SELECTIVITY OF PHOTODIODE ARRAY UV SPECTRA FOR SUBSTANCE IDENTIFICATION IN SYSTEMATIC TOXICOLOGICAL ANALYSIS

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ABSTRACT: An attempt was made to evaluate the selectivity of PDA UV spectra for systematic toxicological analysis (STA). A great spectral variety was found among compounds of toxicological interest. With modern generation PDA detectors even compounds of high structural similarity can be successfully distinguished down to a LOI as low as 10 ng/ml.

KEY WORDS: HPLC-photodiode array detection; UV spectra of toxic compounds; Systematic toxicological analysis.

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INTRODUCTION

Along with GC/MS as the so-called "gold standard", HPLC with photodiode array detection (HPLC-PDA or HPLC-DAD) is regularly employed for substance identification in the context of Systematic Toxicological Analysis [1, 2, 3, 4, 5, 9, 10]. With HPLC-PDA the most important parameters in identifying a compound are its retention time and its UV spectrum.

Critics of the method often question the specificity of UV detection because of poorly structured spectra and broad absorption bands. Therefore a systematic investigation into the selectivity of PDA detection was carried out by analyzing large numbers of UV spectra with respect to their correlation with chemical structure. Furthermore the pros and cons of the method were evaluated determining experimental limits with the latest PDA detectors.

For data analysis the following tools were used:

1. A spectra library [6] of 2076 toxicologically relevant compounds which – at the moment – is being re-recorded on a state-of-the-art PDA detector and is expected to contain about 3000 compounds upon completion. The library is embedded into the chromatography software in a way that spectral similarity is compared nm by nm and a "hit list" is returned to the operator.
2. A database of retention times and specific peak areas at 225 nm for all library entries (MS Access).

3. A database of all molecular structures with an ability for substructure searches (IsisBase™ chemical database software).
4. A structural database of all registered chromophores (IsisBase™).

The term "chromophore" is commonly used to designate the part of a molecule that is responsible for the absorption of ultraviolet and visible light respectively. For this contribution a somewhat narrower definition was chosen according to which a chromophore shall consist of at least two conjugated multiple bonds plus possible one or more conjugated heteroatoms. The chromophore substructure is delimited by hydrogen or sp^3-hybridized carbon atoms and is named after the corresponding isolated compound as shown in Figure 1 for the β-receptor antagonist metoprolol.

While the majority of compounds under investigation possesses only one

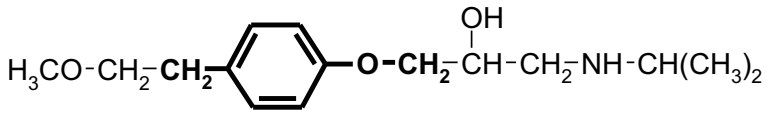

Fig. 1. Metoprolol (chromophore: p-methoxytoluene).

chromophore, substances with up to 6 different chromophores can be found.

METHODS

Instrumental

Pump: Kontron System 520 (flow: 1 ml/min.); degasser: Kontron 3493; autoinjector: Kontron HPLC 560 (standard injection volume: 50 µl); column: Merck LiChrospher RP8ec 250 x 4.0 mm, 5 µm, with 10 mm guard-column of the same type (both from Macherey-Nagel, Germany); PDA detectors: Shimadzu M10Avp (spectra were recorded from 195 to 380 nm) and Kontron HPLC 540. The benzene spectrum of the HP 1100 PDA detector was kindly recorded by Mr. Wohlauf at the German Federal Institute for Drugs and Medicinal Devices (BfArM), Berlin. Software: Kontron KromaSystem 2000, v. 1.61 (operating the hardware) and Shimadzu CLASS-VP 5.03 (for data registration and analysis), MS Access, Isis Base (chemical database software by MDL Inc., Allschwil, Switzerland).

Solvents and reference compounds

Eluent (isocratic): acetonitrile/phosphate buffer (pH 2.3) 37:63 (v/v). Acetonitrile (Uvasol) was purchased from Merck, Darmstadt. The substances in the spectral library were generously donated by the respective manufacturers or purchased from Sigma, Aldrich or Merck.

