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Rapid identification and quantitation of compounds with forensic interest using fast liquid chromatography–ion trap mass spectrometry and library searching

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Abstract

A fast liquid chromatography–electrospray tandem mass spectrometric (LC–ESI-MS–MS) method by using a monolithic column, gradient elution and ion trap mass spectrometer was developed for 14 forensically interesting and chemically different compounds. All compounds were eluted within 2.5 min and the total analysis time was 5 min including stabilisation time required for the next injection. All the compounds, basics, neutrals and acids were efficiently ionised by positive ion ESI. A laboratory library including MS–MS spectra and retention times was developed and tested. Results with 476 standard samples and 50 authentic samples showed that the compounds studied can be unambiguously identified with the library. A quantitative method was developed for the compounds using external calibration. The evaluation process showed good linearity of the method and reasonable repeatability. Limits of detection ranged from 10.0 to 50.0 ng/ml.

Keywords: Forensic analysis; Monolithic columns; Gradient elution; Libraries, MS; Opiates; Benzodiazepines; Lysergic acid diethylamide; Barbiturates

1. Introduction

Samples investigated in a forensic laboratory are commonly prescription drugs with licit origin or illegal cocktails of licit and/or illicit drugs seized by local authorities. Matrices can be various: tablets, capsules and powders, and blotter paper on which the drug is impregnated. Concentrations of the drugs vary from relatively low concentrations to nearly pure synthesis products. However, from the analytical point of view, concentrations of the drugs in forensic samples are high and sensitivity of the method is seldom the problem. On the other hand, physical and chemical properties, such as volatility, polarity, and stability of the drugs vary a lot and the number of the samples investigated in forensic laboratories is continuously increasing. For these reasons, fast and reliable analytical methods capable to identify all the drugs with minimal number of runs are required. For the reasons of law-enforcement

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semiquantitative data rather than quantitative data with high precision is sufficient.

Many analytical techniques have been used in forensic laboratories. The most commonly used methods are thin-layer chromatography (TLC), liquid chromatography (LC) with UV, fluorescence, electrochemical and mass spectrometric (MS) detection or gas chromatography (GC) with flame ionisation (FID), electron-capture (ECD), nitrogen–phosphorus (NPD), and MS detection as described in the handbooks of the field [1]. Among these detection methods, MS is superior over the others due to its extremely good sensitivity and specificity. Commonly used GC–MS is successfully applied for volatile and stable compounds such as cannabinoids [2], and also after derivatisation to compounds, such as opiates, benzodiazepines, lysergic acid diethylamide (LSD), and barbiturates [3]. However, non-volatile, ionic and unstable compounds are difficult and often impossible to analyse with GC–MS. Therefore, LC–MS has gained an increasing interest during the last years, especially after the introduction of electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) [4–6].

LC–MS has been used in forensic analyses, mainly in toxicological studies [7–9] and, e.g. in analysing explosives [10]. Of the atmospheric ionisation methods, ESI has been more popular than APCI. This is because ESI provides very soft ionisation and is suited for thermally labile compounds, which may degrade in APCI. On the other hand, APCI provides ionisation of neutral and relatively non-polar compounds, which might be difficult to ionise with ESI. Due to the high selectivity of MS and especially tandem mass spectrometry (MS–MS), the complete chromatographic separation is not a necessity and the analysis times can be shortened and thus higher throughput of samples is accomplished.

The columns used most often in the analysis of forensic samples with LC–MS are reversed-phase C_8 or C_{18} columns with normal dimensions. With these columns, the run times are long, often more than 10 min. By using shorter RP columns (length $<$50 mm) [11], turbulent LC [12] or a recently introduced monolithic column, the analysis time can be drastically shortened [13–16]. For identification of unknown compounds, several laboratory reference spectra libraries have been developed based on atmospheric pressure ionisation techniques and in-source collision induced dissociation (CID) MS [17–20], MS–MS [21], or high-resolution MS spectra [22,23].

The aim of this study was to develop a fast screening, identification and quantitation method for 14 forensically interesting illicit and therapeutic drugs (Fig. 1) [24], commonly found in forensic samples, using HPLC with a monolithic column and ion trap mass spectrometry. Physical and chemical properties of the compounds differ significantly from each other providing a good series of compounds to test the suitability of LC–ESI–MS for simultaneous analysis of several illicit drugs in forensic samples. A laboratory library based on MS–MS spectra and retention times was developed and tested in the identification of the compounds studied.

2. Experimental

2.1. Chemicals and sample solutions

Methanol, HPLC grade, was purchased from Rathburn (Walkerburn, UK) and formic acid, analytical grade, from Merck (Darmstadt, Germany). Water was purified with a Milli-Q purifying system (Millipore, Bedford, MA, USA).

