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Review

Hyphenated liquid chromatographic techniques in forensic toxicology

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Abstract

The prerequisite of applicability of hyphenated methods in forensic analysis is the achievement of a stage of ''final maturity''. In the field of liquid chromatography, HPLC coupled with diode array detection (DAD) seems to fulfill this criterion, whilst the combination with atmospheric pressure ionization mass spectrometry (HPLC–API-MS) is still in a development stage. HPLC–DAD is broadly used as identification tool in forensic and in emergency toxicology. Two main approaches were observed; development of retention index scales for intra-laboratory exchange of data and establishing of databases only for intra-laboratory use. Using these approaches, several databases were established for toxicological relevant substances (illicit and therapeutic drugs and their metabolites, environmental poisons etc.) in biological fluids. Also, complete HPLC–DAD identification systems are commercially available. Further possibility of progress depends on the on-line combination (''triple hyphenation'') with other detection methods, preferably API-MS. HPLC–API-MS, both in electrospray (ESI) and atmospheric pressure chemical ionization (APCI) options, underwent dramatic development in the last decade and is reaching its final shape. The method was broadly applied for various groups of toxicologically relevant substances, a lot of them unaccessible for other techniques, including GC–MS. Particularly important was application of HPLC–API-MS for detection and quantitation of active, polar metabolites of various drugs and for analysis of macromolecules. APCI seems to be more useful for analysis of less polar compounds, whereas ESI is particularly valuable for determination of polar, large molecules (e.g., toxic peptides, polar metabolites etc.) Up to now, HPLC–API-MS has been mainly applied for dedicated analyses, but the introduction of APCI or ESI in systematic toxicological screening may be expected in the near future. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Forensic toxicology

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1. Introduction

> Hyphenated: noting a naturalized citizen of the US believed to be ambivalent in his loyalty: so called of his tendency to style himself according to his former and present nationalities, using a hyphen (Webster Encyclopedic Unabridged Dictionary)

The Webster's definition cited above fits also very well in chromatography; like freshly naturalized and still ambivalent citizens, newly combined techniques are seldom treated as complete, integer systems. Usually these techniques gather the specialists from two or more different fields, who often feel basically at ease in only one analytical aspect, e.g., separation or detection problems. In the first stage of development, the technical refining and theoretical considerations of the method itself are of the primary interest and importance. When mass spectrometry (MS) and, to a certain extent diode array detection (DAD) were introduced, mainly highly-trained spectroscopists could use these techniques. In the course of consecutive implementation in combination with gas chromatography (GC) or high-performance liquid chromatography (HPLC) and broad commercial availability of low-cost instruments, the techniques started being used by analysts who were definitely less well trained and less interested in methodical aspects. This ''second generation'' of users, which already exists in the case of GC–MS or HPLC–DAD, does not regard these techniques as ''hyphenated'' but takes them for granted. This trend will most probably reach also other combinations, like liquid chromatography (LC)–MS or capillary electrophoresis (CE)–MS, in the future. In conclusion, the term ''hyphenated'' seems to apply to those techniques, which still have not reached a stage of final maturity. As Krull and Cohen [1] stated in their recent review: ''There are at least two fundamental views on the future role of MS in biotechnology HPLC. Is the mass spectrometer an expensive, sophisticated LC detector? Or, is the chromatograph an expensive sample-preparation device for a mass spectrometer? This is a long-standing question that has been debated for years. One of the newer developments in LC–MS is that this distinction will cease to be important in the future''.

It should be kept in mind, that analytical techniques applied for forensic purposes are always subjected to thorough validation and must assure the highest possible level of reliability. This may subsequently mean, that so-called ''hyphenated techniques'' are applicable in forensic sciences only when the hyphenation is not noticed anymore.

The purpose of this paper is to review the forensic applications of these liquid chromatographic techniques, which were, or still are, regarded as hyphenated. For practical purposes, the period of some last 10 years was covered. The electrically driven separations as well as supercritical fluid chromatographic techniques, which have been reviewed recently [2–4] and are covered in other chapters of this volume, were not considered.

2. Application of hyphenated liquid chromatographic methods for screening purposes

2.1. HPLC–DAD

Possible advantages of the combination of HPLC–DAD with other detection methods were assessed already in the early studies. The application of multichannel UV–Vis and fluorescence detectors was described and the methods of peak purity assessment were given [5]. The coupling of HPLC with various spectrometric detection methods was reviewed by Poppe in 1987. The coupling with DAD and MS was already established in practice, and the future applicability of HPLC–Fourier transform infrared spectroscopy (FT-IR), HPLC–nuclear magnetic resonance (NMR), HPLC–fluorescence combinations was assessed. The author advocated the off-line use of the latter combinations, due to interfacing problems [6]. Dual channel detection: DAD and MS particle beam ionization (PBI) combined with HPLC was described and applied for the analysis of steroids. Parallel coupling appeared more versatile and easier than the serial one [7].

The potential of DAD to detect unresolved peaks of substances was a real progress in analysis of forensic biosamples, which sometimes have very complex nature. Several methods of spectra deconvolution were developed. A format for spectral data interpretation employing an absorbance-weighed mean wavelength of a spectrum called the Purity Parameter was developed for HPLC–DAD to reduce spectral data to a single value. Absorbance weighting minimized the effect of noise in the calculation. Purity Parameter was tested for alkane series, olefins (hexene-1, heptene-1, octene-2, nonene-1), aromatics and some drugs (xanthine derivatives and barbiturates) [8]. The Purity Parameter was successfully applied for identification of 10 barbiturates. These drugs present a ''worst case scenario'' in UV spectral identification. The experiments were performed with pure drugs thus the impact of biological matrix was not tested [9,10]. Besides, an automatic peak-purity-control procedure in HPLC–DAD based on principal component analysis was described. The method was applied for spectra identification of unresolved substance peaks. The limit of differentiation was estimated at the ratio of 9:1 in the case of drugs with similar spectra [11]. A method of spectral resolution and quantitation of overlapped peaks, based on the measurement of peak areas of given substances, was elaborated, but the method was applicable only for samples whose composition was known in advance [12]. A rapid method for HPLC–DAD identification of drugs of abuse in urine was presented. The application of mathematical resolution of overlapping peaks (generalized rank annihilation method) allowed the use of very short runs. Some common drugs (opiates, cocaine and metabolites, amphetamines etc.) were eluted and identified, without full separation, within 8 min [13].

The high identification potential of HPLC–DAD has found wide application particularly in emergency toxicology. In the recent review of Lambert el al. [14] the usefulness of this technique in systematic toxicological analysis was stressed. On the basis of their own experience, concerning particularly cases of mixed poisonings, the authors regarded HPLC–DAD as a viable if not better alternative to GC–MS.

HPLC–DAD gives, as any chromatographic technique, the possibility to use chromatographic retention as an identification parameter. In regard to this, two approaches were presented: the use of retention index scales for possible inter-laboratory use and exchange of HPLC–DAD databases, or the expression of retention in time units, mainly for intra-laboratory purposes. Published databases, comprising UV and retention data of toxicologically relevant substances, are shown in Table 1 [15–35]. Further data concerning application of HPLC in toxicological analysis, particularly for detection of drugs of abuse, may be found in a review by Binder [36].

2.1.1. Identification systems based on retention index scales

Establishing of the broadest library of data (UV spectra and retention parameters) is the first and most important step of any HPLC–DAD identification system. It is obvious that one single laboratory cannot establish a database of all relevant toxic substances. This dictates the need of inter-laboratory cooperation. As a consequence, the methods used must be standardized in order to generate the data which may be exchanged and used in many centers.

Table 1
Collections of retention and UV spectral data used in toxicological screening

Content of database, retention parameter used	Column	Elution conditions	Ref.
161 drugs in serum, RRT	RP-8	ACN–H_2SO_4, isocratic	[15]
62 drugs in blood and urine, RRT	RP-18	MeOH–H_3PO_4, pH 2.1, isocratic	[16]
12 diuretics in urine, t_R	RP-18	ACN–buffer pH 5.0, gradient	[17]
21 benzodiazepines in biological fluids, t_R	RP	ACN–buffer pH 5.4, gradient	[18]
48 basic drugs in blood, k	RP-18	ACN–MeOH–buffer pH 2.7, isocratic	[19]
157 acidic drugs and 144 basic drugs, t_R	RP-8, PRP-1	ACN–H_3PO_4 or ACN–NH_4OH, gradient	[20]
48 drugs in biol.fluids, k	RP-18	ACN–sodium perchlorate, isocratic	[21]
17 barbiturates in serum, t_R	RP-18	ACN–MeOH–buffer, pH 2.6, isocratic	[22]
23 diuretics in urine, t_R	RP-18	ACN–buffer, pH 3.0, gradient	[23]
13 diuretics in urine, t_R	RP-18	ACN–buffer, pH 6.8, gradient	[24]
100 basic drugs in urine, t_R	RP-8	ACN–buffer, pH 3.2, gradient	[25]
225 drugs in biological fluids, t_R	RP-18	ACN–buffer, pH 3.0, gradient	[26]
350 drugs in plasma, t_R	RP-18	ACN–buffer, gradient	[27]
27 neuroleptics in biological fluids, k	RP-18	MeOH–THF–buffer, pH 2.6, isocratic	[28]
119 basic drugs in blood, t_R	RP-18, phenyl	ACN–buffer, pH 3.4, isocratic	[29]
469 drugs in biological fluids, RI	RP-8	ACN–buffer, pH 2.2	[30]
383 drugs in biological fluids, RI	RP-18	ACN–buffer, pH 3.0, gradient	[31]
311 drugs in blood, t_R, k	RP-18	MeOH–THF–buffer, pH 2.6, isocratic	[32]
300 drugs in biological fluids, t_R	RP-18	ACN–buffer, pH 3.0, gradient	[33]
600 drugs in biological fluids, t_R	RP-8	ACN–buffer, pH 3.8, gradient	[34]
78 acidic drugs in blood, RRT	RP-18	ACN–buffer, pH 3.2, gradient	[35]

The concept of retention indices, introduced at first in GC [37], has been also implemented in HPLC. Following requirements were suggested for a homologous series of compounds to be widely applicable as a retention index (RI) scale in HPLC [38,39]:

- They should have a strong chromophore in UV range so that they can be added to unknown samples to act as internal standards
- They should not be easily ionized to avoid changes in retention because of pH variations
- A range of members of the series should be readily available at reasonable cost
- The most polar members of the series should be eluted with a similar retention to polar pharmaceuticals
- They could be unreactive and stable in common liquid chromatography solvents
- The relationship between log k and the number of carbon atoms or characteristic functional groups in the homologues should be linear
- They should not specifically interact with silica gel
- The capacity factor (k) values should depend little on the mobile phase composition

A number of homologous series of compounds have been studied as retention index standards for HPLC, among them n-alkanes, n-alkylbenzenes, alkan-2-ones, alkyl-aryl-ketones, 1-nitroalkanes. An in-depth review of retention index scales used in HPLC may be found elsewhere [40]. Three RI scales were applied for identification of toxicologically relevant compounds: alkan-2-ones, alkyl-aryl-ketones and 1-nitroalkanes (Figs. 1 and 2 and Table 2). These applications were thoroughly reviewed [41,42]. The 1-nitroalkane-scale showed some advantages; the lower homologues, in contrary to alkyl aryl ketones, have similar retention factors to polar drugs, and are less reactive and have higher UV absorbance than alkan-2-ones. The other advantage was the applicability of this scale for GC [43] and for micellar electrokinetic chromatography [44]. It must be stressed that the variability of RI values,