Sample preparation

In practical application of the spectra library for the STA of blood/serum samples mostly liquid-liquid extraction with CH_2Cl_2 at pH 2.3 and 9.0 or protein precipitation with acetonitrile were used. An example is shown in Figure 6.

RESULTS AND DISCUSSION

The database of 2076 toxicologically relevant compounds was analyzed with respect to the number and frequency of chromophores present. The results are shown in Table I.

TABLE I. STATISTICAL ANALYSIS OF THE DATABASE

Number of chromophores per compound								
Number	0	1	2	3	4	5	6	Total
Compounds	189	1453	363	64	4	0	3	2076
Chromophore frequencies in the database								
Frequency	1	2	3	4	5	5–10	> 10	Total
Chromophores	897	114	37	26	14	28	22	1138
Different UV absorbing systems								
Number of isolated chromophores contained	1	2	3	4	5	6	Total	
UV absorbing systems	919	264	57	4	–	3	1247	

1887 compounds (90.9%) contained one or more chromophores according to the definition given above, 189 compounds did not show UV absorption above 195 nm. Among the 1887 substances with an absorption 1138 different chromophores were counted, the vast majority of which (78.8%) occurring only once in the whole database. Only 22 chromophores were found more often than 10 times, the “toluene” chromophore being the most frequent with 261 entries both alone (96 compounds) and in combination with other chromophores (116) or with itself (49). Including combinations of chromophores, there was a total of 1247 different chromophoric systems.

As a rule different chromophoric systems lead to different UV spectra, clearly distinguishable for the chromatography software. That means a similarity search in the spectra library will result in the correct compound emerging as hit no. 1. In most cases even compounds with the same chromophore can be distinguished. The 11 phenothiazine derivatives shown in Figure 2 can be classified into two groups depending on the amino group being attached to the β- or γ-carbon, remote from the actual chromophore. In practice by using both spectroscopic and retention data all of these compounds can be unambiguously identified.

Also the 96 compounds containing one toluene chromophore exclusively could be clearly divided into several subtypes according to their UV spectra (Figure 3, the

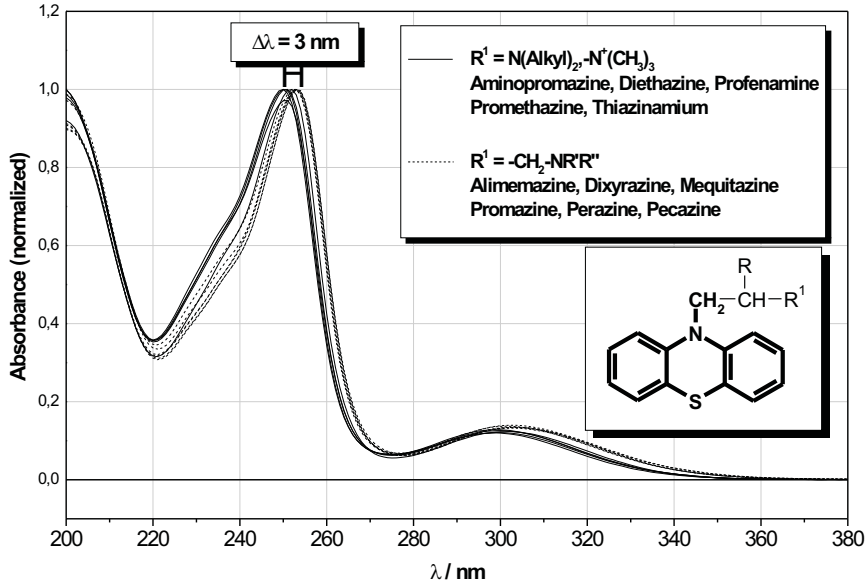

Fig. 2. Spectra of 11 phenothiazine derivatives.

