See Colour Plate 122.

See also: **II/Chromatography: Gas:** Column Technology; Detectors: Mass Spectrometry; Headspace Gas Chromatography; Historical Development; Theory of Gas Chromatography. **Extraction:** Solid-Phase Extraction; Solid-Phase Microextraction.

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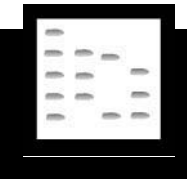
TOXICOLOGICAL ANALYSIS: LIQUID CHROMATOGRAPHY



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Introduction

With the introduction of high performance liquid chromatography (HPLC) coupled to detection systems providing spectral information (e.g. photodiode array detection and mass spectrometric detection, HPLC-DAD and LC-MS) the development of HPLC methods for broad-spectrum drug screening has attracted great interest in forensic laboratories. The information obtained by these methods is two-fold, i.e. retention data and spectral information, creating a powerful identification system. The growing literature describing HPLC as a broad-spectrum technique demonstrates its unique and essential position in toxicological investigations.

A number of important parameters in toxicological screening by HPLC include column packing material, column dimensions, detection, standardization and peak purity assessment. These topics will be treated while the applicability will be demonstrated by presentation of selected examples of general screening and specific detection of a limited number of compounds.

Column Packing Materials

Underivatized Silica

Unmodified silica can retain drugs by a weak cation exchange mechanism and was used for broad-spectrum drug screening as early as 1975. The main problem, however, with the use of underivatized silica is the substantial variability of this material. Different brands of silica and even different batches

of the same brand of silica packing material often result in different retention of selected basic drugs. As a consequence, chromatographic conditions (same batch of same brand of packing, eluent composition, temperature control) need to be exactly defined and strictly followed before reproducible retention times or *retention factors* (k) can be obtained in one laboratory or in different laboratories (the latter being even more difficult). The impact of all these parameters on retention is more substantial in an adsorption system than under reversed-phase conditions. Due to these difficulties, the use of underivatized silica in the application of adsorption chromatography to systematic toxicological analysis (STA) remains rather limited.

Bonded-phase Packing Material

Bonded-phase chromatography, and more especially reversed-phase chromatography on octyl- or octadecylsilica, is by far the most popular liquid chromatographic technique used in STA. In the early 1980s valuable methods for basic drugs on modified silica began to appear. Also practical solutions to the tailing problem were established and refinements were under investigation. Free silanol functions are known to have a marked influence on retention behaviour of different drugs. These silanol effects can be reduced by changing the pH of the eluent or by addition of competing aliphatic bases (amine modifiers) as surface masking agents. Various manufacturers have launched specially prepared columns claimed to be free of silanol effects and providing more reproducible retention times. This is mainly achieved by deactivation of the free silanols by various endcapping procedures and by elimination of the trace metals from the silica support.

Alternatively, polymeric stationary phases have also been introduced. However, although the ability to run these packing materials at pH values even higher than 9 permits the analysis of basic drugs as un-ionized compounds without tailing, these polymeric phases have only a limited application in systematic toxicological analysis.

Recently, again in an effort to eliminate chromatographic problems due to residual silanol groups and to prevent the incorporation of buffer salts in the eluent, an alumina-based packing material coated with polybutadiene has also been used for broad-spectrum drug screening purposes. The inherent absence of silanol functions on this packing material simplifies the retention mechanism, eliminates the need for addition of amine modifiers and prevents irreversible adsorption of co-extracted impurities. In addition, aluminum oxide as well as the polybutadiene coating are stable in the pH range of 2 to 12. This polybutadiene coating has hydrophobic properties comparable to reversed-phase packing materials. Consequently the same solvent mixtures can be used as in reversed-phase chromatography. By incorporation of NaOH in the eluent ($0.0125\ \text{mol L}^{-1}$), basic drugs can be chromatographed without tailing. Of course, this high pH results in poor retention of phenolic compounds (e.g. morphine) or carboxylic acids (e.g. benzoylecgonine). The latter compounds need to be chromatographed on a second and classical reversed-phase packing material. This approach of using two different and complementary packing materials is certainly not unique in systematic toxicological analysis.