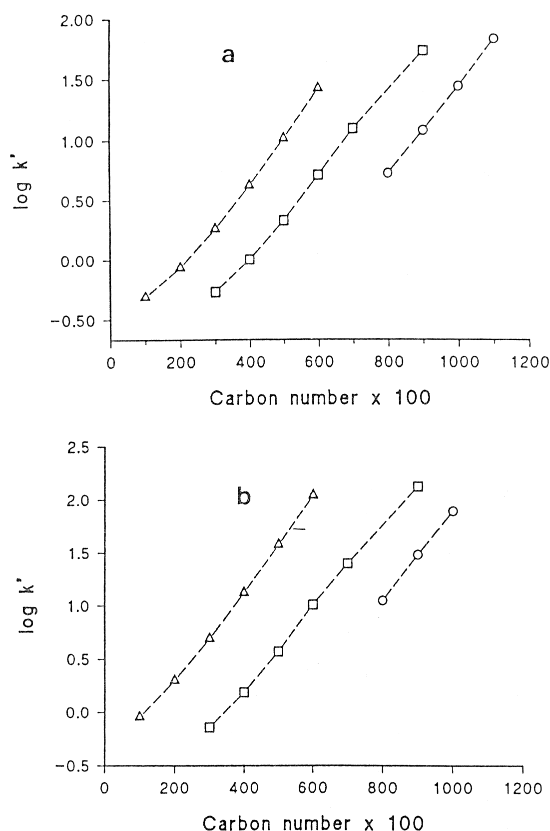

Fig. 1. Relationship of log k to carbon number×100 for the three homologous series, alkyl aryl ketones, alkan-2-ones and 1-nitroalkanes. Reproduced from Ref. [41] with permission from Elsevier Science and the authors.

particularly in gradient elution conditions, is much smaller than that of other retention parameters (Fig. 3).

The first studies with 1-nitroalkanes showed that the RI values of acidic drugs, separated on C_{18} column, decreased distinctly with increasing concentration of organic modifier (acetonitrile) in the mobile phase (Fig. 4) [45]. Basic drugs showed opposite trend. The RI data obtained with different isocratic conditions and with gradient elution were not transferable [46]. Therefore, the concept of "corrected retention indices" was introduced and applied at first for alkyl aryl ketone scale, then for 1-nitroalkane scale [47]. In this approach, it was assumed that the differences in RI values obtained for the same substances on different columns could be normalized by scaling the results using selected, standard reference drugs with known retention indices. Two scales were used, separately for acidic/neutral and for basic compounds. Inter-laboratory studies showed that the corrected RI values of 47 selected drugs, determined on seven different C_{18} columns showed much smaller variability than that of RI values calculated with 1-nitroalkanes [48]. In consequence, some large databases of RI values were established [26,30,31]. The comparison of RI values from three databases, comprising together 1149 drugs, showed very good agreement between the results obtained in two labs using the same column packing. The comparison of the values obtained in the laboratories using different columns and slightly different elution conditions showed some deviations, particularly for drugs with pK_a values around their pH of the mobile phase. The search window of ± 10 RI units was recommended for laboratories using standardized elution conditions [49]. Elliott and Hale [50,51] introduced recently retention index scale based on five acidic/neutral and five basic drugs, with arbitrary assigned value of 100–500 RI units in each scale (Fig. 5). The elution conditions were almost identical as in the studies of Bogusz and co-workers [26,31,49], a mixed-phase octadecyl/cyanopropyl column was used. Very good long-term reproducibility of RI values, also for extracted drugs, were observed (Table 3). The system was applied in clinical and forensic casework.

2.1.2. *Identification systems based on retention time scales (absolute or relative)*

The rationale behind these systems was to develop own search library, tailored to local needs and technical possibilities. The authors advocating this approach noted that inter-laboratory exchange of retention data alone is of limited importance, in the view of the fact that the transfer of UV spectra taken with different instruments is hardly possible [33].

One of the first large databases was developed by Hill and Langner [20], who used "basic system" (Zorbax C_8 column, gradient elution in acetonitrile–phosphoric acid) for separation of 144 drugs and "acidic system" (PRP-1 column, gradient elution in acetonitrile–ammonia) for 157 drugs. Retention time was expressed in t_R; the authors, however, expressed their concern about the reproducibility and advocated

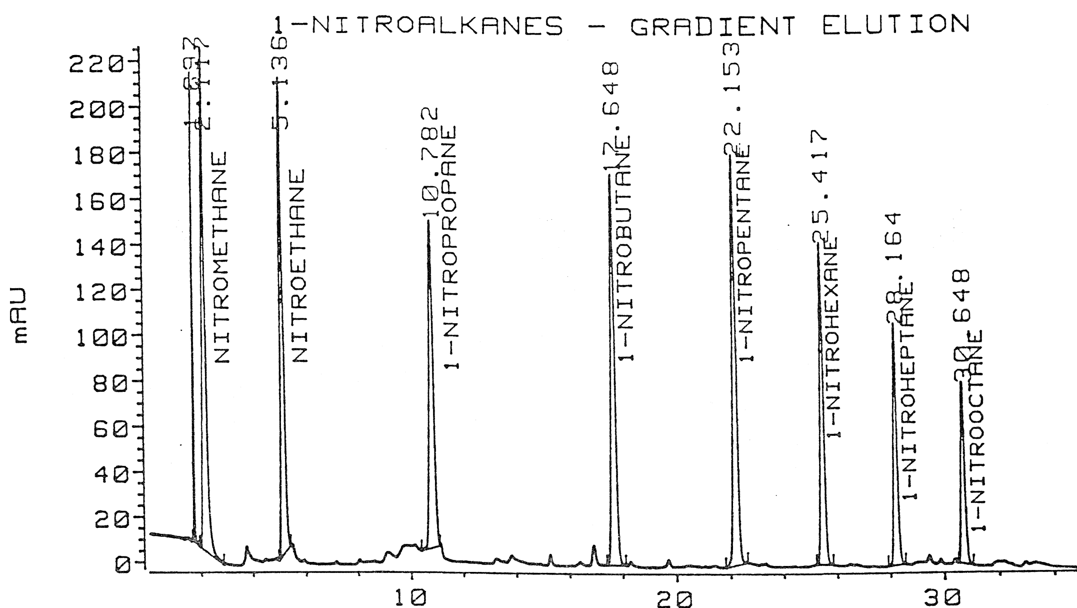

Fig. 2. Chromatogram of the 1-nitroalkane mixture in a gradient of acetonitrile–triethylammonium phosphate buffer (pH 3.0). From Ref. [26] with permission of Preston Publications.

the use of relative retention times (RRTs) or RIs. Logan et al. [25] published data for 100 basic drugs, extracted from urine and subjected to HPLC–DAD screening. The authors stressed that the t_R values may differ between laboratories due to local conditions, even when the method is exactly followed. In a conclusion, they recommend to prepare the own database of t_R values and UV spectra. The database of Turcant et al. [27] (Fig. 6) comprised over 350 drugs and was used for toxicological screening of plasma samples. Narrow bore HPLC column (2.1 mm I.D.) was applied. A shift in t_R of drugs caused by column aging was noted as a cause of difficulties in identification. A UV-spectra search without time parameter was recommended to detect some metabolites of similar spectral properties. Two-column identification system was applied by Koves and Wells [29] (Fig. 7) for post-mortem blood screening. Beside retention data, the purity parameters were given for 119 drugs. An identification system, consisting of two isocratic setups with different mobile phases and two detectors (rapid scan and variable wavelength) was applied in emergency toxicology together with the library of over 200 drugs [52]. Data for 311 drugs were published by Tracqui et al. [32]. Retention was expressed in t_R and k values. Thirty-five pairs of drugs showed similarities both in t_R and UV spectra. Particularly difficult appeared differentiation of some opiates. Nevertheless, the authors assessed the method as simple, rapid, highly specific and useful for emergency screening. The database of Lai et al. [33] comprised 300 drugs and metabolites and was applied for emergency toxicological screening of blood or urine samples. The preference of urine screening was stressed. Comprehensive database of 600 drugs (which, according to private information, was increased to over 800 compounds) was published by Gaillard and Pepin [34] (Fig. 8) and applied for serum and urine screening. The authors found high reproducibility of established t_R values, which was regarded to improved quality of columns and instrumentation. The method was adapted also for quantitation. The same authors applied the combination of HPLC–DAD and GC–MS (electron impact ionization, EI) as screening procedure for drugs in human hair [53] (Fig. 9). HPLC–DAD screening was applied for the diagnosis of poisoning by traditional African medicines

Table 2
Comparison of retention indices calculated using different retention index scales (reprinted from Ref. [41] (Table 3.3) with permission from Elsevier Science and the authors)[a]

Compound	Retention index scale		
	Alkan-2-one I(CO)	Nitroalkane $I(NO_2)$	PhCOR I(AAK)
Alkan-2-ones			
Acetone	300[b]	118	(520)
Butan-2-one	400	219	(596)
Pentan-2-one	500	319	(689)
Hexan-2-one	600	422	(796)
Heptan-2-one	700	520	904
Nonan-2-one	900	(676)	1076
1-Nitroalkanes			
Nitromethane	(257)	100[b]	(507)
Nitroethane	342	200	(577)
Nitropropane	426	300	(669)
Nitrobutane	564	400	(772)
Nitropentane	676	500	882
Nitrohexane	798	600	992
Alkyl aryl ketones			
Acetophenone	619	426	800[b]
Propiophenone	(723)	516	900
Butyrophenone	(833)	(607)	1000
Valerophenone	–	(700)	1100
Column test compounds			
N-Methylaniline	589	412	785
2-Phenylethanol	582	404	777
p-Cresol	603	425	799
Nitrobenzene	626	448	825
Toluene	794	593	985
Drug compounds			
Aspirin	(170)	(<0)	(420)
Paracetamol	308	124	(526)
Theophylline	327	147	(540)
Barbitone	421	242	(616)
Salicylamide	452	273	(644)
Caffeine	400	221	(596)
Phenobarbitone	504	324	(693)
Phenacetin	579	401	(774)

[a] Conditions: column, ODS-Hypersil; eluent, methanol–phosphate buffer, pH 7.0 (40:60). Indices in parentheses have been calculated by extrapolation.
[b] Index standards I=carbon number$\times$100.

(poisonous plants), but no data were given [54]. Recently, retention data and UV spectra of 65 drugs have become available on the Internet (http://chrom-.tutms.tut.ac.jp/JINNO/DRUGDATA).

