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Potential of high-performance liquid chromatography with photodiode array detection in forensic toxicology

Willy E. Lambert, Jan F. Van Bocxlaer, André P. De Leenheer*

Laboratorium voor Toxicologie, Universiteit Gent, Harelbekestraat 72, B-9000 Gent, Belgium

Abstract

The potentials and limitations of high-performance liquid chromatography–photodiode array detection are highlighted in respect to its use in the analysis of different biological matrices followed by the identification of unknowns. The logical analytical approach used in clinical and forensic toxicology, vital for the identification of one or more toxic substances as a cause of intoxication, is largely based on both simple and fast "general unknown screening" methods which cover most relevant drugs and potentially hazardous chemicals. In this field of systematic toxicological analysis, a literature overview shows that HPLC can play a substantial role. Both column packing material and eluent composition have their impact on intra- and interlaboratory reproducibility. In view of the sometimes different retention characteristics of various HPLC columns, several possibilities are addressed to enhance the discriminating power of primary retention parameters. The advantages of photodiode array detection as compared to UV detection have been of paramount importance to the success of HPLC in toxicological analysis. Dedicated libraries with spectral information and searching software are powerful tools in the process of identification of an unknown substance. In the present paper, these aspects are also verified in a number of real cases, i.e., trazodone and dothiepin, ,azide, chloroquine and cocaine, in which we illustrate from our own experience the potentials of HPLC–photodiode array detection in systematic toxicological analysis.

Keywords: Toxic compounds

1. Introduction

The analysis of different biological matrices (e.g., blood, urine, stomach contents, tissues...) to achieve the identification of "unknown" potentially toxic compounds is a challenging task in forensic toxicology. The number of these compounds currently reported in literature exceeds seven million, each of which is a possible candidate in any case of acute poisoning. Therefore, a rational chemical-analytical approach, also called systematic toxicological analysis (STA), is needed for the identification and determination of the toxic compound(s) responsible of the poisoning.

The success of a systematic toxicological analysis largely depends on the quality of the analytical system used. A number of analytical techniques, such as thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and a variety of immunoassay tests are available to the toxicologists, which can be coupled (on-line or off-line) with highly selective detectors such as mass spectrometers (MS) and Fourier transform infrared (FTIR) spectrometers for GC and fast-scanning or photodiode array (DAD) spectrometers and mass spectrometers for HPLC.

In this overview we will only focus on HPLC–

*Corresponding author.

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DAD. Not only the potentials but also the pitfalls of this combination will be reviewed, especially with respect to the applicability in STA. HPLC–DAD used as a general unknown screening tool should cover as many drugs and toxicants as possible, but should be also very selective, sensitive and reliable. A survey of the literature in this field is presented in this paper, and some of the analytical approaches are compared and applied in a number of real cases.

2. The application of liquid chromatography for STA

2.1. Column packing materials

2.1.1. Underivatized silica

The use of underivatized silica as a stationary phase for the separation of a number of basic drugs was already described in 1975 [1] and in 1984 [2]. Later, other researchers evaluated the retention behaviour of basic drugs on different silica packing materials using a rather unusual eluent composition based on methanol and ammonium nitrate. Different brands of silica and even different batches of the same brand of silica packing material often resulted in different retention of selected basic drugs [3]. As a consequence of the basic principles of retention mechanisms in adsorption chromatography [4], a number of conditions must be fulfilled before reproducible retention times or capacity factors (k') can be obtained in one single laboratory or (and this is even more difficult) in different laboratories. In this way, chromatographic conditions (same batch of same brand of packing, eluent composition, temperature regulation) should be exactly defined and strictly followed. Even under reversed-phase chromatographic conditions small changes in these parameters result in other retention times, however, the impact on an adsorption chromatographic system is more substantial. Due to these stringent conditions, the application of adsorption chromatography to systematic toxicological analysis remained rather limited.