Chromatographic Conditions

Due to the large differences in polarity of the compounds encountered in broad-spectrum screening and in view of simultaneous chromatography of parent drugs and metabolites in nearly all reversed-phase chromatographic systems, gradient elution is used. The few systems based on adsorption chromatography apply isocratic elution.

In LC-MS the choice of the solvent composition is limited. The use of nonvolatile mobile phase constituents (e.g. phosphate buffers) is absolutely prohibited. This limits the practical use of LC-MS by excluding techniques like ion pair and ion exchange liquid chromatography.

Offline sample preparation procedures based on liquid–liquid extraction or solid-phase extraction are not really the subject of this article. However, automation of solid-phase extraction coupled directly with injection and chromatographic analysis and online enrichment based on the use of two or more high pressure columns or cartridges (column switching) are already commercialized for broad-spectrum screening in toxicological analyses and therefore worthy of mention.

Detection Systems

Besides retention data, spectral information is essential for the positive identification of an unknown substance. Therefore, detection systems not providing spectral information (e.g. fixed wavelength UV detection, electrochemical detection) have found only limited application in toxicological laboratories such as for repetitive analysis of a small group of structurally similar compounds (e.g. epinephrine, norepinephrine in the case of electrochemical detection).

Photodiode Array Detection

The introduction of diode array and fast-scanning absorption detectors allowed the acquisition of UV (and visible) spectral data during the chromatographic process. This combination of the discriminatory power of the chromatographic retention parameters (which is lower for HPLC than GC) with that of the UV spectral data increased the overall reliability of an HPLC analysis in the area of toxicology. Standard reference spectra can be stored in a database tagged with parameters of retention in order to restrict the search into a window around each retention parameter.

The major problem in the identification of unknown compounds by a UV spectral match is the lack of fine structure in the UV spectrum of many compounds. The identification of metabolites is also difficult because biotransformations do not always result in a drastic change of the UV spectrum. Several studies from the area of chemometrics have provided models for UV spectral matching methods used for toxicological drug analysis. Peak maxima, calculation of differences between normalized spectra and between first-derivative spectra are thereby essential data for estimations of similarity or dissimilarity.

Ideally, each toxicological laboratory should build up its own library of UV spectra recorded under stringent chromatographic conditions. Analysis of unknown samples and recording unknown UV spectra should then be performed under exactly the same chromatographic conditions (column, eluent composition, gradient, pH, etc.) because at least the last three of these parameters can affect the observed absorbance. Another source of library variability is detector-to-detector variation. Because different photodiode array detectors use different numbers of diodes, a UV library development on one system may not be able to meet the same criteria on another instrument.

Co-elution of drugs with other drugs or with endogenous co-extracted substances remains one of the major causes of errors in HPLC analysis. Erroneous conclusions can be drawn if a co-eluting compound mimics the UV spectrum of a known compound or when the co-elution of two compounds results in a spectrum that does not match any library spectrum. Therefore, before running a library search, peak purity assessment is essential. This can be done either manually by comparison of the spectrum at different positions of the emerging peak, or alternatively some computer programs automatically indicate the peak purity under each peak in the chromatogram. The software of the more sophisticated systems even allows peak deconvolution of two co-eluting compounds, resulting in the specific UV spectrum and quantitative contribution of each compound. Other software systems claim to be able to determine the individual drugs from a UV spectrum even if this is a result of up to six compounds eluting at the same retention time. However, it should be notified that in the latter case previous information on the probable co-eluting drugs is essential. This can be obtained from other chromatographic techniques.

Mass Spectrometric Detection

The combination of liquid chromatography and mass spectrometry (LC-MS) offers a major improvement regarding drug identification compared with the above-mentioned HPLC-DAD combination.