2.1.3. Commercially available HPLC–DAD identification systems for toxicological screening

Three HPLC–DAD identification systems are commercially available. The oldest and most popular

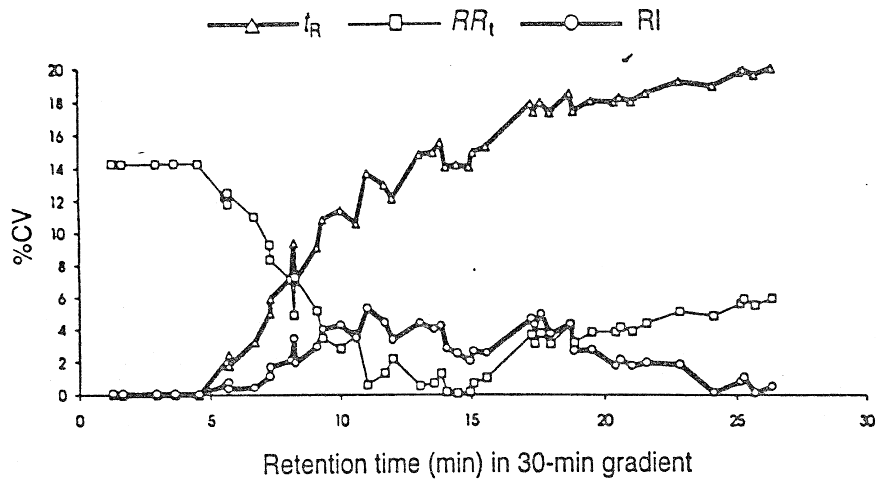

Fig. 3. RSD of retention time t_R, relative retention time (RRT) and retention index (RI) of selected drugs between the 20- and 30-min gradients plotted against the drug retention time in the 30-min gradient. From Ref. [30] with permission of Preston Publications and the authors.

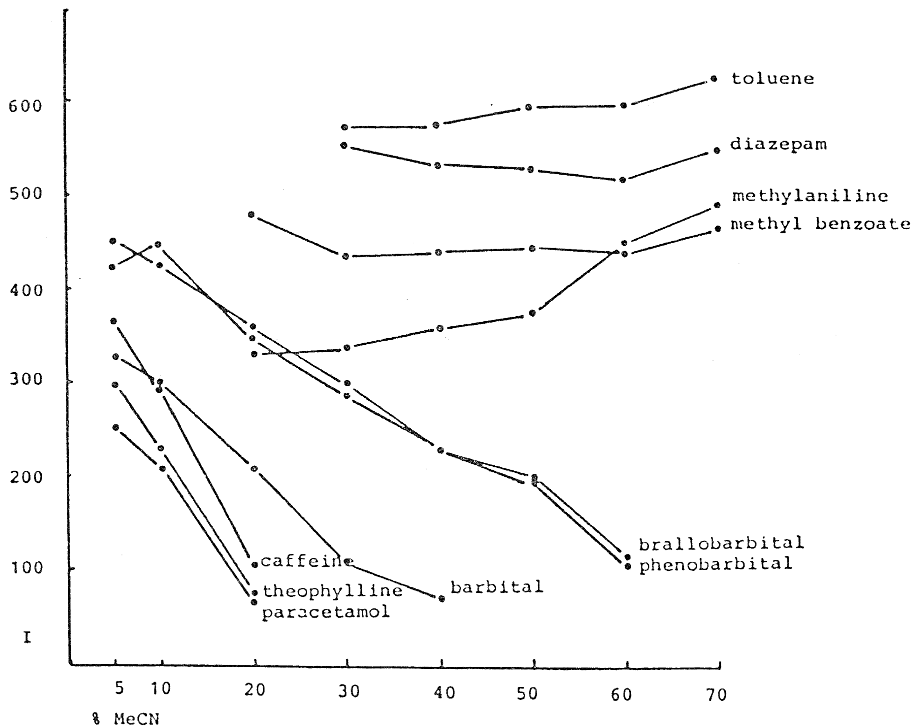

Fig. 4. The retention indices of the test compounds calculated with reference to 1-nitroalkanes at different concentrations of acetonitrile–phosphate buffer (pH 3.2). From Ref. [45] with permission from Elsevier Science.

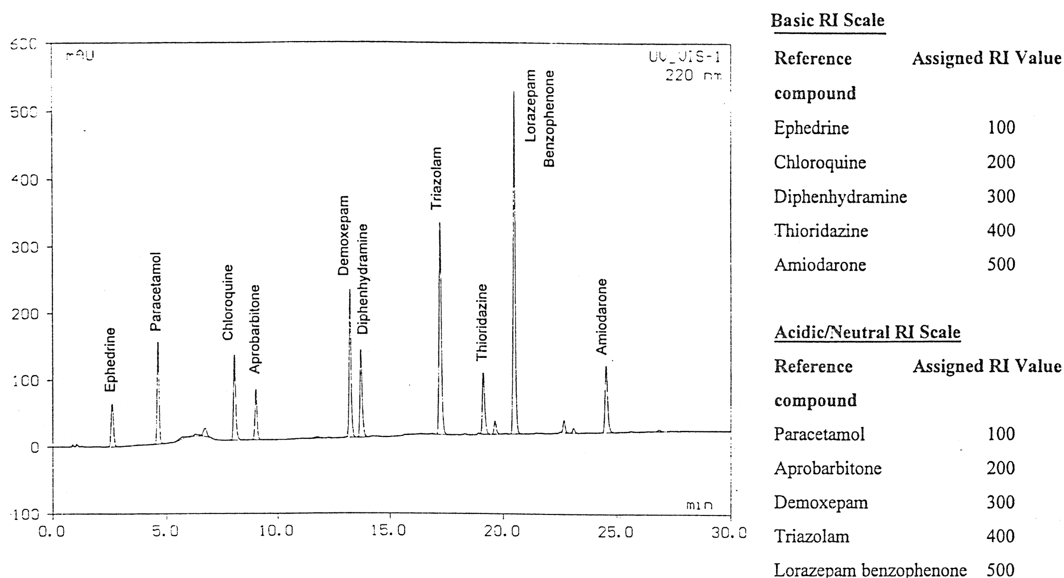

Fig. 5. Chromatogram of the reference drugs used to calculate the basic and acidic/neutral RI scales. From Ref. [50] with permission from Elsevier Science and the authors.

one is REMEDi (Bio-Rad Labs., Hercules, CA, USA). This isocratic system was described by Binder et al. [55] (Fig. 10). The system includes automatic extraction on polymeric pre-columns (PRP-1 and Aminex A-28), isocratic separation on RP-8 and silica columns and DAD. The identification is performed through the comparison of UV spectra and relative retention times (calculated with two internal standards). The library of the recent version (REMEDi HS) comprises almost 800 drugs. The system was primarily developed for screening of basic compounds in urine. Recently, an alternate version was introduced for benzodiazepine screening, with 33 compounds in the database. A report based on evaluation of 116 samples (78 urines and 38 stomach contents) demonstrated that the REMEDi largely expands the range of drugs covered by the immunoassays, since in 53 emergency cases additional compounds were detected [56]. Chen et al. stated that in 67 out of 96 emergency cases no drugs were detected. In 23 cases the results of REMEDi were compatible with clinical appearance [57]. According to Ohtsuji et al., who analyzed 18 emergency cases and 24 autopsy cases, the REMEDi library was not

Table 3
Comparison of retention index (RI) values of various acidic and basic drugs in pure methanolic solution and following extraction from post-mortem blood and urine specimens [reprinted from Ref. [50] with permission from Elsevier Science and the authors]

Compound	Library RI value	RI_{pure} MeOH solution[a]	RI_{blood} acid extract	RI_{blood} basic extract	RI_{urine} basic extract
Caffeine	192	194 ± 0	192	192	192
Dothiepin sulfoxide	228	229 ± 0	ND[b]	229	229
Nordothiepin sulfoxide	221	N/A[b]	ND	ND	220
Nordothiepin	325	323 ± 1	ND	325	325
Dothiepin	336	335 ± 1	ND	336	336
Temazepam	357	358 ± 1	360	ND	ND
Mefenamic acid	488	490 ± 1	491	ND	ND

[a] Mean±standard deviation calculated from three duplicate determinations.
[b] N/A=Not available; ND=not determined.

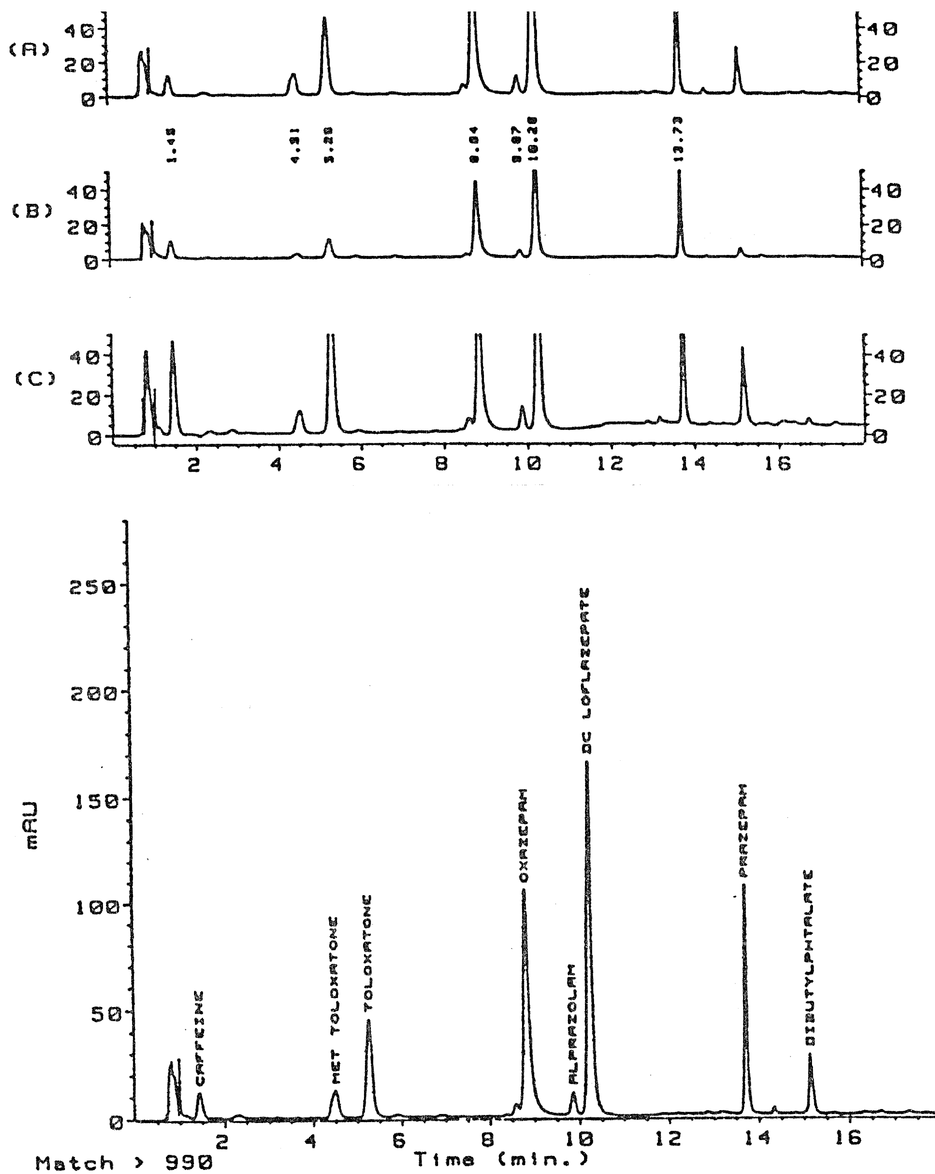

Fig. 6. Multi-wavelength chromatogram (A=230 nm, B=254 nm, C=210 nm) of the plasma extract of intoxicated patient and final presentation of identified drugs. From Ref. [27] with permission of the American Association for Clinical Chemistry and the authors.