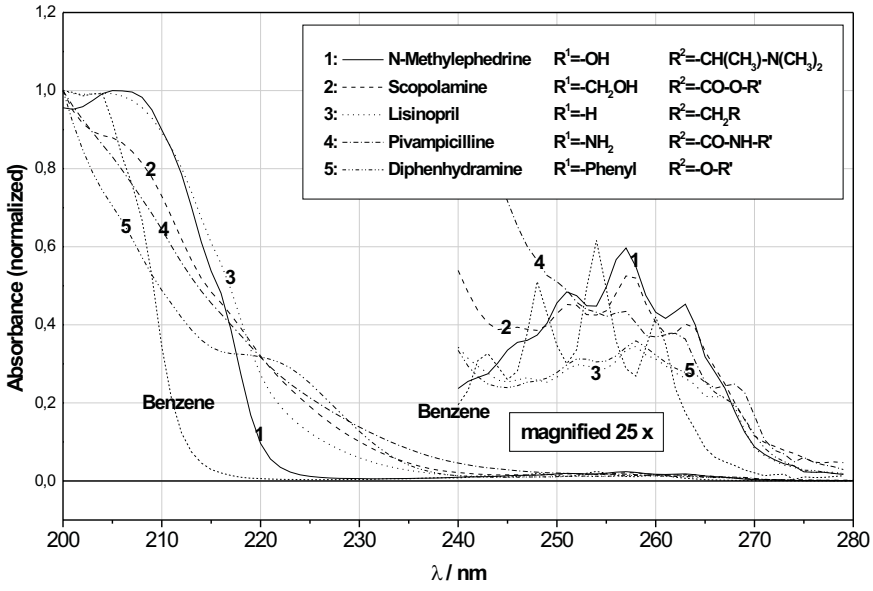

Fig. 3. Toluene chromophore subtypes.

spectrum of benzene is shown in comparison). Significant differences in absorbance were observed between 200 and 240 nm as well as in the intensity and position of the vibrational fine structure around 254 nm. Even within a given subtype, considerable spectral variation could be found among 18 differently substituted phenylacetic acid esters. Introduction of oxygen or nitrogen at the acetic acid α-carbon resulted in a clearly changed vibrational fine structure.

Under practical conditions several factors can limit the identification power of PDA detection:

1. In general, the information content of a UV spectrum varies depending on the number of absorption bands present as well as on the overall absorption range and is related to the complexity of the chromophore.
2. The shape of the UV spectrum itself is influenced by the spectral resolution of the detector in use; for many compounds also a change in pH would result in a modified curve.
3. Detection Limits depend mainly on the specific intensity of light absorption, determined by the nature of the chromophore (molar extinction coefficients, specific peak areas) as well as by the signal-to-noise ratio of the detector.
4. Correct spectra can only be derived from sufficiently resolved chromatographic peaks.

Modern generation PDA detectors surpass their predecessors both in terms of sensitivity and spectral resolution. Figure 4 shows the good agreement of benzene spectra recorded on different manufacturer's devices. Absorption band position as well as the quality of spectral resolution are highly reproducible.

Spectra of injected substance amounts as small as 50 pg can still be analyzed with the correct library spectrum being found among the first 3 hits of the search result list, as shown in Figure 5 for the spectrum of flunitrazepam. With the help of efficient smoothing algorithms, even noisy spectra are clearly recognizable.

In our laboratory HPLC-PDA is used daily for the investigation of intoxications with a great variety of toxic compounds at blood levels of a very different scale. Figure 6 shows a lidocaine intoxication case, in which beside lidocaine at a blood level of 11 µg/ml diazepam was identified at 40 ng/ml with a good agreement of sample and library spectrum even at such a low concentration.