The improved resolution and the higher separation efficiency together with the desire to interface HPLC with mass spectrometric detection have been the major driving forces behind the development of capillary LC. Unlike GC, interfacing problems between LC and MS are still a challenge for researchers. Since the early 1970s interfaces have been constructed each applying a different technique to eliminate the chromatographic eluent, which of course cannot be introduced directly into the high vacuum region of a mass spectrometer. At least seven major interfacing techniques exist, i.e. moving belt (MB), particle beam (PB), direct liquid introduction (DLI), fast atom bombardment (FAB), thermospray (TS), electrospray (ES) and atmospheric pressure chemical ionization (APCI).

It is beyond the scope of this contribution to give an extensive overview of these different techniques. However, the respective advantages and/or disadvantages of a number of these techniques, especially in view of their application to broad-spectrum screening, will be presented. Both the DLI and the MB techniques have only historical interest and have virtually disappeared from the area of toxicological analysis. Particle beam and FAB proved to be valuable for specific applications such as the detection of steroids (PB) or the detection of compounds with high molecular mass (FAB). However, in other applications sensitivity is often a problem for these two techniques. Of primary interest for toxicological analysis are the three remaining techniques: thermospray, electrospray and atmospheric pressure chemical ionization. Because TS is able to handle flow rates of conventional HPLC systems (1–2 mL min^{-1}) it became the first popular HPLC-MS interface to be used in many fields with a high sensitivity. Because the ion production in this technique is dependent on the solvent composition, the application of TS with gradient elution can result in difficulties.

Electrospray operates without heat in the spray ionization step which makes this technique suitable

for thermolabile compounds, such as sulfate conjugates of drugs.

Both ES and APCI have found a wider use during the last decade with APCI having an excellent sensitivity especially for hydrophobic compounds. Electrospray has the advantage of being applicable to a wide range of analyte polarity.

Looking to the number of applications and keeping in mind that ES and APCI have not yet been exploited to their full potential, these two techniques together with TS are the most interesting techniques for toxicological analysis. The three techniques are based on a relatively soft ionization process so the mass spectra obtained sometimes lack the fragment ions necessary for confirmation of the identity of an unknown compound.

Quadrupole mass spectrometers are used most frequently because of their ruggedness, however, ion trap instruments are becoming more and more common in STA. Coupling to an ion trap spectrometer is interesting for a variety of reasons, e.g. economical aspects, sensitivity and the ability to run MS-MS experiments. Other techniques, such as collision-induced dissociation in tandem mass spectrometric configurations are also becoming available.

Reliability of Retention

As already mentioned, the efficiency of a HPLC system is considerably less than that of a capillary GC set-up so the risk of co-elution is greater. In addition, the retention behaviour of a compound (together with the spectral information, a pivotal criterion for identification of an unknown substance) is often imprecise. Batch-to-batch variation and variation of packing materials between different manufacturers result in inconsistent retention data. In addition, coating of the active sites by irreversible adsorption and loss of the bonded-phase by ageing of the column can also contribute to changes in retention. Of course this problem can be overcome by injection of the authentic standard directly after the tentative identification of a compound. This procedure, however, is time consuming and presupposes prior identification even without a perfect match of the retention time and the availability of a pure standard. Several studies have evaluated the use of homologous hydrocarbon series, multiple drug reference standards and nitroalkanes to minimize the effect of irreproducible HPLC retention data. Although these relative retention procedures improve the reproducibility, it is difficult to obtain linear relative retention scales by using a homologous series of compounds during gradient elution, the latter being the most popular technique for liquid chromatographic toxicological screening purposes. The use of multiple drug standards instead of non-drug compounds such as nitroalkanes results in a more effective correction for retention shifts. Both principles can also be combined by calculation of retention indices of compounds from their retention times by linear interpolation between standard drugs, whose retention indices have been previously determined on a nitroalkane scale.

