# Identifying Toxins Using an Extensive, Fast and Automated HPLC Spectral Library

## Application Note

Pharmaceutical and Toxicological Analysis

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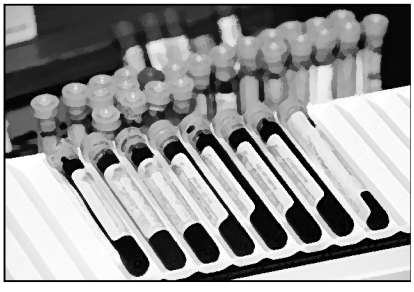

The Institute of Legal Medicine at Humboldt University in Berlin, Germany, initiated development of a spectra library to help clinical and forensic toxicologists identify poisons as quickly as possible. The library contains more than 1,600 UV spectra of compounds relevant to pharmacology and toxicology. The spectra were measured at the Forschungsgesellschaft für Lungen- und Thoraxerkrankungen (FILT) in Berlin and tested with extracts of human serum, spiked full blood or urine samples and samples taken during the investigation of many real cases of poisoning. The library is divided into sublibraries that enable faster peak identification. Problem-oriented preselection of the matching sublibrary allows identification of an analyte with a higher degree of accuracy. Using this library and a diode-array detector software, analysts can compare the spectrum of an unknown peak with those in the library in a very short time. With relatively simple sample preparation methods, analysts can use this library to identify poisons and their metabolites fast and confidently. The library also offers a link to a database that gives each compound's nonproprietary name, CAS number, effect or use, retention time and spectroscopic maxima, minima and shoulders. An easy-to-use manual provides the same data, a printout of each spectrum and the structural formula of each compound. All of this makes the library a valuable tool in forensic, clinical and toxicological analysis.

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## Introduction

During the past decade, High Performance Liquid Chromatography (HPLC) has become one of the most important analytical techniques in the chemical, biochemical and medical practice. HPLC is particularly effective for separating and identifying compounds used in pharmacology and toxicology. Applications include controlling environmental and food contamination and determining the levels of active agents in medicinal drug candidates.

Almost all compounds of interest in the chemical and medical fields absorb light in the UV spectral region. Therefore, most HPLC analyses use UV-visible detectors. The introduction of the diode-array detector and the similarly-operating rapid-scan UV-visible detector opened another dimension in UV detection. These detectors allow each chromatographic peak to be identified not only by retention time but also by its UV spectrum. The UV spectrum of a compound is very reproducible and its full shape is much more compound-specific than generally assumed.

A prerequisite for identifying compounds with diode-array detection is a library containing the UV spectra of as many compounds of interest as possible. Such a library is laborious to generate. And because many compounds are not available, creating a comprehensive library is often expensive and time-consuming. Most such libraries, therefore, remain very limited.

The Institute of Forensic Medicine at Humboldt University in Berlin, Germany, initiated development of a spectra library that contains more than 1,600 UV spectra relevant to pharmacology and toxicology, including a large variety of metabolites, which are particularly challenging to identify. The library was created because clinical and forensic toxicologists need to diagnose poisonings as quickly as possible. The large number and variety of potential poisons make diagnosis challenging, particularly when there is little information about the type and amount of poison ingested.

Spectra were measured and tested at the Forschungsgesellschaft für Lungen- und Thoraxerkrankungen (FILT) in Berlin and tested with human serum, spiked full blood or urine samples or samples taken during the investigation of real poisonings. The identification and correctness of the spectra were thoroughly controlled by multiple measurements and comparisons with the literature.

In addition to peak identification, this library is a valuable tool for designing optimal detection and quantification conditions for new analysis methods.

In addition to the spectra files, the library offers a link through each compound's CAS number to a database that contains each compound's nonproprietary name and CAS number, pharmacological or toxicological use or effect, relative retention time and UV spectroscopic maxima, minima and shoulders. These data can be read by Microsoft Excel®, Microsoft Access® or Borland dBase®. The library also comes with an easy-to-use manual that includes a printout of each spectrum and the structural formula together with instructions for the proper use of the library.

Furthermore, the user can quickly generate a new library extracted from the entire library with only the user's spectra of interest. Relative retention times, based on internal standards, indicate the expected elution order, helping to overcome the well known difficulties of reproducibility even with the same type of column and the same mobile phase.

This application note summarizes the development of the toxicological library and provides examples of its use.

## Experimental

### Selected compounds

The Institute's HPLC spectra library contains approximately 1,600 spectra that match a broad user range in pharmacological and toxicological analysis. All kinds of drugs were considered, with drugs that are most used included without question. A large number of metabolites were included, as available. Illegal drugs, such as opiates, cannabinoids, cocaine, amphetamins, commonly used hallucinogenes and other so-called designer drugs were also incorporated into the library. Various drug manufacturers generously provided the pure compounds.