2.1.2. Bonded-phase packing material

Bonded-phase chromatography and especially reversed-phase chromatography on octyl- or octadecylsilica is by far the most popular technique used in STA. Several papers already described the applicability of these packing materials to forensic toxicology [5–11]. Two of these papers compared different reversed-phase packing materials [7,10] and stressed on the influence of free silanol functions on the retention of different drugs. Such silanol effects can be reduced by changing the pH of the eluent or by adding amine modifiers to the eluent. Alternatively, 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 elimination of trace metals from the silica support and by deactivation of the free silanols by various endcapping procedures. Again, it became clear that in reversed-phase chromatography also, although to a lesser extent than in chromatography on underivatized silica, the mobile phase composition, its pH, ionic strength and temperature seriously influence the retention of drugs. As an alternative, polymeric stationary phases have also been introduced. To eliminate intra- and interlaboratory variations Bogusz et al. [12] and De Zeeuw et al. [13] proposed to replace primary retention parameters (retention times, capacity factors) by secondary parameters of retention, such as relative retention times and retention indexes. This concept can result in more reliable retention data within one single laboratory and even in the development of a retention information data base for interlaboratoy use.

Retention indexes (RI-values) based on neutral reference homologues (alkane-2-ones, alkylarylketones and 1-nitroalkanes) were still sensitive to slight variations in chromatographic conditions because these neutral reference homologues behave differently from drugs of toxicological relevance. Later it became clear that separate sets of correction standards should be used for acidic/neutral and for basic/neutral drugs. Based on these observations Bogusz and Wu [14] developed a standardized HPLC–DAD system on a Superspher RP-18 column for the chromatography and identification of over two hundred toxicologically relevant substances. They used the same chromatographic conditions for acidic and for basic drugs (i.e., gradient elution with a mixture of acetonitrile and a triethylammonium phosphate buffer pH 3.0) combined with the 1-

nitroalkane retention index scale and a number of drugs as retention index markers. Obviously, the acidic pH suppresses the ionization of acidic silanol groups while the amine modifier further improved the chromatographic behaviour of basic drugs.

In an effort to eliminate chromatographic problems due to residual silanol groups and to prevent instrumental problems due to the incorporation of buffer salts in the eluent, we developed a HPLC separation of more than two hundred toxicologically relevant substances using totally different chromatographic conditions [15]. An alumina based packing material coated with polybutadiene was eluted in a gradient mode with a mixture of methanol and water, both containing 0.0125 *M* NaOH. The inherent absence of silanol functions on the new packing material (Aluspher RP-Select B from Merck, Darmstadt, Germany) simplified the retention mechanism, eliminated the need for amine modifiers and prevented irreversible adsorption. In addition, aluminum oxide as well as the polybutadiene coating are stable from pH 2 to 12. This means that basic drugs can be chromatographed under alkaline conditions proceeding in a drastic decrease in peak tailing. The polybutadiene coating gives to the packing material hydrophobic characteristics comparable to reversed-phase stationary phases. Of course, the high pH of the eluent resulted in poor retention of compounds carrying a phenolic function (e.g., morphine) or a carboxylic acid function (e.g., benzoylecgonine). Hence, the latter compounds needed another chromatographic approach on a silica-based chromatographic packing material with superior end-capping characteristics, i.e., the Hypersil BDS C18 column (Alltech, Deerfield, IL, USA) [16]. The column was eluted with a mixture of 0.045 *M* solution of ammonium acetate in HPLC-grade water (80%), methanol (10%) and acetonitrile (10%), as solvent A, and a mixture of 0.045 *M* solution of ammonium acetate in methanol (40%), acetonitrile (40%) and HPLC-grade water (20%), as solvent B. We used linear gradient conditions from 100 to 47.2% A in approximately 20 min, while the effluent was monitored with a photodiode array detector. The latter method was complementary to the former, because a large number of compounds not chromatographing under strong alkaline conditions (opiates, acids and several benzodiazepines) eluted with an excellent peak shape and a high plate number under acidic conditions. In this way, coelution of compounds due to a too high number of substances that can be chromatographed was also minimized.

The same approach of using different column systems (and different extraction procedures) in the analysis of "multiple drug overdose" cases is also described by Koves [10]. The described reversed-phase chromatographic systems (Supelcosil LC-DP, LiChrospher 100 RP-8, APEX ODS and Nova-Pak Phenyl) are rather unique because all columns are eluted isocratically. Indeed, due to the large differences in polarity and in view of a simultaneous determination of parent drug and metabolites, all other systems except those based on adsorption chromatography, use gradient elution.