Applications

A number of recently developed broad-spectrum screening procedures based on HPLC-DAD are brought together in **Table 1**. They all use

Table 1 Operating conditions of HPLC-DAD in toxicological broad-spectrum screening

Column	*Eluent*	*Flow rate (mL min^{-1})*	*Standardization*	*Number of compounds*	*Year*
Superspher 100 RP18 4 μm; 125 × 4 mm	Acetonitrile : triethylamine phosphate; gradient	1	1-Nitroalkanes 18 standard drugs	383	1994
Symmetry C8 5 μm; 250 × 4.6 mm	Phosphate buffer (pH 3.8) : acetonitrile; gradient	1 to 1.5	None	600	1997
Supelcosil LC-DP 5 μm; 250 × 4.6 mm	Acetonitrile : phosphoric acid : triethylamine; isocratic	0.6	None	272	1995
Lichrospher 100 RP8 5 μm; 250 × 4 mm	Acetonitrile : phosphoric acid : triethylamine; isocratic	0.6	None	280	1995
Hypersil C18 5 μm; 150 × 4.6 mm	Acetonitrile : phosphate buffer (pH 3.0) : sodium octyl sulfate : triethylamine; gradient	1	None	>300	1997
Aluspher RP-select B 5 μm; 125 × 4 mm	Methanol : water containing 0.0125 mol L^{-1} NaOH; gradient	1	None	>150	1995
Spherisorb S5 ODS-2 5 μm; 150 × 3.8 mm	Acetonitrile : phosphate buffer (pH 3.1); gradient	1	*p*-Methylphenyl-phenylhydantoin	130	1993

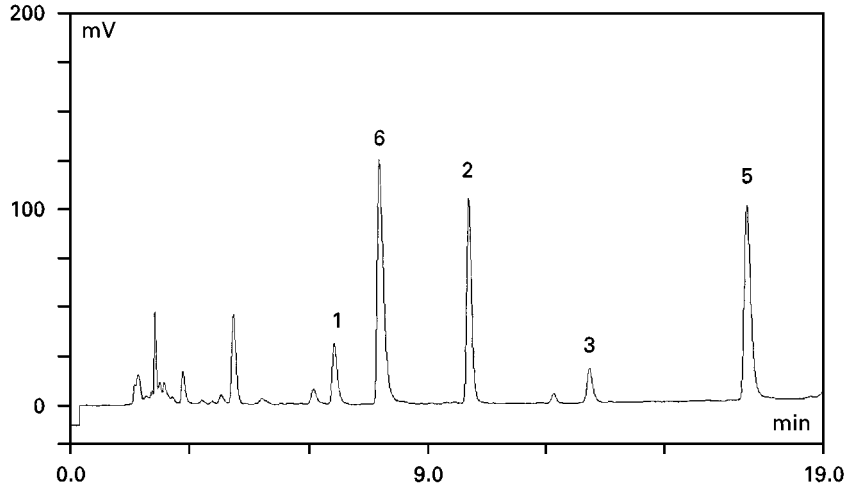

Figure 1 Chromatogram of a real postmortem whole blood sample. Peaks: (1) benzoylecgonine; (2) 2′-methylbenzoylecgonine; (3) cocaine; (5) 2′-methylcocaine; (6) 3,4-methylenedioxy-*N*-ethylamphetamine. Levels: (1) 0.1; (2) 0.65; (3) 0.1; (5) 0.65; and (6) 1.3 $\mu g\,mL^{-1}$. (Reproduced with permission from Clauwaert *et al.*, 1997.)

reversed-phase packings on either a silica or an alumina matrix. All except two procedures apply gradient elution and standardization of the retention is rather an exception (two procedures out of seven). The authors present lists of a large number of drugs and toxicologically relevant compounds (ranging from 130 to 600) and state that this is not a limitation but that other compounds can also be added to these lists.