Most herbicides, wood preservatives and pesticides became part of the library. Environmental toxins were restricted to a series of polycyclic aromatic hydrocarbons, polychlorinated biphenyls and some phenols. Alcaloids such as strychnine, brucine and nicotine were included.

Nomenclature of the compounds applies international names, when available, or IUPAC nomenclature. Many of the compounds were used in their salt form for spectral analysis, that is, as hydrochloride or sodium salt. The identification code was generated with the first letter of the compound's name and a three-digit number. Caffeine, for example, became C042. Compounds analyzed using a different mobile phase have an additional "b" added to their names, such as A018b for Amiodarone.

### Instrumentation and analytical conditions

Table 1 lists the instrumentation and conditions used in the development of the pharmaceutical and toxicology library. Certainly these analyses can be done on a Agilent 1100 Series HPLC system as well.

| | |
|---|---|
| HPLC system | HP 1090 Series Liquid Chromatograph with Agilent ChemStation |
| HPLC column | Lichrosorb RP-8, 5.0 µm, 240 x 4.6 mm |
| Mobile phase A | • 156 g (=200 ml) acetonitrile (UV grade)<br>• 340 g phosphate buffer, pH=2.3, made with 4.8 g $H_3PO_4$ and 6.6 g $KH_2PO_4$ in 1 L water, with pH control by glaselectrode.<br>Retention times were calculated as 5- (4-Methylphenyl)-5-phenyl hydantoine (MPPH, code M000, RRT=1.000), as standard |
| Mobile phase B for a smaller number of compounds having retention times that exceeded 30 minutes (RRT>3 in mobile phase A) | • 625 ml acetonitrile<br>• 375 ml phosphate buffer, pH 2.3<br>Retention times were calculated based upon p-Phenylbenzophenone (Code P191b, RRT=1.000) as standard |
| Degassing | Helium or vacuum |
| Column temperature | 40 °C |
| Flow rate | 1 ml/min |
| Injection volume | 1 µg of each compound (10 µl of a solution of 0.1 mg/ml in the mobile phase or acetonitrile) |
| Detector | Diode-array detector |
| UV spectra | Taken at the peak apex between 195 and 380 nm after background correction. |

**Table 1**
**HPLC system configuration and conditions**

The library contains corrected relative retention times calculated according to the general formula:

$$RRT = (RT_{compound} - \text{dead time}) / (RT_{standard} - \text{dead time})$$

Dead time (the time of an unretained peak) was determined by injection of histamin hydrochlorid, which showed no retention in the acidic mobile phase. The absolute retention time is stored under **Acquisition** in the data file.

## Results and discussion

### Parameters for spectral library search and quantification

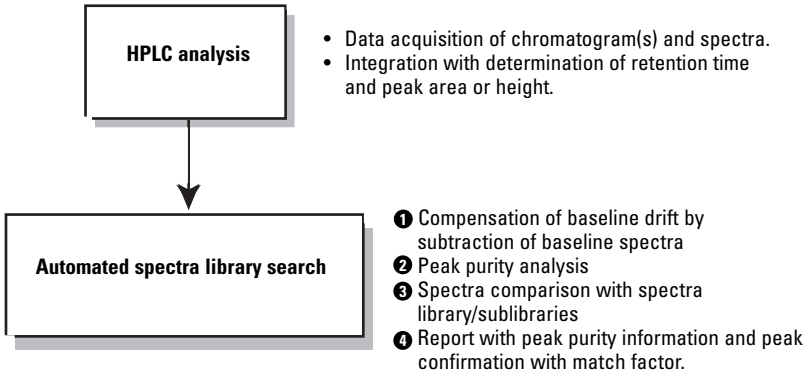
The LC 3DChemStation evaluates each set of raw data automatically. This occurs immediately after the chromatographic run. Once retention time and peak area or height have been determined by integration, the library search begins on these integrated peaks.

First, to compensate for baseline drift caused by solvent composition changes across the gradient (especially at wavelengths shorter than 220 nm), the spectral contribution of the baseline either before or after the peak is subtracted from each peak's spectrum.

Second, a check on peak purity provides the analyst with a purity level (maximum value 1,000) that reflects how closely the spectra match each other along the peak's elution. Values above the predefined threshold of 990 indicate peak purity; values below 990 indicate impurities, which are then marked in the library search report.