CONCLUSIONS

In summary it can be said that PDA detection is a valuable and selective tool for substance identification. Toxicologically relevant compounds display a great variety of chromophoric systems showing significantly different UV spectra. In most cases even UV spectra of compounds sharing the same chromophore are distinguished due to structural differences in neighboring groups. With modern PDA detectors, UV spectra are recorded with high qualitative and quantitative reproducibility down to a LOI of

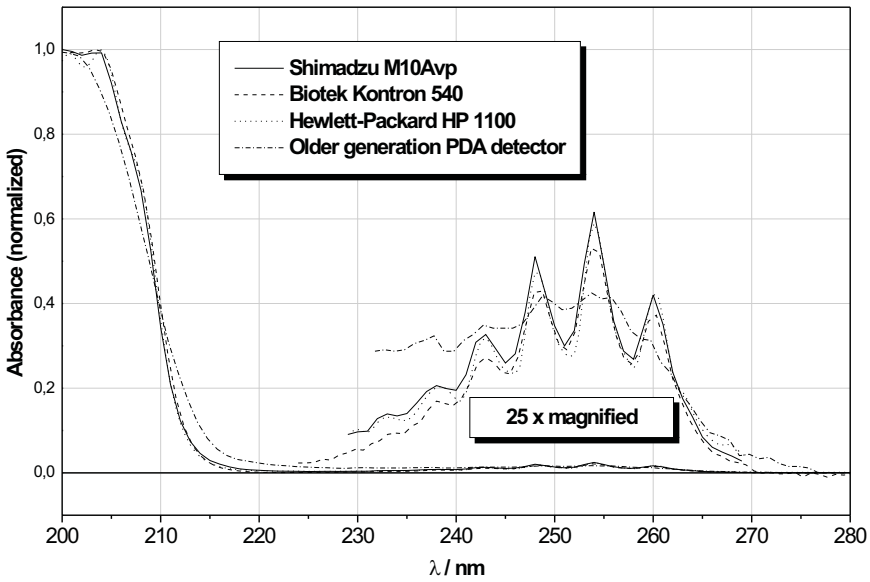

Fig. 4. Spectra of benzene recorded with different PDA detectors.

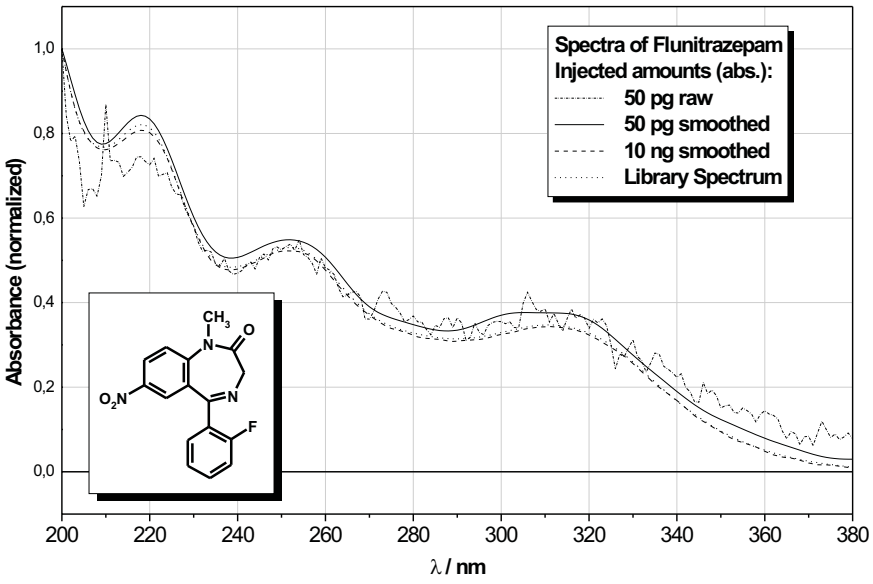

Fig. 5. Spectra of flunitrazepam at low concentrations.