2.2. Photodiode array detection

Since the early eighties, the introduction of diode-array and fast-scanning absorption detectors allowed the acquisition of UV (and visible) spectral data during the chromatographic process. This has offered a new dimension of analytical data to HPLC. This appeared especially useful in view of applications to systematic toxicological analysis. Combining the discriminatory power of chromatographic retention parameters with that of the spectral data was shown to increase the overall reliability of the HPLC method [17]. Ideally, standard reference spectra could be stored in a data base tagged with parameters of retention in order to restrict the search into a window around each retention parameter [18].

Coelution of two (or more) compounds remains one of the major causes of errors in chromatographic analysis. Erroneous conclusions are produced either by an interferent coeluting compound that mimics the UV spectrum of a known compound or when coelution of two compounds results in a UV spectrum that does not match any library spectrum. Therefore, before running a library search, it is absolutely necessary to check the peak of interest for purity. This can be done manually by comparing the UV spectra at different positions of the peak, or alternatively using some devices that automatically indicate the peak purity (whereby the number of horizontal lines under a peak corresponds to the

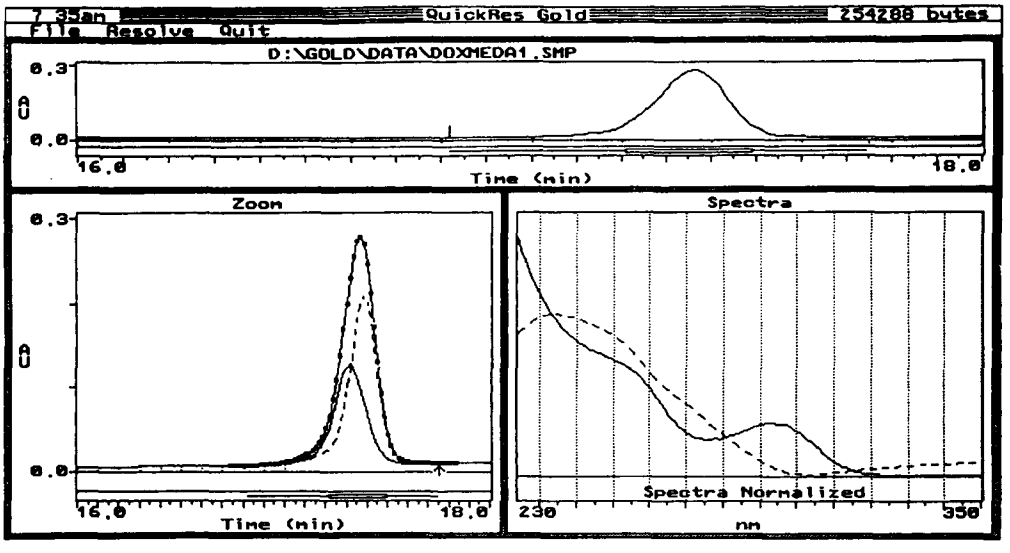

Fig. 1. Example of peak deconvolution. Upper trace: cochromatography of two compounds at 17.4 min. Lower left: the same peak with the partial contribution shown for medazepam (- - -) (58%) and for doxepin (———) (42%). Lower right: reconstructed UV spectrum of medazepam (- - -) and of doxepin (———).

number of constituents). The software of the more sophisticated systems allows peak deconvolution of two coeluting compounds, resulting in the specific UV spectrum and in the identification of each compound (Fig. 1).

3. Applications of HPLC to STA

The applicability and the potentials of HPLC in STA will be exemplified by a number of case reports already published by our group. For a more detailed description we refer to the original papers.

3.1. Case 1: a fatal case of trazodone and dothiepin poisoning

A 22-year-old female was found dead on the bank of a canal. An autopsy was performed within 24 h. Vascular congestion of all organs was noted, together with dysaeration and edema of the lungs. Upon dissection of the back muscles, an aromatic smell was perceived. All other autopsy findings on the woman were unremarkable. Because no cause of death could be found, postmortem specimens were taken from blood, urine, stomach contents, liver and kidney for further toxicological analysis [19].