Finally, the actual spectra search is contained within a search window of ±4 percent of the compound's retention time, to find compounds with a retention time close to the one of the unknown peak. Peaks that match an entry in the library can be considered identified when the search results are better than a significance limit of 990, as calculated by the LC 3DChemStation. If peak height is very small and background absorbance is relatively high, then values of between 950 and 990 can possibly constitute an identification. Visual control of the overlay spectra is advised to avoid errors.

Detection limits will vary depending on differences in analytes and their sample matrices. In general, identification of compounds with absorption in the high wavelength range is easier when sample matrices have fewer compounds, such as with fresh serum. Ideal concentrations for identification are about 100 ng/ml.




### Spectra sublibraries

The entire set of spectra is divided into sublibraries to make peak identification faster. Moreover, through problem-oriented preselection of the matching sublibrary, identification can occur with a higher degree of certainty.

Table 2 shows the sublibraries of the toxicology library. These sublibraries were created according to the range of effects of individual drugs. The Agilent ChemStation makes it easy to modify these sublibraries, customizing the library to user needs.

Metabolites are in the same sublibrary as the parent drug. However, because of possible multiple effects, some compounds reside in sublibraries that are somewhat arbitrary.

| Sublibrary | Effect/Usage (listed in the database) | # |
|---|---|---|
| **TOX01**<br>Agents with addiction potential, illegal drugs, psychoactive agents, hypnotics (see also TOX02) | Analgetic (Btm), anesthetic, anoretic, antihypnotic, barbiturate, central stimulant, hallucinogen, hypnotic, illegal drug, narcotic, opiate, opiate antagonist, psycholeptic, psychostimulating agent, psychotonic, sedative | 122 |
| **TOX02**<br>Psychopharmaceuticals, antiepileptics and similar effects (see also TOX01) | Analeptic, anticonvulsant, antidepressant, antiepileptic, anxiolytic, benzodiazepine, myotonolytic, neuroleptic, spasmolytic, tranquilizer | 201 |
| **TOX03**<br>Analgetics, antirheumatics, antitussives and similar effects (see also TOX01) | Analgetics (except for Btm), antiarthritic, antigout agents, antimigraine, antineuralgetic, antipyretic, antirheumatic, antiphlogistic, antitussive (except for opiate), bronchiolytic, expectorant, local anesthetic, muscle relaxant, mucolytic agents, secretolytic | 138 |
| **TOX04**<br>Antihistaminics, antiallergics, further CNS-effective agents with different effects | MAO-inhibitor, antiallergic, anticholinergic, antidot, antiemetic, antihistaminic, antiparkinsonian, cholinergic, cholinesterase inhibitor, dopamine antagonist, mydriatic, parasympatholytic, serotonin antagonist, a-sympathomimetic, ß-sympathomimetic | 134 |
| **TOX05**<br>Heart-blood circulation-remedies (see also TOX04 and TOX06) | Antiarrhytmic, antihypercinetic, antihypertonic, antihypotonic, beta-blocker, calcium antagonist, cardiac glycoside, cardiac, cardiotonic, vasoconstrictor, vaodilator, venodynamic, venotonic | 150 |
| **TOX06**<br>Diuretics, remedies for blood coagulation, for the digestion system, other remedies with different effects | ACE-inhibitor, ALDH-inhibitor, anticataractic, anticoagulant (see also rodenticide antidiabetic), antidiarrhoeal, antifibribrinolytic, antihypoxemic, carbonic anhydrase inhibitor, carminative, choleretic, ecbolic, gastric ulcer therapeutic, hepatotherapeutic, diuretic, hemostyptic agent, hyperemisation agent, laxative, lipid lowering agent, peristaltic stimulant, platelet aggregation inhibitor, remedy against varicose veins, rubefacient, remedy against ulcus, saluretic, uricostatic, vulnery, X-ray contrast agent | 137 |
| **TOX07**<br>Remedies with steroid structure, hormones, endogenous agents, vitamins | Aldosterone antagonist, anabolic, androgenic, antiandrogenic, antiestrogenic, estrogenic, glucocortcoide, gonatotropine inhibitor, hormones, prostaglandines, vitamin, thyreostatic, endogenous agents | 146 |
| **TOX08**<br>Cytostatics, antibiotics, other agents with anti-micro-organism effects | Antibacterial, antibiotic, antimalarial, antiprotozoic, chemotherapeutic, cytostatic, gramicinide, immunostimulating agent, immunosuppressant, opthalmic, tuberculostatic, virostatic | 179 |
| **TOX09**<br>Fungicides, disinfectants, stabilizer for drug preparation | Anthelminic, antimycotic, antiparasitic, antiscabatic, anti-septic, disinfectant, fungicide, stabilizer for drug preparation | 116 |
| **TOX10**<br>Insecticides, acaricides, nematicides, etc. | Acaricide, acetylcholin esterase inhibitor, fungicide (see also W09), insecticide, nematicide, pesticide, repellant, rodenticide (see also W06) | 158 |
| **TOX11**<br>Herbicides | Herbicide, growth regulator for plants, wood preserver | 108 |
| **TOX12**<br>Ecotoxicological agents (see also TOX10 and TOX11) | Polychlorinated biphenyls, polycyclic aromatic hydrocarbons, internal standards | 32 |