completely suitable for Japanese conditions and a number of drugs were not detected, even in toxic concentrations [58]. Kalasinsky et al. applied REMEDi in forensic cases not only for urine screening (236 cases) but also for whole blood (35 cases) and tissues (17 samples), after liquid–liquid extraction. REMEDi was found as useful complementary screening method and in some cases allowed to find drugs which were not detected by GC–MS [59] (Fig. 11). Recent evaluation of REMEDi, based on the results of 469 blood samples and 95 gastric content samples showed good sensitivity of the system for tricyclic antidepressants and lower for barbiturates and benzodiazepines. The usefulness of REMEDi as

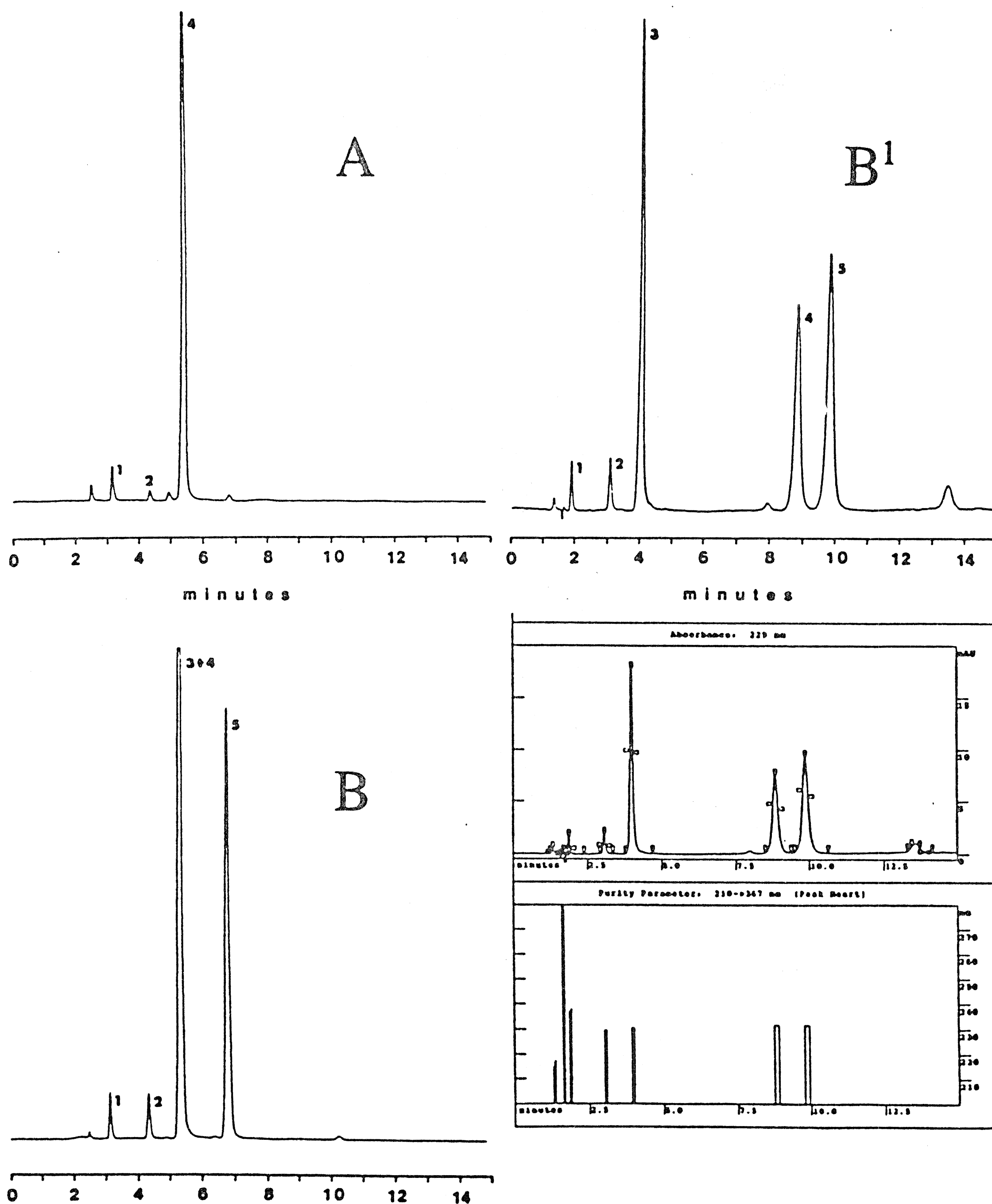

Fig. 7. Chromatograms and double report of a case blood extract: A=Sulphuric acid fraction, column 1, B and B1=HCl fraction on columns 1 and 2. Peaks: 1=caffeine, 2=possible temazepam metabolite, 3=temazepam, 4=nordiazepam, 5=diazepam. From Ref. [29] with permission from the ASMS and the authors.

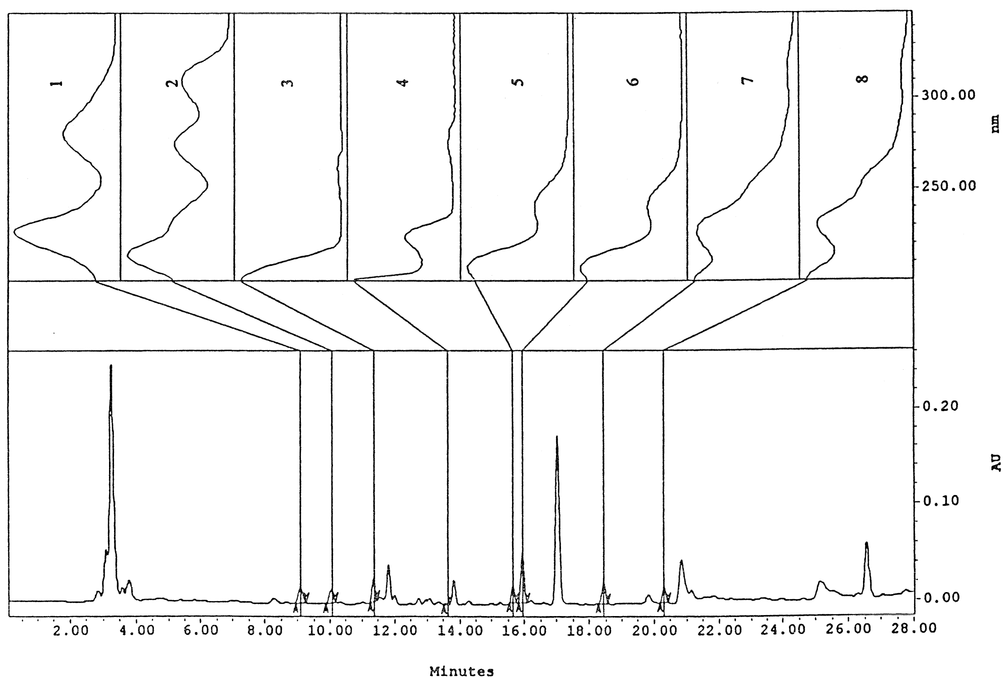

Fig. 8. Chromatogram (210 nm) and corresponding UV spectra of an extract of whole autopsy blood. Peaks: 1=amisulpiride, 2=metoclopramide, 3=fluconazole, 4=betaxolol, 5=notriptyline, 6=amitriptyline, 7=nordazepam, 8=diazepam. From Ref. [34] with permission from Elsevier Science and the authors.

complementary tool in emergency toxicology was stressed [60]. Sadeg et al. analyzed results obtained among of 354 poisoned patients with REMEDi, immunoassays and thin-layer chromatography (TLC). The positive predictive value of REMEDi (the probability of true positives) was 92%, the negative predictive value (the probability of true negatives) was 72%. Lower probability of true negative results (i.e., higher probability of false negatives) was associated with low sensitivity of DAD for some drugs with low UV absorptivity. The authors suggested the combination of different analytical techniques as the best strategy in emergency toxicological screening [61]. Beside general unknown analysis, REMEDi may be used for quantitative analyses and was applied in therapeutic drug monitoring [62] or emergency toxicology [63]. The off-line combination of REMEDi with direct-probe MS was described for confirmation of demoxepam in urine. In this approach, REMEDi was used in semi-preparative way [64]. It may be generally stated that the REMEDi system found broad application in clinical and forensic toxicology, mainly because of integration of all analytical steps: isolation, detection and automatic interpretation of results (“black box principle”). The drawbacks are: applicability only to basic drugs and some limitations related to isocratic elution.

The second commercially available HPLC–DAD identification system used the nitroalkane retention index scale introduced by Bogusz and co-workers [26,31]. The system is available from E.Merck (Darmstadt, Germany) as a part of Merck Tox Screening System (MTSS), which comprises TLC, GC and HPLC–DAD data [65]. Over 1100 acidic, neutral and substances were included. The system was used also for therapeutic drug monitoring in gradient elution [66] (Fig. 12) and isocratic mode [67]. The system assures very good resolution of drugs and is applicable to acidic and basic drugs. Nevertheless, in comparison with highly sophisticated REMEDi machine the MTSS is less developed technically and the handling is more difficult.