In our STA procedure, the urine was screened for opioids, cannabinoids, amphetamines, methaqualone, methadone, cocaine, propoxyphene, phencyclidine, benzodiazepines, barbiturates, tricyclic antidepressants, acetaminophen, salicylates and caffeine using a homogenous enzyme multiplied immunoassay technique (EMIT). LSD, Fentanyl and cotinine were tested using a radioimmunoassay technique (RIA). TLC was used for an overall screening according to the procedure described by Sunshine [20]. Simultaneously, extracts were prepared from blood, stomach contents and (hydrolyzed) urine by a liquid–liquid extraction procedure using *n*-hexane–ethyl acetate (7:3, v/v). Extracts were redissolved in the chromatographic solvent and injected into a HPLC–DAD system. Reversed-phase separation was performed on a Chromspher C_8 column (10×0.3 cm I.D., 5 μm) using a MeOH-H_2O (containing 0.125% isopropylamine) gradient of 30:70 to 75:25 (v/v) within 15 min.

The EMIT screening for urine proved positive for amphetamines, benzodiazepines, opioids, tricyclic antidepressants and caffeine. In the blood sample, the

presence of benzodiazepines and tricyclic antidepressants was demonstrated. In the TLC analysis, dothiepin was tentatively identified from a standard, but a highly fluorescent spot was seen for all matrices extracted under alkaline conditions. The HPLC–DAD general screening first led to the identification of this compound in blood (Fig. 2) and urine as trazodone, based on its UV spectrum. The co-elution of a standard corroborated this identification. Also the presence of dothiepin (Fig. 3) was confirmed as well as the identification of opiates and benzodiazepines (EMIT positive) as codeine, lorazepam and nordiazepam, respectively. From the HPLC–DAD analysis, it also became clear that the positive result, as obtained from the EMIT analysis for amphetamines, was a "false positive" due to cross-reactivity with β-phenylethylamine and tryptamine, two compounds often encountered as a result of the putrefaction process.

We have already highlighted that RP-HPLC can be advantageous in the detection of metabolites in cases of intoxication. In the present case, a peak was found with a similar UV spectrum of dothiepin, eluting about 4 min earlier. Mild oxidation of a dothiepin standard (using H_2O_2) and injection in the HPLC system resulted in a peak, co-chromatographing with the unknown. The UV spectra of dothiepin and of dothiepin sulphoxide are shown in Fig. 4. Further investigation, including mass spectrometry, identified the compound as dothiepin sulphoxide, a naturally occurring metabolite of dothiepin.

The results of the STA clearly indicated the intake of trazodone and dothiepin as the cause of death. In this approach, HPLC played a paramount role. It not only actually identified the causal toxicants but the same procedure, extended with internal standardiza-

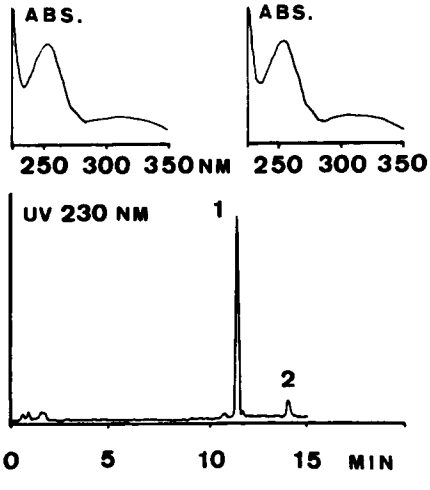

Fig. 2. HPLC–DAD trace (displayed at 230 nm) of a blood sample extract. Peak identification: (1) trazodone and (2) dothiepin. Upper left: UV spectrum (225 to 350 nm) of peak 1. Upper right: UV spectrum of trazodone standard. Reproduced from J. Anal. Toxicol. by permission of Preston Publications, a division of Preston Industries, [19].

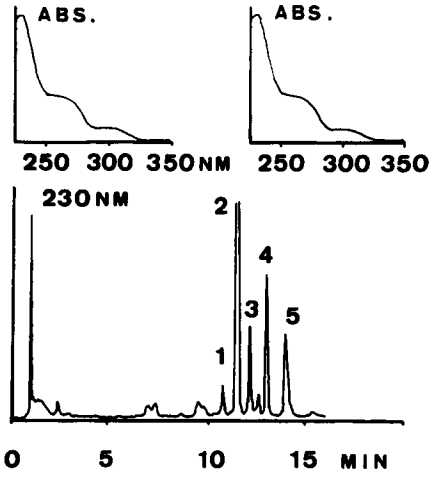

Fig. 3. HPLC–DAD trace (displayed at 230 nm) of a hydrolyzed urine sample extract. Peak identification: (1) dothiepin sulfoxide, (2) trazodone, (3) metabolites of nordiazepam and lorazepam (hydrolyzed), (4) metabolite of diazepam (hydrolyzed),and (5) dothiepin. Upper left: UV spectrum (225 to 350 nm) of peak 5. Upper right: UV spectrum of dothiepin standard. Reproduced from J. Anal. Toxicol. by permission of Preston Publications, a division of Preston Industries, [19].