It is not possible to give a similar table showing the operating conditions of LC-MS applied to broad-spectrum screening in forensic sciences. Screening the literature rapidly demonstrates that to date LC-MS has only been applied to selected compounds or groups of compounds such as steroids, thiourea pesticides, mycotoxins, tricyclic antidepressants and 10 illicit drugs (all by TS), diuretics, non-steroidal anti-inflammatory drugs, carbamate pesticides (by ES) and β-agonists, carbamate pesticides and alkaloids by APCI. For detailed information on the operating conditions of these various applications we refer to the specialized literature in this field.

Application

The applicability of HPLC coupled to photodiode array detection as well as to mass spectrometric detection in the field of forensic sciences will be demonstrated by the analysis of cocaine and some of its metabolites by both techniques. Cocaine, benzoylecgonine and cocaethylene have been determined by HPLC-DAD using 2 mL of blood, serum or urine under reversed-phase gradient conditions. The quantitative limit, defined as that concentration that can be determined with an acceptable reproducibility ($\leqslant 6\%$), is $50\,ng\,mL^{-1}$ for benzoylecgonine and cocaine and $25\,ng\,mL^{-1}$ for cocaethylene (using 2 mL of body fluid) (**Figure 1**).

On the other hand, cocaine, benzoylecgonine, ecgonine methylester, ecgonine and norcocaine have been quantified in urine (1 mL) with an LC-MS system based on step-gradient elution of a large (250×7.6 mm) steric exclusion column followed by atmospheric pressure chemical ionization–mass spectrometry. The detection limits (signal-to-noise ratio = 3) under selected ion monitoring (SIM) mode conditions were 320, 200, 200, 20 and $60\,ng\,mL^{-1}$ for ecgonine, benzoylecgonine, ecgonine methyl ester, cocaine and norcocaine, respectively. Unfortunately, quantitative limits were not reported for this method (**Figure 2**).

Both systems used solid-phase extraction for sample preparation. Due to their non-UV-absorbing properties ecgonine and ecgonine methyl ester were not detected in the HPLC-DAD system. This system was, however, able to detect and to chromatograph other toxicologically relevant compounds while for the LC-MS system this is not mentioned.

Conclusion and Perspectives

Besides further optimization of both the liquid chromatographic and the spectrometric parts of the described configurations, a great challenge for the future is the automation of those systems. Complete automation of an analytical procedure including online sample pretreatment is always advantageous