**Table 2**
**Sublibraries—divided according to the agents' effect/usage**

## Validity of spectra

Below 200 nm, spectra are influenced by noise and are not very reproducible. Therefore the wavelength range below 200 nm was not included in the spectra library search.

Acidic acetonitile/phosphate buffer mixtures were used for the mobile phase for three reasons: because of their UV transparency down to 195 nm, because of the suitable retention times of the toxicologically-relevant compounds, and because of the high resistance of RP-phases to these mobile phases. The amount of acetonitrile buffer, which is valid for both neutral and acidic compounds, can be varied widely without significant changes in spectra. The validity of this buffer was proven through many measurements in both mobile phases A and B. However, the acidic pH conditions must be maintained for all compounds that underlie acid-base-equilibria because the dissociation of -COOH, -OH or -SH groups or the protonation of basidic (=alkaline) N-atoms often leads to significant changes in light absorption.

## Minimal sample preparation

In many cases, analysts can use the LC system with a simple sample preparation. For a general toxicological analysis with minimal equipment, the user can follow this typical procedure:

- 0.5 ml blood + 0.5 ml phosphate buffer pH=2.3, or 0.5 ml blood + 0.5 ml carbonate buffer pH=9.4
- Extraction with 0.5 ml methylenchloride
- Extraction with 0.5 ml ethylacetate/ether
- Unify the organic phases and evaporate to dryness and dissolve with eluent.