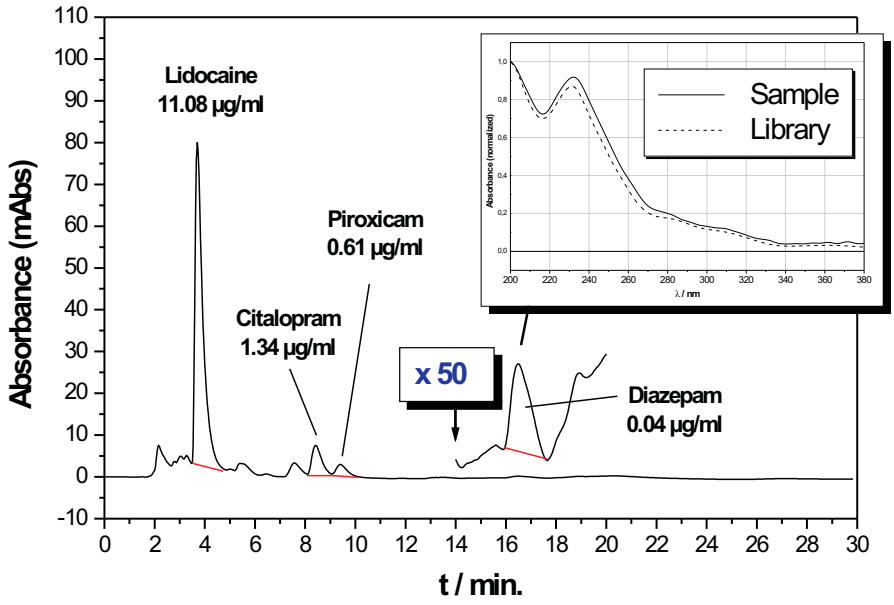

Fig. 6. Blood extract of a lidocaine intoxication case, chromatogram at 225 nm.

10–20 ng/ml (blood). This is sufficient for the identification of therapeutic or at least toxic concentrations of a large group of toxicologically relevant compounds [7, 8]. Using highly selective and reproducible spectral data in combination with retention times, HPLC-PDA is successfully employed as a principal method in systematic toxicological analysis.

References:

1. Gaillard Y., Pépin G., Use of high-performance liquid chromatography with photodiode-array UV detection for the creation of a 600-compound library. Application to forensic toxicology, *Journal of Chromatography A* 1997, vol. 763, pp. 149–163.
2. Herre S., Pragst F., Shift of the high performance liquid chromatographic retention times of metabolites in relation to the original drug at an RP-8 column with acidic mobile phase, *Journal of Chromatography B* 1997, vol. 692, pp. 111–126.
3. Koves E. M., Use of high-performance liquid chromatography-diode array detection in forensic toxicology, *Journal of Chromatography A* 1995, vol. 692, pp. 103–119.
4. Lambert W. E., Van Bocxlaer J. F., De Leenheer A. P., Potential of high-performance liquid chromatography with photodiode array detection in forensic toxicology, *Journal of Chromatography B* 1997, vol. 689, pp. 45–53.
5. Pragst F., Erxleben B. T., Herre S., Aberger K., HPLC in der Systematischen Toxikologischen Analyse, *Chromatographie* 1994, Bd. 2, S. 92–96.
6. Pragst F., Erxleben B. T., Herre S., UV-Spectra of toxic compounds. Photodiodearray-UV-spectra library of pharmaceuticals, illegal drugs, pesticides, ecotoxic compounds and other poisons. Software and manual, version I/97, Institute of Legal Medicine, Humboldt-University, Berlin 1997.
7. Regenthal R., Krüger M., Köppel C., Reiß R., Zu den Möglichkeiten und Grenzen von therapeutischen und klinisch toxikologischen Referenzwerten für Plasma-/Serum-/Vollblutkonzentrationen von Arzneimitteln bei akuten Vergiftungen – eine Übersicht, *Anästhesiologie & Intensivmedizin* 1999, Bd. 40, S. 129–144.
8. Schulz M., Schmoldt M., Therapeutic and toxic blood concentrations of more than 500 drugs, *Pharmazie* 1997, vol. 52, pp. 895–911.
9. Tracqui A., Kintz P., Mangin P., Systematic toxicological analysis using HPLC/DAD, *Journal of Forensic Sciences* 1995, vol. 40, pp. 254–262.
10. Turcant A., Premel-Cabic A., Cailleux A., Allain P., Toxicological screening of drugs by microbore high-performance liquid chromatography with photodiode-array detection and ultraviolet spectral library searches, *Clinical Chemistry* 1991, vol. 37, pp. 1210–1215.