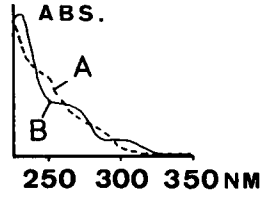

Fig. 4. Superposition of (A) the UV spectrum (225–350 nm) of peak 1 in Fig. 3 identified as dothiepin sulfoxide and (B) the UV spectrum (225–350 nm) of peak 5 in Fig. 3, identified as dothiepin.

tion and calibration allowed a complete quantitative assessment of this fatal overdose.

3.2. Case 2: a fatality involving azide

A 25-year-old laboratory technician was found without signs of life in a cloakroom at her place of work. The young woman was immediately transferred to a university hospital. Despite intensive resuscitation, including administration of typical cardiac medication and peritoneal dialysis, cardiorespiratory standstill persisted and she was pronounced dead 35 min after admission. Emergency blood analysis revealed severe metabolic acidosis. The autopsy was carried out 36 h after death. On internal examination, both lungs were edematous and congested. Both pleural cavities contained a viscous fluid with a brownish tinge and the blood was equally of a brownish color. The stomach mucosa was quite hemorrhagic and eroded, the contents had a persistent but not specific smell. An intoxication was strongly suspected and post-mortem specimens were taken to perform STA [21].

In accordance with our laboratory's operating procedures, a comprehensive test for volatiles and drugs was performed on the postmortem specimens. This included headspace GC analysis, EMIT, RIA, TLC and GC–MS (see Case 1, Section 3.1). Furthermore, blood, stomach contents and a kidney homogenate were subjected to HPLC screening for basic drugs. This method is based on extraction under alkaline conditions and RP-HPLC analysis on an Aluspher RP-select B phase, eluted in a gradient mode with methanol and water both containing 0.0125 *M* NaOH [15]. It was intriguing to find that all toxicological tests were negative, although the anatomo-pathological results as well as the criminalistic findings at the scene all strongly suggested a suicidal overdose. Thorough investigation of all pieces of evidence revealed that a beaker, containing a crystalline residue, was found near the victim. The anatomo-pathological indications and a positive ferricchloride color test [22] on the crystalline residue initiated the search for azide.

A quantitative analysis of azide on all submitted postmortem biological samples was undertaken based on a precolumn derivatization with 3,5-dinitrobenzoyl chloride followed by isocratic HPLC [21]. We used an Ultrasphere ODS column (15×0.46 cm I.D., 5 μm) with a mobile phase consisting of a 1:1 (v/v) mixture of acetonitrile and water. The detection was by DAD using display wavelengths of 240 and 254 nm. Sample preparation basically consisted of dilution in a K_2CO_3 solution and acetonitrile, adjusting the pH to 5 and derivatization for 10 min at room temperature with 3,5-dinitrobenzoyl chloride. Blood, bile, stomach contents, kidney and liver all contained azide in large concentrations. A representative chromatogram is illustrated in Fig. 5. As can be seen, 3,5-dinitrobenzoyl azide can advantageously be chromatographed using a reversed-phase packing material. Identification of the azide peak in chromatograms of the derivatized postmortem samples was mainly based on identical retention behaviour as compared with a derivatized azide standard. The spectral information provided by the DAD is in this case of lesser importance as it is largely dominated by the spectral characteristics of the derivatizing label. Nevertheless, it is still very useful for peak purity evaluation and, if need, for peak deconvolution.