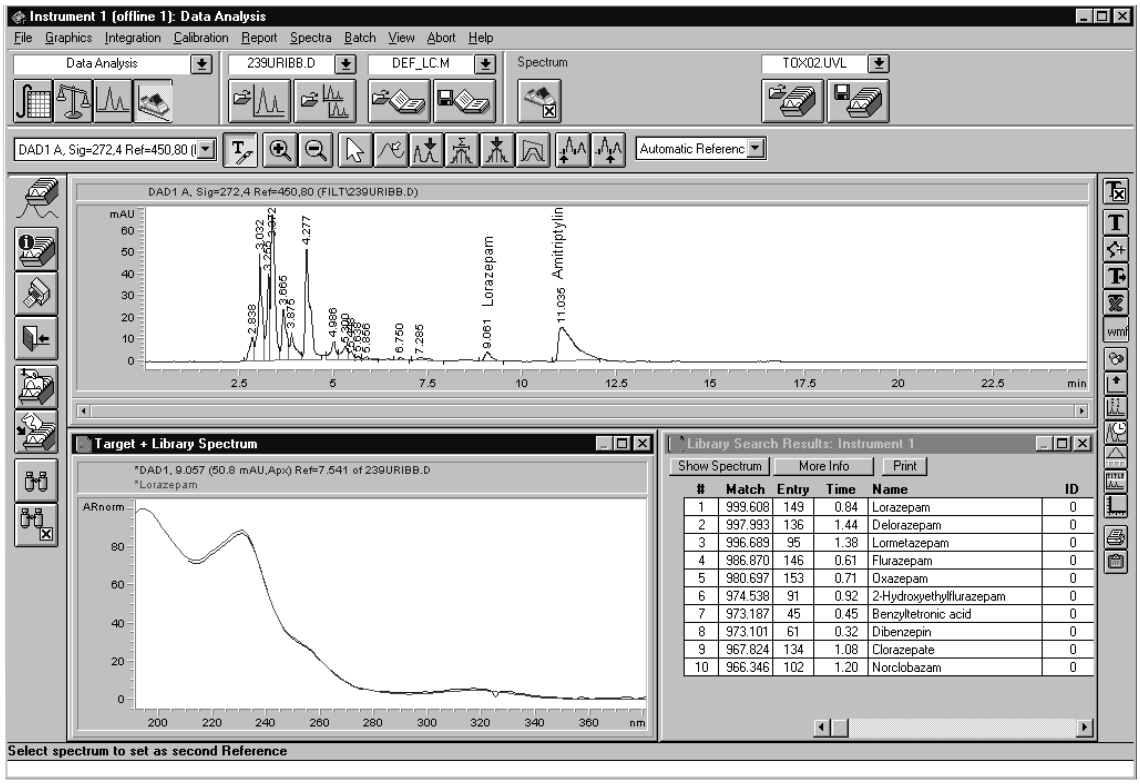



**Figure 1**
**ChemStation screen with the HPLC chromatogram and the peak identification of a urine sample. 1 mL of urine was enzymatically (glucuronidase/arylsulfatase) hydrolyzed and extracted with ethylacetate at pH=9.4. Urine extracts contain a significantly higher number of matrix compounds than blood or serum extracts. Therefore, under the chromatographic conditions given above, only compounds of high concentration or high retention can be analyzed well. The sample displayed in figure 1 was spiked with Amitriptyline and Lorazepam.**

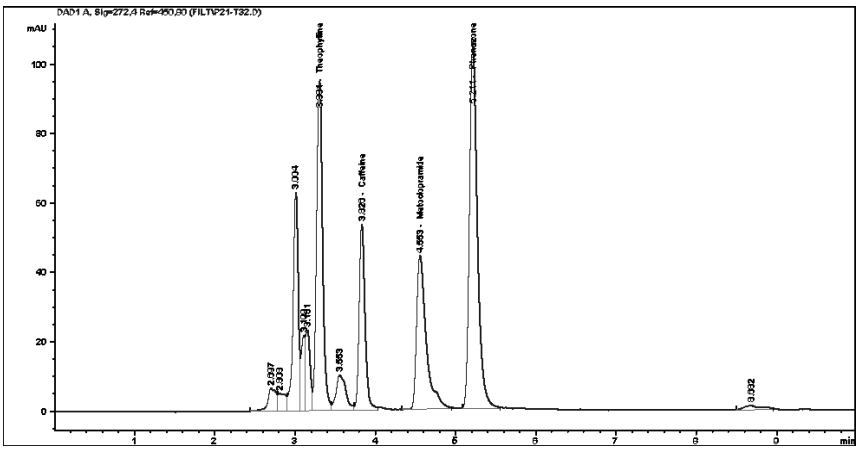



**Figure 2**
**Chromatographic separation of a native serum sample. 1 ml serum was extracted with RP18-solid phase extraction at pH=7.4. The extraction process results in very clean extracts with peaks that can be well characterized. The sample mainly contains theophylline, caffeine, metochlopramide and phenazone.**

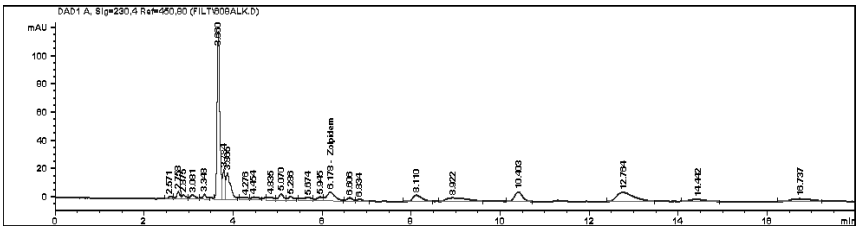



**Figure 3**
**HPLC analysis of postmortal full blood sample. 0.5 ml were extracted with Dichloromethane at pH=9.4. As can be seen from the small peak at 6.178 min, the concentration of the compound of interest is very low and the spectra are very noisy because of strong background. Nevertheless this compound can be identified as Zolpidem by comparison with the UV spectra library.**

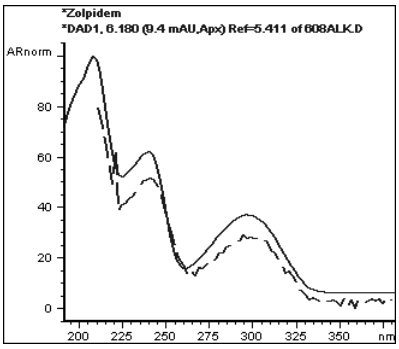



**Figure 4**
**Noisy spectrum of the chromatogram's Zolpidem peak with only low UV absorption and the pure library Zolpidem spectrum, both normalized and overlayed.**

## Conclusion

The spectral library developed by the Institute of Forensic Medicine at Humboldt University in Berlin is a useful and efficient tool for identifying toxic compounds. With minimal sample preparation, analysts can use this library to identify poisons and their metabolites quickly and confidently.

Moreover, the ability to link to a database using Microsoft Access® or Microsoft Excel® makes the library a valuable tool for chromatographic method development.

## Ordering Information

The spectral library is commercially available. “UV Spectra of Toxic Compound” Authors: F. Pragst, M. Rothe, B.T. Erxleben and S. Herre.

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