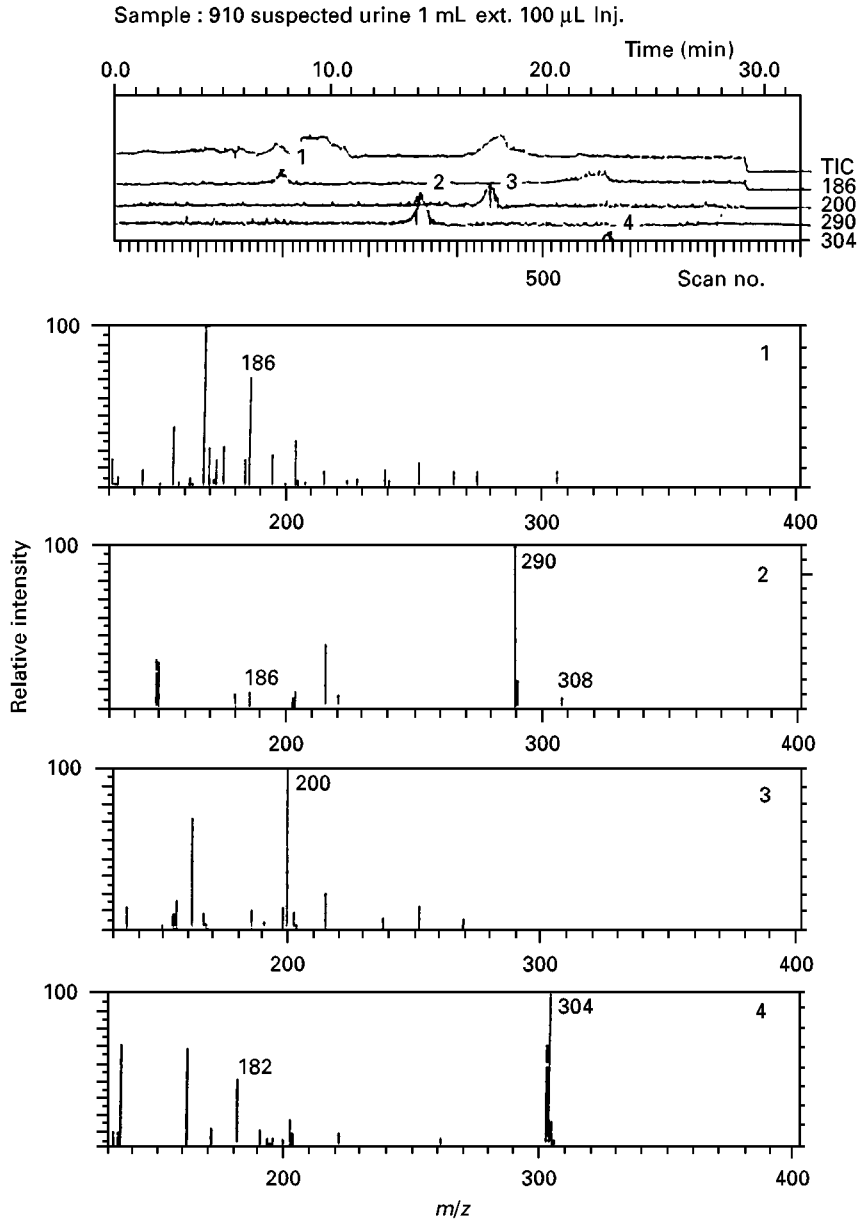

Figure 2 Mass chromatograms and mass spectra of extracts obtained from suspected urine. Peaks. (1) ecgonine; (2) benzoylecgonine; (3) ecgonine methyl ester; (4) cocaine. Levels: (1) 3.2; (2) 4.8; (3) 3.6; and (4) 0.8 µg mL^{-1}, respectively. (Reproduced with permission from Nishikawa *et al.*, 1994.)

in STA. Because the optimum performance of HPLC-DAD and more especially of LC-MS are determined by the simultaneous optimization of a large number of interrelated parameters (flow, pressure, temperature, voltage …), expert systems should be optimized allowing fully automated tuning and control.

The different detection principles as well as the comparable sensitivity demonstrate the complementary character of both HPLC-DAD and LC-MS. In the future, both techniques will undoubtedly gain in interest and will play an essential function in forensic sciences.

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TOXINS: CHROMATOGRAPHY

See **III / MARINE TOXINS: CHROMATOGRAPHY; NEUROTOXINS: CHROMATOGRAPHY**

TRACE ELEMENTS BY COPRECIPITATION: EXTRACTION

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In separation by precipitation, contamination with other elements by coprecipitation is undesirable. However, since the publication by Bonner and Kahn of a summary on the separation of carrier-free radioactive tracers by coprecipitation in 1951, this technique has found wider application to the separation and preconcentration of trace elements in various kinds of samples, such as natural water, treated wastewater, high purity metals and geological and biological materials.

In modern textbooks, coprecipitation is recommended for separation and preconcentration of a single trace element or a group of trace elements when the concentration is too low to be directly precipitated or the amount is too small to be handled. In general, coprecipitation of trace elements is carried out with inorganic and organic precipitants attaining high degrees of concentration, so that subsequent determinations can be performed by using the precipitate itself.

Mechanism

Depending on the nature of the solid phase produced in a solution and the experimental conditions, coprecipitation occurs by different mechanisms. Although the various types of coprecipitation cannot be distinguished clearly, they may be classified according to the following mechanisms: (i) the formation of mixed crystals and mixed chemical compounds, (ii) surface adsorption and the occlusion and (iii) mechanical inclusion of trace components into the other compounds during crystal formation. However, these processes often proceed concurrently, making the precipitation process quite complicated.