This case again illustrates the versatility of HPLC. Demonstration of azide is mostly based on a color reaction after addition of ferricchloride or cerium(IV) ammonium nitrate and it is well known that color reactions are prone to false positive results. Even in the case of the inorganic sodium azide salt, HPLC provided a tool for a highly reliable, sensitive

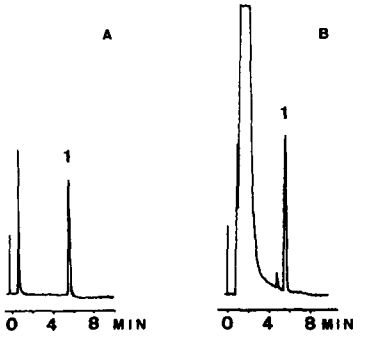

Fig. 5. HPLC–DAD chromatograms (displayed at 240 nm) of (A) the deceased's appropriately diluted bile; peak 1 (t_r: 5.70 min): 3,5-dinitrobenzoyl azide; azide level, 1283 μg/ml. (B) An undiluted 5-μg/ml sodium azide standard; peak 1 (t_r: 5.65 min): 3,5-dinitrobenzoyl azide. Reproduced from the J. Anal. Toxicol. by permission of Preston Publications, a division of Preston Industries, [21].

and specific method, without even the need of rare or sophisticated columns, eluents or detectors. At the same time this case illustrates the intrinsic limitations of a general unknown screening. Whatever the number of drugs and toxicants covered in the operating procedure, the vast number of candidate toxic chemical entities and their wide variety in physico-chemical nature inherently make it incomplete.

3.3. Case 3: unexpected suicide by chloroquine

A 40-year-old female with a history of psychological problems was implicated in a car accident. She had managed to leave the car, which was only moderately damaged, but was found dead lying at the side of the road. Considering the circumstances, a suicidal intoxication was strongly suspected and a postmortem blood sample was taken for toxicological analysis, pending a full autopsy. As described before, a comprehensive screening for volatiles and drugs was performed on the blood sample. None of the tests was positive except for the HPLC screening procedure for basic drugs [15] in which a peak was found, which could be identified as chloroquine on the basis of retention behaviour and its characteristic UV spectrum obtained by DAD. The chloroquine identity was later confirmed by GC using nitrogen–phosporus detection and mass spectrometry. It was quantified using the same HPLC–DAD procedure in which quinine was used as internal standard, obtaining a chloroquine concentration of 31.0 μg/ml.

Chloroquine is primarly used as a suppressive prophylactic for malaria infections. Although malaria is not endemic in our region, a high number of people travel to malarious areas, requiring prescriptions of antimalaria drugs. As such, our initial hesitation and surprise in finding a chloroquine overdose was definitely inappropriate.

In this case, our routine HPLC–DAD screening method promptly led us to solve this forensic problem.

3.4. Case 4: cocaine, polydrug abuse and forensic evidence

A question which is frequently asked to any forensic laboratory is to provide evidence of illicit drug use. In this particular case a young male was charged with drug trafficking and suspected of cocaine abuse. A urine sample was made available to our laboratory and initial results using combinations of immunoassays proved positive for cocaine and amphetamines. It is generally accepted that positive results of an immunoassay are to be confirmed by an independent analytical method. As usual in forensic analysis, this implies that the chemical entity has to be distinguished from endogenous, food-related substances and eventual medication in a complex biological matrix, which in itself is subjected to considerable interindividual variation. For this particular challenge, it is common practice to use GC–MS as the method of choice. However, for the quantitative part a dedicated HPLC–DAD analysis procedure for cocaine was adopted [16].

The urine sample was extracted using solid-phase extraction on a double mechanism phase, which extracts substances on the basis of polarity and charge (Varian Bond Elut Certify, Varian, Harbor City, CA, USA). This particular procedure has been optimized for cocaine and two of its metabolites, cocaethylene and benzoylecgonine. In view of the quantitative demands, two internal standards with great structural resemblance to benzoylecgonine (a carboxylic acid) and to the two esters, cocaine and cocaethylene were used. Chromatographic separation was performed on a Hypersil BDS C18 column (15×0.46 cm I.D., 5 μm), using a mobile phase consisting of a 0.045 *M* solution of ammonium acetate in HPLC-grade water (80%), methanol (10%) and acetonitrile (10%) (solvent A) and in methanol (40%), acetonitrile (40%) and HPLC-grade water (20%) (solvent B). The substantial differences in retention characteristics of the various compounds required a gradient elution from 100% A to 47.2% A within 19 min. The diode array detector was set to collect spectra every 21 ms over a 220–400 nm range, display wavelength was 230 nm (4-nm bandwidth). In Fig. 6, the resulting chromatogram is shown. Retention time information combined with spectral comparison of unknowns with library spectra immediately identified cocaine and its metabolites.