HPLC–DAD database developed in isocratic con-

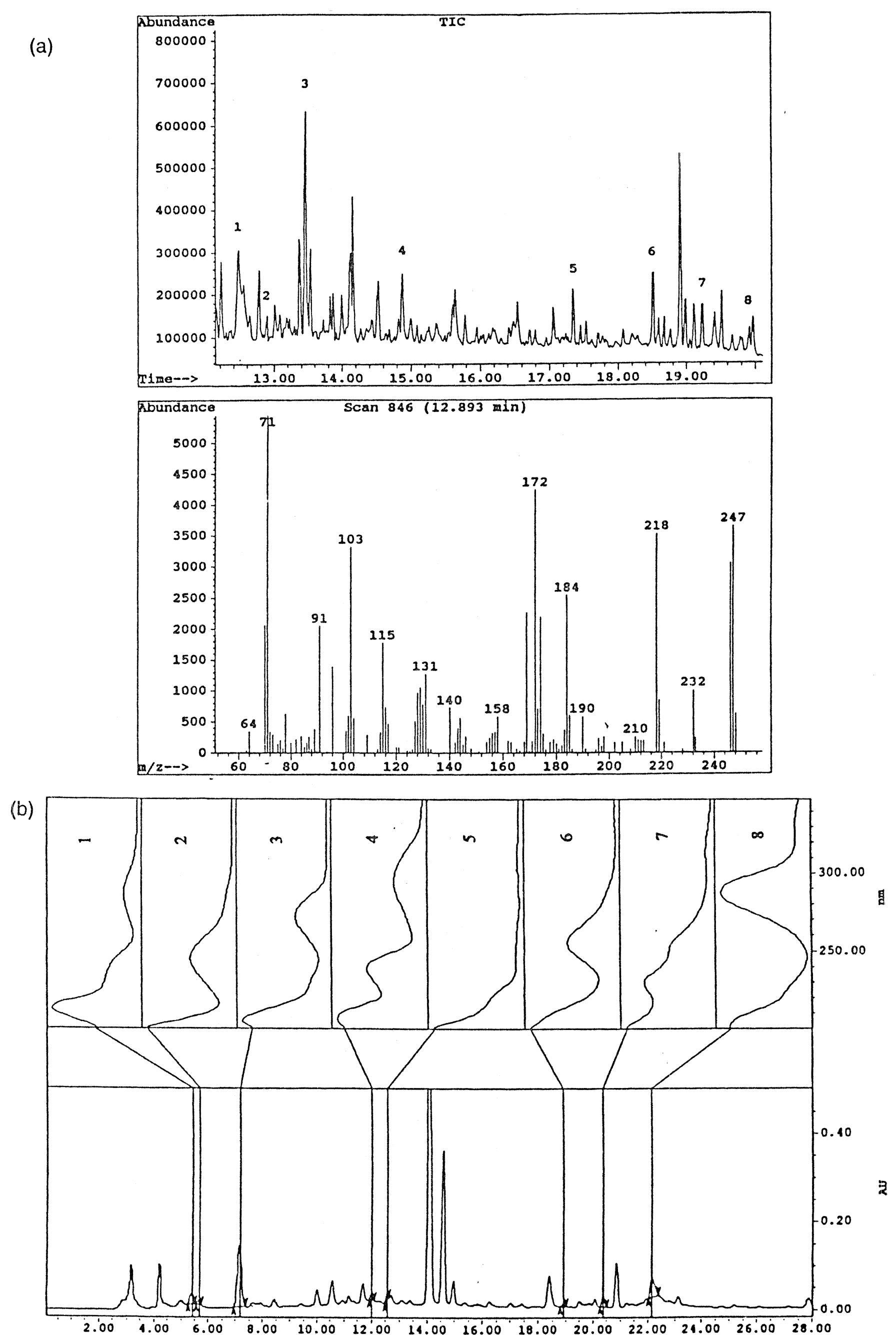

Fig. 9. Chromatograms of an extract of 75 mg of powdered hair. (a) GC–MS analysis, peaks: 1=paracetamol, 2=pethidine, mass spectrum below, 3=caffeine, 4=nefopam, 5=diazepam, 6=promethazine, 7=thiapride, 8=niflumic acid. (b) HPLC–DAD analysis, peaks: 1=tiapride, 2=paracetamol, 3=caffeine, 4=zolpidem, 5=nefopam, 6=ketoprofen, 7=diazepam, 8=niflumic acid. From Ref. [53] with permission from Elsevier Science and the authors.

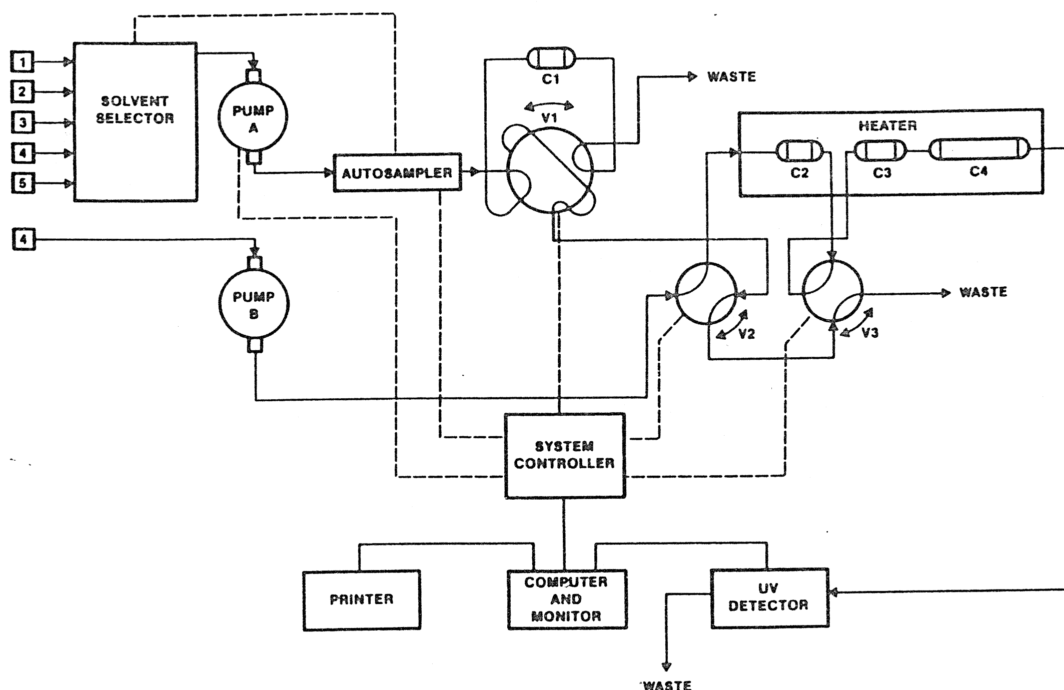

Fig. 10. Scheme of automatic analyzer REMEDi. From Ref. [55] with permission from Elsevier Science and the authors.

ditions by Pragst et al. [68] is commercially available together with Shimadzu SPD-MXA DAD system. The database comprises over 1100 spectra of drugs and environmental poisons.

2.2. HPLC–MS

Several reviews of LC–MS, written in the last decade, show how crucial this period was in the evolution of this technique.

Tomer and Parker [69] in their 1989 review stressed that of all detectors used in LC, mass spectrometer is the most universal but not the most sensitive. The limit of molecular mass for LC–MS was then defined at ca. 20 000–30 000 u. Direct liquid introduction and moving belt interfaces were already abandoned, and mostly used techniques were thermospray ionization (TSP), PBI and fast atom bombardment (FAB). Atmospheric pressure ionization (API) was shortly mentioned as a promising but quite exotic technique. Main papers describing atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) were published shortly before [70,71]. In their review on LC–MS Garcia and Barcelo [72] in 1993 covered the last 10 years and compared TSP, FAB, APCI and ESI. Best perspectives were given to both API interfaces. TSP was assessed as old, but still robust technique. Gelpi [73], reviewing the period 1991–1994, defined the introduction of API techniques as a revolution, which made possible to analyze a lot of previously inaccessible compounds. As shortcoming of ESI the applicability only for low flow-rates was mentioned. The drawback was already overcome through the introduction of pneumatically-assisted ESI. The second drawback, which concerns both API techniques (i.e., ESI and APCI), is the limitation of using volatile mobile phase buffers. In 1997 Hoja et al. [74] in their review on the applications of LC–MS in toxicology stated that PBI and FAB were already of secondary interest, whereas TSP and API (both in ESI and APCI options) were of major importance. The future – according to authors – belongs to API interfaces. Careri et al. reviewed the applications of

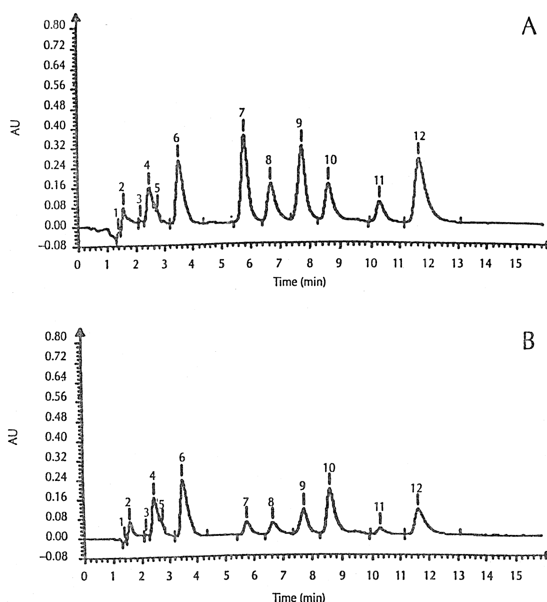

Fig. 11. Chromatograms of a whole blood sample spiked with nordiazepam (4), lidocaine (7), notriptyline (8), doxepin (9), quinidine (10) and codeine (11). Internal standards: *N*-ethyl-nordiazepam (6) and chlorpheniramine (12). REMEDi system at 205 nm (A) and 235 nm (B). From Ref. [59] with permission of Preston Publications and the authors.

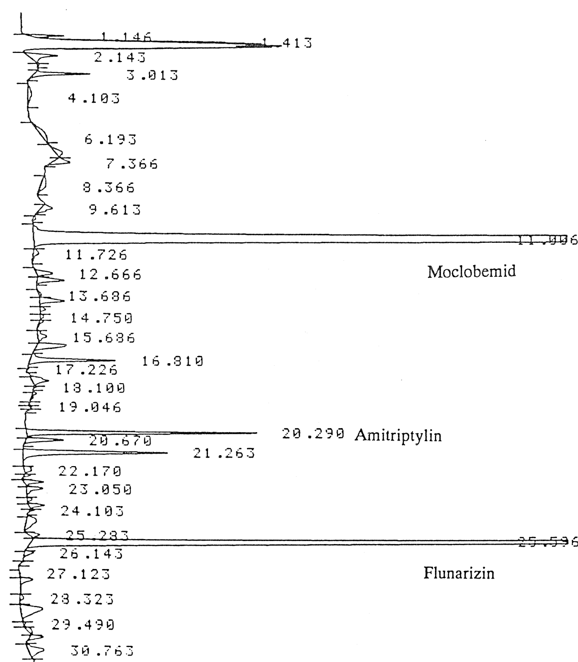

Fig. 12. Chromatogram of 1 ml serum extract spiked with 2.5 μg moclobemide, 250 ng amitriptyline and 1 μg flunarizine. MTSS system. From Ref. [66] with permission from GIT Verlag and the authors.

LC–MS interfacing systems in food analysis in 1996 and in 1998. In the first review, devoted to xenobiotic substances, LC–API-MS was defined as very promising, but still not a routine, widespread technique [75]. In the second review, on naturally occurring substances in food it was concluded that both APCI and ESI became a standard, robust methods, and the development of customized databases of the mass spectra is expected [76]. In his recent review, Niessen [77] has stated that LC–MS has became a broad applicable technique, due to development of numerous robust and easy to operate instruments. All these instruments use API interfaces. In the future LC–MS may find much wider applicability that GC–MS. The comparison of number of contributions per interface in the years 1989–1996 showed huge increase in papers devoted to ESI, distinct increase of APCI-related papers and vanishing number of those devoted to other interfaces, like TSP or FAB.

It is clear from the above mentioned reviews that the last decade has brought a breakthrough in technical development of LC-API interfaces, which in consequence has determined the further direction of the LC–MS. As Niessen [77] wrote, the mass spectrometrist may feel somewhat overwhelmed by the speed of development of new instruments which allow to collect previously unthinkable amount of data and are contrasting sharply with the ‘‘hectic fights of the past’’. Under this term Niessen meant obviously the unreliability and unreproducibility of earlier API instruments.

