entered into the computer, together with the data for the reference mixtures used to calibrate the chromatographic systems. The latter allow the computer to correct the R_F values and/or to calculate the RI values. After pressing the "Search" key, the computer then compares the values for the unknown(s) with the values in the data bases to find possible matches within the allowed error windows. It can do this for up to five unknown spots or peaks per sample and it automatically

checks all possible configurations (14) for combinations of spots and peaks. It is also able to deal with situations where the number of spots and peaks do not match (e.g. two TLC spots and three HPLC peaks). Finally, the computer prints out a list of substances that give acceptable matches for all the analytical parameters entered, in decreasing order of possibility. Also, a similarity index is given for each listed substance, indicating the difference between the measured data and those in the data base. Obviously, when data for only one analytical system are entered, the list of candidates will be very long, but the length of the list is drastically decreased when data for a multitude of systems are entered (as already seen under the MLL evaluations). Of course, the ultimate is reached when the final list contains only one substance with an acceptable similarity index. If the list continues to contain more than one candidate it means that additional systems and detection modes must be applied. The best choice is strongly dependent on the substances involved. On the other hand, it should be realized that there is no need to use all the techniques and/or systems in the MTSS system. It may suffice to run the GC system and combine that with suitable MS-information. Or, if one does not have access to HPLC, one can run the appropriate TLC and GC systems with the available detection options and then see how long the list of possible candidates will be. If it remains difficult to come to an acceptable identification, one may look for other tests or techniques that can provide further differentiation. It may also occur, that no response is obtained in a given technique, for example in TLC because the concentration present is below the detection limit, or in HPLC–DAD because the analyte does not have suitable UV-absorption. In those cases the computer will skip those techniques which will usually result in a relatively long list of possible candidates. Another factor is the reproducibility of measurement that the system takes into account. These parameters have been entered for the various techniques and systems on the basis of interlaboratory observations. Yet, if the user feels that the entered values may be too small for a particular case (e.g. when dealing with an extract that shows large amounts of matrix interferences in the TLC chromatogram that may have affected the R_F-value of the unknown analyte) the reproducibility window can be enlarged for that system to prevent that it will be excluded in the data base search. Of course, this will result in an extended list of candidates.

Future versions of the MTSS may include additional HPLC systems and immunoassay systems. Work on the evaluation of the latter is in progress.

The use of a computerized search is indispensable in cases in which no prior information is available, such as the so-called general unknowns. However, it should be stressed that also in cases that appear to be quite routine and where abundant prior information is available, the MTSS may be very valuable. It may hint at a substance that is not seen very often but whose data are similar to a better known substance. Also, even when the identity of at least one toxicant is already known, the computer will check the likelihood of the presence of all other substances in the data base, so that no substance is being overlooked. This satisfies the ultimate aim of STA, to exclude all substances except the one(s) present.

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