It is vital that such libraries are "home-made", the spectra have to be collated from injections of standards under the same chromatographic conditions. This is especially true for toxicants containing ionizable functional groups because these can sub-

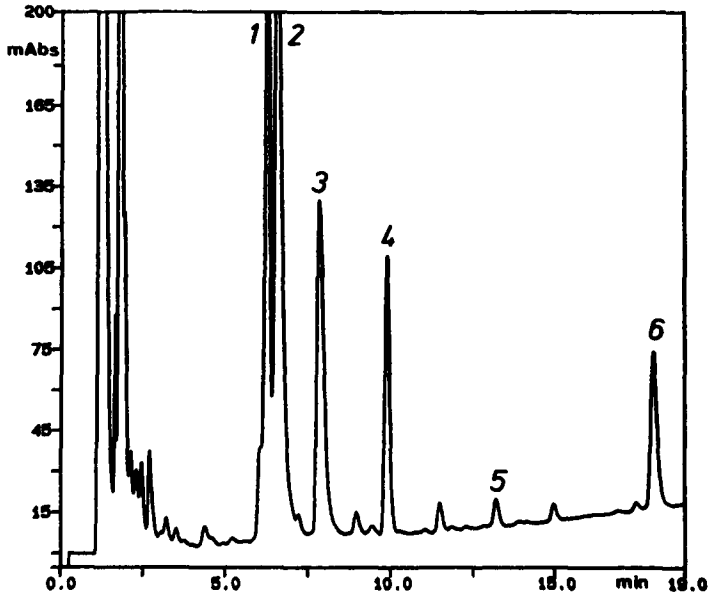

Fig. 6. HPLC–DAD trace (displayed at 230 nm) of the urine extract. Peak identification: (1) benzoylecgonine; (2) 3,4-methylenedioxy methamphetamine (XTC); (3) 3,4-methylenedioxy ethylamphetamine (EVA); (4) (I.S.$_1$) 2′-methylbenzoylecgonine; (5) cocaine; (6) (I.S.$_2$) 2′-methylcocaine.

stantially influence the spectrum as a result of pH, which is of course dependent on the chromatographic eluent composition. Correlation of the peak area ratios with those of a set of standards revealed a benzoylecgonine level of 10.5 μg/ml urine and unmetabolized cocaine at 1.4 μg/ml. Further inspection of the chromatogram and evaluation of the UV spectra, a process which can be completely automated in modern chromatography software, revealed additional clues to complete this drug inquiry. Some of the minor peaks could be identified as 3,4-methylenedioxy methamphetamine (MDMA, "XTC") and 3,4-methylenedioxy ethylamphetamine (MDEA, "EVA"), thus explaining the positive EMIT result for amphetamines. In fact this combination did not surprise us, because this kind of poly-drug abuse is frequently encountered in what is called the "recreational drug abuse" scene. This case, again, exemplifies the main role of HPLC–DAD within the STA approach. It presents us with an interesting combination of possibilities, suited for screening as well as for accurate quantitative analysis, without exhaustive sample preparation and derivatization, which is almost mandatory in GC.

4. Conclusion

The identification of the toxicant responsible for an acute intoxication, either in a clinical or forensic context, is a very challenging task. The above-mentioned issues should make it very clear that a screening procedure which covers most relevant drugs and toxicants is required to achieve a valid and reliable result. To this aim, HPLC–DAD offers many advantages in terms of specificity, sensitivity, speed and ruggedness. The data produced, comprising both retention behaviour and absorption spectra of eluting chemical entities, result in an identification power at low cost and with widened availability through many hospital laboratories. In addition, the above mentioned examples show a great versatility in application fields and excellent quantitative potential.

The fast progress in DAD detector technology, computer and software power and HPLC packing material quality have led to an exponential rise of the number of reports on the use of HPLC–DAD in STA. The advent of routine use of HPLC–MS will probably promote HPLC as a viable if not better alternative to GC–MS.

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