Many modern applications of LC–API-MS demonstrate the high relevance of this technique for analytical toxicology. On one hand, APCI makes possible selective and sensitive determination of small molecules, which are too thermolabile for GC–MS (e.g., drug glucuronides), and, on the other hand, ESI allows to handle large, ionized molecules (e.g., peptides) of toxicological relevance. Both options give not only molecular mass data, but also structural information. This is possible using LC–MS–MS or

more simple by in-source collision induced dissociation (CID), sometimes denoted dryly as ''poor-man MS–MS'' [78–83]

Recently, the multi-API interface (atmospheric pressure spray with electron impact ionization, APEI, APCI, atmospheric pressure spray ionization, APSI, ESI and sonic spray ionization, SSI) was described by Hitachi scientists [84]. These ionization sources may be applied for various groups of compounds, from very polar (e.g., proteins) to lipophilic (e.g., hydrocarbons). The authors observed, that in the same mobile phase different major ion species $(M+H)^+$, $(M+NH_4)^+$ and $(M+Na)^+$ were formed for different compounds in APCI. That may mean that the development of APCI-based mass spectra library might be a very challenging task.

Several publications dealt with the comparison of various LC–MS techniques. Edlund et al. [85] applied ESI and APCI for determination of methandronostenolone and its metabolites in equine urine. APCI performed better for neutral compounds and ESI for sulfate metabolites. The comparison of APCI, ESI, TSP and PBI for carbamate pesticides showed that APCI was best, followed by ESI and TSP. PBI was not sensitive enough [86]. Five β-agonists were determined with GC–MS, TSP and ESI. Both GC–MS and ESI gave 50-fold lower limit of detection (LOD) than TSP [87]. TSP, APCI and ESI were compared for drug metabolites of various polarity. APCI and TSP performed better for hydrophobic compounds, ESI was applicable for wide range of polarity [88]. The comparison of ESI, APCI, TSP and FAB for determination of plant metabolites of various classes of 100–3000 u demonstrated that no one interface allows the optimum ionization of all the metabolites within a single crude plant extract. The API techniques allowed the ionization of the broadest range of compounds but showed an important selectivity for some groups and were sensitive to the use of modifiers [89]. This paper showed some possible limitations in use of LC–API-MS in systematic screening. Verweij and Lipman [90] have compared TSP, ESI and APCI in determination of methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDE). The lowest detection limits were obtained for TSP. APCI appeared ca. 20–30-times more sensitive than PBI, both in positive and negative ionization mode, for determination of a priority pesticides in environmental water [91].

Two general conclusions may be drawn from these studies: both API techniques are superior to all others, and APCI due to its active ionization mode is more suitable for less polar compounds than ESI.

2.2.1. Identification systems with TSP

LC–MS-TSP procedure was developed for screening for ten corticosteroids in urine and applied in doping control. The LOD ranged from 10–50 ng/ml in the full scan mode and 1–5 ng/ml in their selected ion monitoring (SIM) mode [92]. More recently, Tatsuno et al. [93] applied LC–MS-TSP method for simultaneous determination of amphetamines, methamphetamine, ephedrine, methylephedrine, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), morphine, 6-acetylmorphine, cocaine and benzoylecgonine (BE) in urine after solid-phase extraction (SPE). The LODs ranged from 2 to 40 ng/ml in SIM mode and 50 to 400 ng/ml in full scan mode. The same group applied LC–MS-TSP for identification and quantification of bromvalerylurea in the bone marrow taken from completely skeletonized body [94]. Verweij et al. [95] developed an identification system for various sedative/hypnotic drugs (benzodiazepines, thioxanthenes, butyrophenones and methadone derivatives) in blood, using LC–MS–MS-TSP. The LODs were in the range 0.05–0.5 ng/ml.

2.2.2. Identification systems using API source (ESI or APCI)

Bogusz et al. [96] applied LC–MS-APCI and HPLC–DAD for determination of 14 amphetamines as phenylisothiocyanate derivatives. LC–ESI-MS, LC–ESI-MS–MS and LC–APCI-MS were applied for detection and identification of degradation products of organophosphorus warfare agents (nerve agents). ESI was one order of magnitude more sensitive that APCI. A rapid screening procedure for the analysis of aqueous samples was developed [97]. An automated LC–ESI-MS was developed for characterization of compound libraries. Compounds falling below a given threshold level of purity were

subjected to on-line preparative LC–MS. Real-time spectrometric ion signals were used to trigger fraction collection according to given masses [98]. The LC–UV–NMR–MS-ESI system, defined as ''triple hyphenated'' was applied for identification of various glucuronated and sulfated metabolites of paracetamol. Also, a number of endogenous metabolites was analyzed, which was previously impossible using only a ''single hyphenation'' [99]. A combined LC–UV–NMR–MS (ESI) system was developed for structure elucidation of compounds. The system was tested using a mixture of commercially available peptides and enable to obtain UV, NMR and MS data in a complex mixture within a single LC run [100]. The same research group used the combination of LC–UV–NMR–MS for the identification of paracetamol metabolites in human urine [101].

It must be stressed, that all identification systems cited above were developed for relatively small groups of usually related substances. None of these systems can be compared with the available databases for LC–DAD or GC–MS-EI techniques. Therefore, the LC–MS methods up to now were not applied for general toxicological screening (''general unknown'' analysis).

3. Use of hyphenated liquid chromatographic techniques for dedicated toxicological analyses

3.1. Drugs of abuse

LC–MS procedures, which encompassed several groups of drugs of abuse, were relatively scarce. Miller et al. [102] applied LC-ESI for drug screening (cocaine, BE, codeine, morphine and 6-MAM) in methanolic hair extracts. One hundred and fifteen hair samples were taken during autopsy from homicide, suicide or accident victims. Positive results for cocaine and opiates were recorded in 65% of cases. A method for isolation and determination of opiate agonists (morphine, M3G, M6G, codeine, C6G, methadone, dihydrocodeine, dihydromorphine, buprenorphine, tramadol, ibogaine), cocaine and its metabolites (BE, ecgonine methyl ester, EME), and lysergic acid diethylamide (LSD) in body fluids based on SPE and LC-APCI (SIM) was described by Bogusz et al. [103]. The method was applied in routine forensic casework.

Weinmann and Svoboda [104] demonstrated the usefulness of flow injection ESI-MS–MS (without chromatographic separation) for detection and quantification of several opiates, cocaine, BE, EME and methamphetamine.

Hyphenated LC–MS procedures concerning defined groups or some individual drugs of abuse were more often published in last years. Table 4 summarizes the most important data from these studies.

3.1.1. Opioids

Among the studies devoted to determination of opiate agonists almost exclusively the LC–API-MS methods has been found. One exception is a paper by Polettini et al. [105], who determined heroin, morphine, M3G, M6G, 6-acetylmorphine, codeine and acetylcodeine in blood and urine by LC–TSP-MS–MS. In the first application of LC–APCI-MS for opiate analysis, the urine samples were extracted with Sep Pak C_{18} cartridges and subjected to analysis on M3G and morphine in SIM and full scan mode [106].

Pacifici et al. [107] applied ESI for determination of morphine, M3G and M6G in serum after SPE with C_2 cartridges. Naltrexone and codeine were used as internal standards. In the study of Tyrefors et al. [108] morphine, M3G and M6G were extracted from serum using C_{18} SPE cartridges and subjected to HPLC (gradient elution) with ESI detection. External standardization was applied, which according to the authors assured better accuracy and precision. The effects of mobile phase composition on the signal intensity was studied. Heroin and its potential metabolites: morphine, M3G, M6G, codeine and MAM were determined after experimental administration of heroin in mice. Nalorphine was used as internal standard. Serum samples were extracted with C_2 SPE cartridges and subjected to LC–MS (ESI) examination in SIM mode [109]. Morphine, M3G, M6G, 6-MAM were determined in autopsy blood, urine, cerebrospinbal fluid and vitreous humor taken from 21 heroin victims. Body fluids were extracted with C_{18} SPE cartridges and morphine-d_3 were used as internal standard. APCI-

Table 4
Drugs of abuse analyzed by LC–MS

Drug	Source	Matrix	LOD[a]	Ref.
Cocaine, BE, EME, E, ME	APCI	Urine	1–10 ng	[116]
BE	APCI	Blood stains	2	[117]
Cocaine, BE, cocaethylene	ESI	Hair	25 ng/g	[118]
MDMA, MDA, MDE	TSP, ESI, APCI	Pure drugs	0.1–1 ng	[90]
13 Phenethylamines	APCI	Blood, urine	1–5	[97]
LSD	ESI	Urine	0.5	[121]
LSD	ESI	Urine	0.05	[123]
M3G, Morphine	APCI	Urine	1–3	[105]
M3G, M6G, Morphine	ESI	Serum	10–100	[107]
M3G, M6G, Morphine	ESI	Serum	0.8–5	[108]
M3G, M6G, Morphine, heroin, codeine	ESI	Serum	0.5–4	[109]
M3G, M6G, Morphine, MAM	APCI	Biological fluids	0.1–1	[110]
M3G, M6G, Morphine, MAM, codeine, C6G	APCI	Biological fluids	0.5–100	[111]
M3G, M6G, Morphine, MAM, codeine, C6G, DHC, DHM, Buprenorphine, cocaine, BE, EME, ibogaine, LSD	APCI	Biological fluids	0.1–100	[103]
Buprenorphine	ESI	Blood	0.1	[112]
Buprenorphine	ESI	Blood	0.05–0.1	[113]
Methadone, EDDP	ESI	Hair	0.1–0.2 ng/mg	[115]
Cannabinoids	PBI	Cannabis plant	200–1060	[120]
Cannabinoids	APCI	Cannabis plant	0.55–2.1 ng	[119]
Nicotine	APCI	Serum	50	[125]

[a] ng/ml When not otherwise stated.

MS (SIM) was applied [110]. In the next paper of this group, morphine, M3G, M6G, codeine, C6G and 6-MAM were determined in body fluids after SPE by APCI-MS (SIM) in flow-rate gradient. Deuterated analogs were used as internal standards for each substance involved [111] (Fig. 13).

Two studies were devoted to buprenorphine and norbuprenorphine determination with ESI-MS in biological fluids and hair samples [112,113]. Liquid–liquid extraction procedures were used (Fig. 14). An LC–ESI-MS–MS method for determination of buprenorphine in plasma showed higher sensitivity than GC–MS assay (the LOQ was 0.1 ng/ml against the 0.5 ng/ml). The CID of buprenorphine molecule was studied [114]. *R*- and *S*-enantiomers of methadone and its metabolite (EDDP) were determined in human hair samples by ESI. The predominance of the biologically active *R*-form was observed. The method was as sensitive as GC–MS [115].

3.1.2. Cocaine

Cocaine and its four metabolites (BE, EME, ecgonine and norcocaine) were extracted from urine using SPE cartridges and determined by LC–APCI-MS in SIM mode [116]. Sosnoff et al. [117] applied LC–APCI-MS–MS for determination of BE in dried blood spots. The method was applied in epidemiological screening study involving newborns as confirmatory analysis after immunoassays. Cocaine, BE and cocaethylene were extracted from human hair samples taken from dead drug addicts and subjected to HPLC separation. Column eluent was split and analyzed by ESI-MS–MS and fluorescence detection [118].

3.1.3. Cannabinoids

Two published studies dealt with determination of cannabinoids [tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN)] in cannabis preparations such as hashish or marijuana. Backström et al. determined cannabinoids by supercritical fluid chromatography coupled with APCI-MS. The method was superior to GC–MS and HPLC–UV [119] (Fig. 15). Hashish constituents (THC, CBD, CBN) were determined by LC–PBI-MS. The LODs ranged from 200 to 1060 ng/ml [120]. To our knowledge, no papers were published

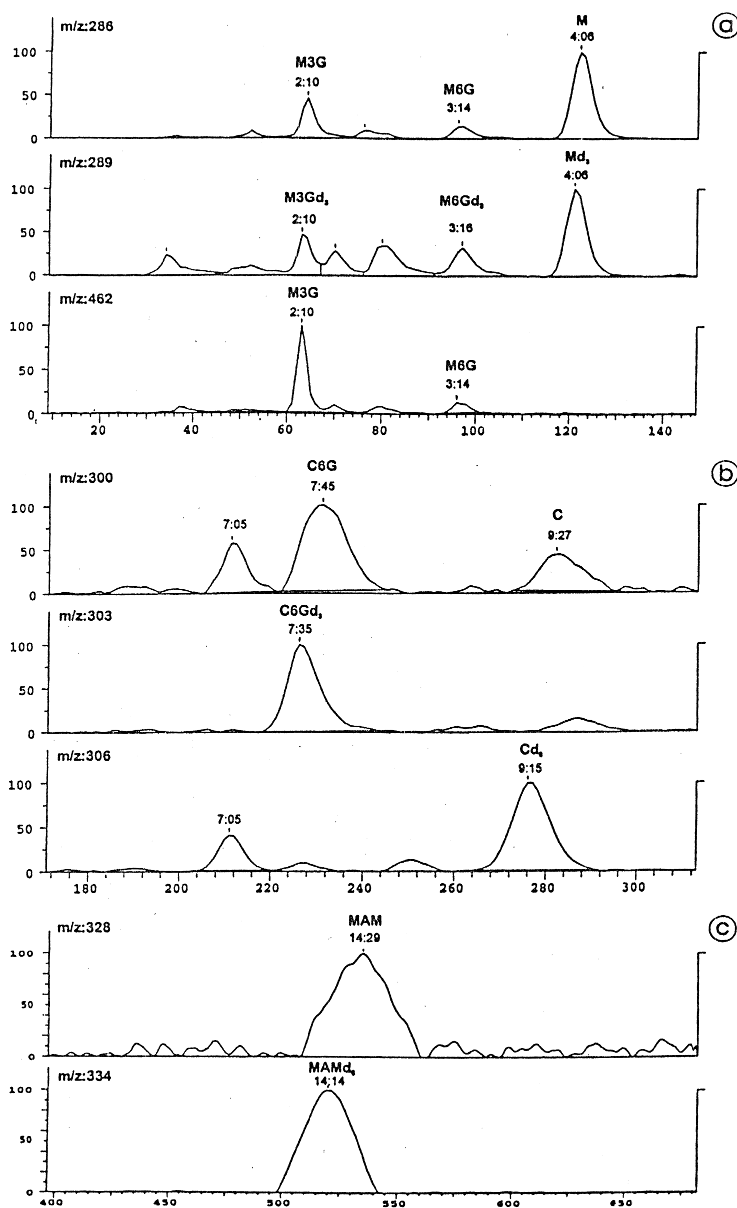

Fig. 13. LC–APCI-MS Chromatograms of serum extract spiked with morphine (20 ng/ml), M3G, M6G and codeine (100 ng/ml), C6G (200 ng/ml) and 6-MAM (5 ng/ml). From Ref. [111] with permission from Elsevier Science.

devoted to analysis of cannabinoids in biological fluids.

3.1.4. Amphetamines

Amphetamines (amphetamine, methamphetamine, MDMA, MDE, MDA and others) were subjected to LC–MS investigations with TSP, ESI and APCI sources [90,96].

3.1.5. LSD

A novel immunoassay of LSD in urine was developed and the confirmation analysis was done by

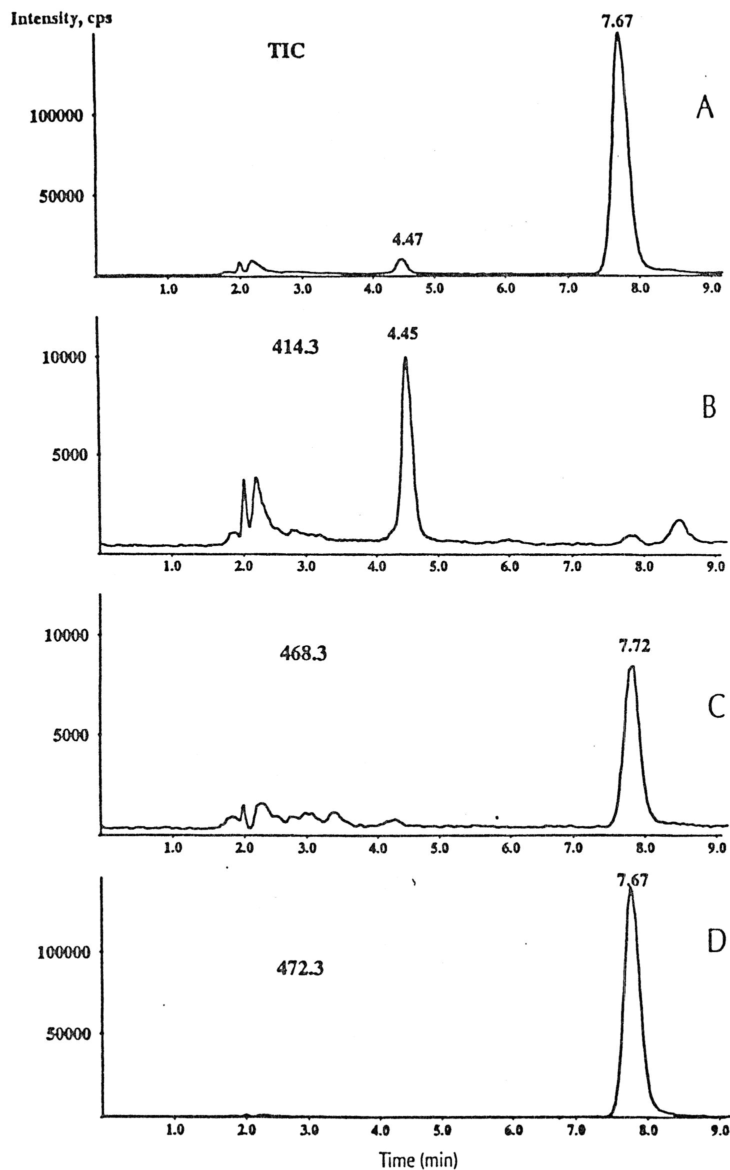

Fig. 14. LC–ESI-MS Chromatograms of whole blood extract containing 3.0 ng/ml buprenorphine (*m/z* 468.3), 3.1 ng/ml norbuprenorphine (*m/z* 414.3) and buprenorphine-d_4 (*m/z* 472.3). From Ref. [112] with permission of Preston Publications and the authors.

ESI [121]. The same research group determined LSD in urine after SPE by ESI with methysergide as internal standard. LSD-d_3 was rejected because of the common *m/z* 281 ion with the parent drug. *N*-Demethyl-LSD was also identified in real sample [122] (Fig. 16) Hoja et al. [123] determined LSD and *N*-demethyl-LSD in urine by means of ESI after Extrelut extraction. The LODs were 0.05 and 0.1 ng/ml for LSD and *N*-demethyl-LSD, respectively. Metabolism of LSD in human liver microsomes was

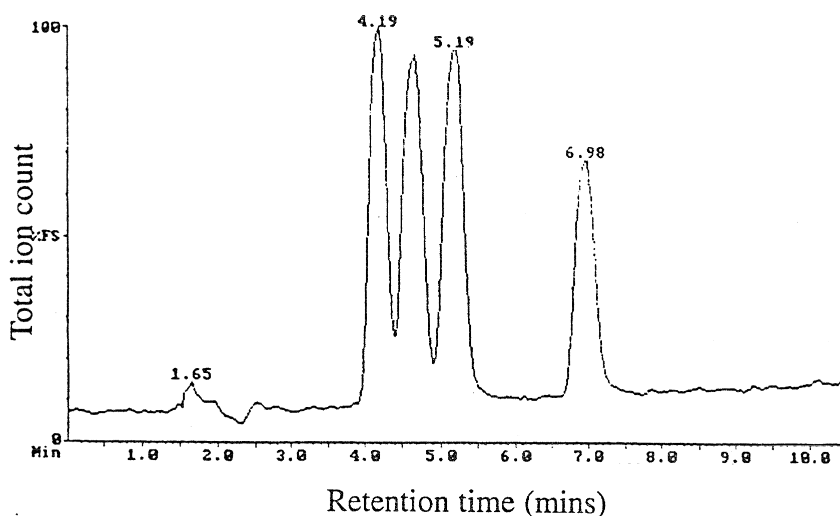

Fig. 15. Separation of cannabinoids by SFC–APCI-MS. Elution order: cannabidiol, D8-THC, D9-THC, cannabinol. From Ref. [119] with permission of the Forensic Science Society and the authors.

investigated using LC–MS–MS (ESI) and CE–MS–MS (ESI). Sixteen metabolites were identified, two of them for the first time [124].

3.1.6. Nicotine

A method for determination of nicotine in serum of smokers and nonsmokers by LC–APCI-MS with the daily output of 100 samples was reported [125].

3.2. Doping agents and related compounds

Several contributions were devoted to determination of steroid compounds. In an early paper of Sandra et al. [7] HPLC with dual channel detection system consisting of DAD and PBI was applied to the analysis of testosterone esters. The comparison of ESI and APCI for assay of methandronostenolone and its metabolites in equine urine demonstrated that APCI was better for neutral compounds and ESI for sulfate metabolites [85]. Park et al. [126] developed an LC–MS-TSP screening system for 10 corticosteroids in urine. The LOD ranged from 10–50 ng/ml in the full scan mode and 1–5 ng/ml in their SIM mode. Steroid sulfates and glucuronides were determined in urine with ESI-MS–MS. The LOD of 20 pg on-column was achieved [127]. On-line coupled immunoaffinity chromatography–reversed-phase (RP) HPLC with PBI and quadrupole ion trap was applied for determination of corticosteroids (dexamethasone and flumethasone) in equine urine. The LODs were 3–4 ng/ml [128].

The advent of β_2-agonists as stimulating and anabolizing agents in sports was associated with development of various LC–MS detection methods, which have recently been reviewed by Polettini [129]. Five drugs of this group were determined with GC–MS, TSP and ESI. Both GC–MS and ESI gave 50-fold lower LOD than TSP [86]. Five β-agonists (fenoterol, metaprotorenol, terbutaline, sambutamol and clenbuterol) were extracted from human plasma and determined by APCI-SIM. LODs at low ng/ml levels were reported [130].

Reserpine was determined in equine plasma by LC–ESI-MS–MS after SPE, with a LOD of 0.01 ng/ml [131].

3.3. Therapeutic drugs of toxicological relevance

Table 5 shows selected papers dealing with application of hyphenated liquid chromatographic techniques for therapeutic drug analysis. Usually, API-MS techniques were used. The exception is the

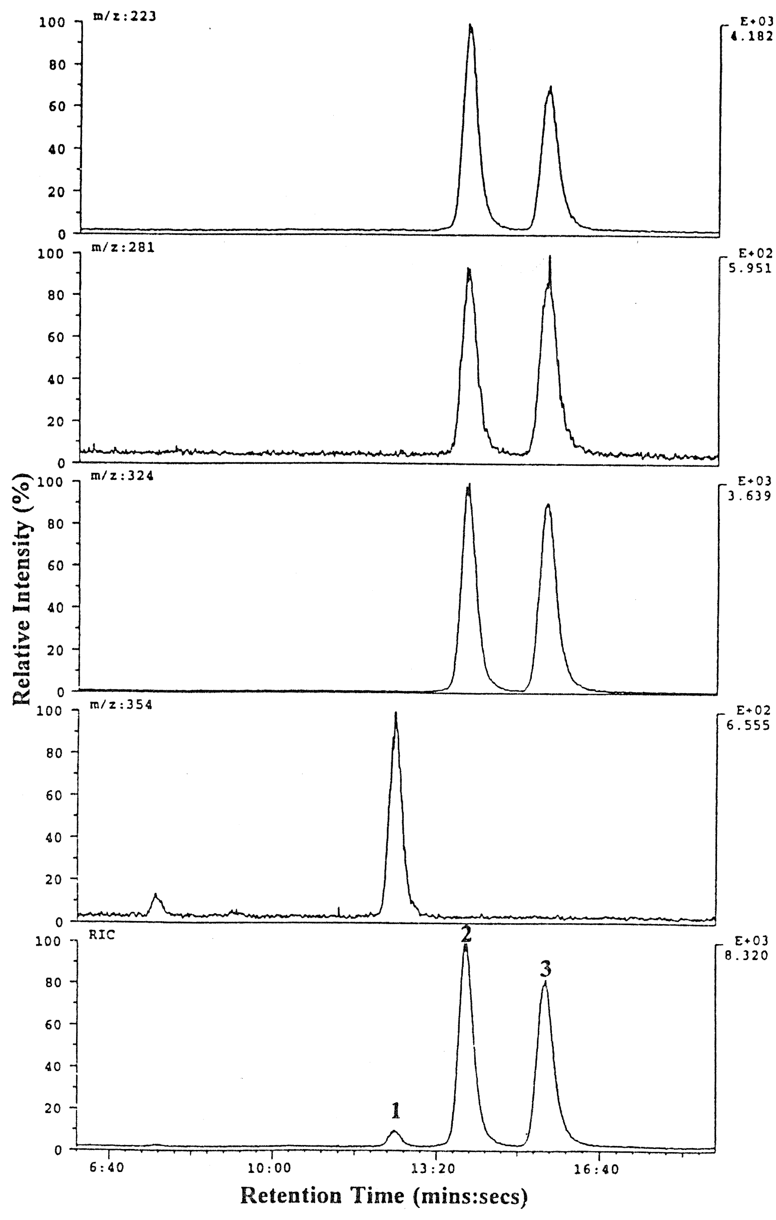

Fig. 16. LC–ESI-MS chromatograms of LSD, LAMPA (*m/z* 329, 281, 223) and methysergid (*m/z* 352). From Ref. [122] with permission from Elsevier Science and LGC (Teddington) Ltd.

publication of Verweij et al. [95], who applied HPLC–TSP-MS–MS for determination of different psychotropic drugs (benzodiazepines, thioxanthenes, butyrophenones, diphenylbutylpiperidines and methadone). The LODs ranged from 0.05 to 0.5 ng/ml of whole blood for most drugs.

Several platinum anticancer drugs were determined by HPLC-ESI in plasma ultrafiltrates.

Table 5
Therapeutic drugs of forensic relevance analyzed by LC–MS

Drug	Source	Matrix	LOD (ng/ml)	Ref.
10 Benzodiazepines	TSP	Blood	0.05–1	[95]
4 Butyrophenones	TSP	Blood	0.05–0.5	[95]
4 Thioxanthenes	TSP	Blood	0.5–50	[95]
Organic Pt-compounds	ESI	Plasma	3	[132]
Ranolazine and 11 metabolites	APCI	Plasma		[133]
Amlodipine	APCI	Serum	0.014	[134]
Cardiac glycosides	ESI	Blood	0.15–0.6	[135]
Flunitrazepam (F), 7-AF, N-DF, 3-OH-F	APCI	Serum, urine	0.2–1	[138]

$(M+K)^+$ and $(M+Na)^+$ adducts were identified as main ions [132]. Tracqui et al. [135] developed a HPLC–ESI-MS method for determination of cardiac glycosides: digoxin, digitoxin, lanatoside C and acetyldigitoxin. The method was less sensitive but more specific than radioimmunoassay. The enantioselective determination of several chiral drugs (verapamil, norverapamil, oxybutynin, sotalol, doxazosin) by APCI-MS–MS was described. The method combined the convenience of normal-phase chiral separation with high specificity and sensitivity of APCI [136]. The same group developed enantioselective determination of α_1-adrenoreceptor antagonist terazosin by ESI in human plasma after a 5 mg single oral dose [137]. Flunitrazepam and its polar metabolites (7-aminoflunitrazepam, *N*-desmethylflunitrazepam and 3-OH-flunitrazepam) were extracted from serum and urine with SPE cartridges and determined by APCI [138].

ESI-MS–MS was used for determination of glucuronides of nitrocatechol derivatives, acting as catechol-*O*-methyltransferase inhibitors and used as antiparkinson drugs. Negative chemical ionization (NCI) was applied, and deprotonated molecular ion was chosen as precursor ion. CID resulted in the loss of glucuronide moiety and in the appearance on negatively charged drug molecule. This behavior seems to be typical for all glucuronated drug metabolites [139]. The bench-top ESI-ion trap MS was applied for the structural characterization of glyburide metabolites, which were obtained in vitro during incubation with liver microsomes of various origin [140]. APCI-MS–MS was used for examination of a new antipsychotic agents 2-amino-*N*-{4-[4-(1,2-benzisothiazol-3-yl)-1-piperazinyl]butyl}-benzamide, both for quantification and identification of metabolites in human urine and plasma. Plasma samples were extracted with SPE, urine was only filtered prior to direct analysis [141].

3.4. Environmental poisons

In an early paper Kawasaki et al. [142] published a rapid LC–APCI-MS method for simultaneous analysis of methylcarbamate pesticides in serum in poisoning cases. Some problems concerned with the production of cluster ions originating from the mobile phase solvent were noticed. Most papers were devoted to determination of polar pesticides, like organophosphates [97,143], imidazolinone herbicides [144], phenylurea herbicides [145,146] in water samples. Two papers, mentioned already in the Section 2.2, were devoted to comparison of various LC–MS methods in pesticide analysis [86,91]. Table 6 presents selected LC–MS method applied for environmental xenobiotics and natural compounds.

3.5. Natural toxins

In the early paper of Arai et al. 42 compounds, occurring in traditional Japanese plant medicines, were analyzed by HPLC with DAD and MS [148]. The kind of LC–MS interface was not mentioned. UV spectra and retention data were given. Sixteen alkaloids from *Aconitum japonicum* were simultaneously determined with LC–APCI-MS [149]. ESI-MS was applied for elucidation of structure of 18 taxanes obtained from *Taxus* extracts [150]. Fifty

Table 6
Environmental poisons and natural compounds analyzed by LC–MS techniques

Drug	Source	Matrix	LOD (ng/ml)	Ref.
Organophosphates	ESI, APCI	Water	<50	[97]
8 Carbamates	APCI	Serum	12–60	[142]
10 Organophosphates	ESI	Water	0.01–0.2	[143]
6 Herbicides	ESI	Water	1	[144]
12 Pesticides	APCI	Water	1–5	[145]
Herbicides	ESI	Water	10	[146]
14 Herbicides	APCI	Water	0.1–1	[147]
Aconitum-alkaloids	APCI	Plant		[149]
Colchicine	ESI	Blood, urine	0.6	[152]
α,β-Amanitins	ESI	Urine	10	[153]

kinds of spider venoms were analyzed by HPLC–FAB-MS [151]. Tracqui et al. determined alkaloid colchicine in blood, plasma and urine by HPLC-ESI, with a LOD of 0.6 ng/ml [152]. α- and β-amanitin were extracted from urine samples in the cases of *Amanita* poisoning and analyzed with ESI. The LOD was 10 ng/ml urine [153].

4. Perspectives and expectations for the future

In order to minimize the errors in a risky task of prophecy, it may be reasonable to summarize the present situation concerning the application of hyphenated liquid chromatographic techniques in forensic toxicology. As a criterium of broad application may serve the abandonment of the denotation ‘‘hyphenated’’. This is observed for two kind of techniques:

(1) HPLC–DAD methods have found broad application as simple and reliable tools of toxicological screening. These methods seem to be less useful for dedicated purposes, due to rather limited sensitivity. Since HPLC–DAD has been very intensively used in toxicology over the last two decades, it is hardly possible to expect major technical improvements in this technique. More attractive is the parallel coupling LC–DAD–API-MS for simultaneous examination of retention, UV and MS properties of substances. This ‘‘double-hyphenated’’ instruments may be welcome among toxicologists. During the last 46th ASMS Conference, Fitzgerald et al. presented a poster showing successful coupling of automatic HPLC–DAD analyzer (REMEDi) with ion trap LC–ESI-MS instrument [154]. It seems therefore, that this combination will be introduced in the near future.

(2) HPLC–API-MS methods, both in APCI and ESI options, have became first choice in examination of polar substances (drugs and metabolites) and are gradually replacing GC–MS procedures also for non-polar drugs. In contrast to HPLC–DAD, there is still a large room of improvement for LC–API-MS techniques. There are several unexplored fields, concerning particularly intra- and inter-laboratory reproducibility of mass spectra, and systematic studies in this area must be undertaken. The results of these studies may be of decisive importance for the future of LC–API-MS techniques as identification tools in toxicology. First successful application of LC–ESI-MS in ‘‘general unknown’’ toxicological analysis was presented recently by Marquet et al., who established a library of more than 1000 toxicologically relevant compounds [155].

The recent review of Smits [156], concerning the future of hyphenated techniques in industrial laboratory, indicated the need of sophisticated couplings, like LC–NMR–MS, LC–DAD–MS, and introduction of low-cost, bench-top LC–, GC– and CE–MS instruments. The same trend may be expected in forensic toxicology. Also, the on-line combinations of all analytical steps (isolation, separation, detection, identification, quantification), together with development of commercially available LC–DAD–API-MS instruments, will be probably just a matter of time. An interesting, although very futuristic approach has recently been formulated by Thomson [157], who has observed that most molecules of

interest are ''happy'' in the liquid phase. Therefore, instead of forcing the ions to leave their natural environment, instruments should be developed which would able to measure the ions in solution.

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