Buprenorphine and LSD (USP, Rickville, MO, USA), stanozolol, amphetamine, salbutamol and clenbuterol (Sigma, St. Louis, MO, USA), nandrolone, metandienone (Steraloids, Newport, RI, USA), testosterone (Fluka, Buchs, Switzerland), 3,4-methylenedioxymethamphetamine (3,4-MDMA) (RBI, Natick, MA, USA), morphine (University's Pharmacy, Helsinki, Finland), phenobarbital, temazepam (Radian International, Austin, TX, USA) and psilocybine (Lipomed, Arlesheim, Switzerland) were purchased as pure reference materials.

The standards were first dissolved in methanol (1.0 mg/ml, stock solution). The dilutions for HPLC analysis were done with 0.1% formic acid. For infusion experiments, the stock solution was diluted with methanol:water:formic acid (49.95:49.95:0.1, v/v, pH 2.66) to the final concentration of 10 to 50 μg/ml, depending on the compound. All solutions were stored at −20 °C.

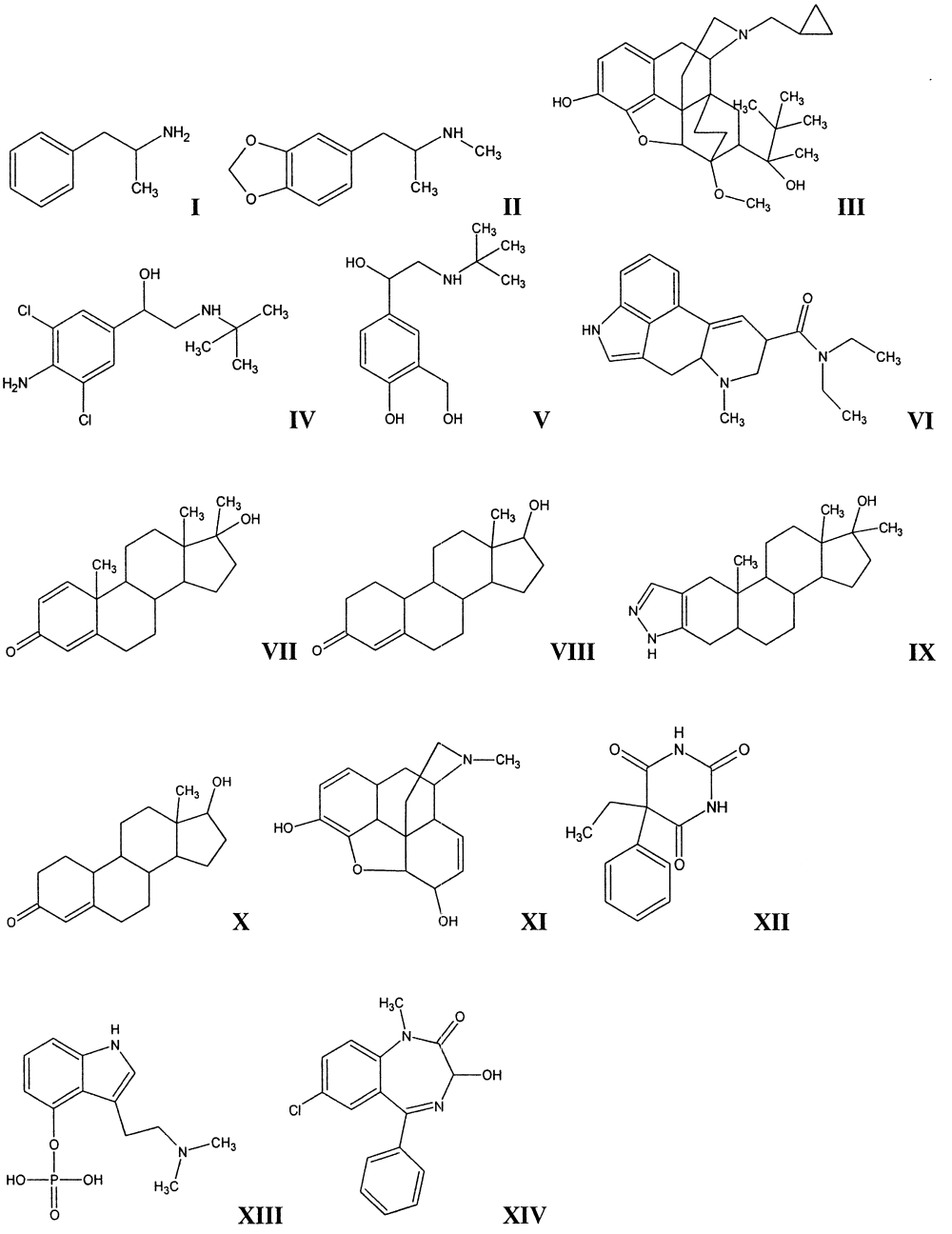

Fig. 1. Structures of the compounds studied. (I) = amphetamine, (II) = 3,4-MDMA, (III) = buprenorphine, (IV) = clenbuterol, (V) = salbutamol, (VI) = LSD, (VII) = metandienone, (VIII) = nandrolone, (IX) = stanozolol, (X) = testosterone, (XI) = morphine, (XII) = phenobarbital, (XIII) = psilocybine, and (XIV) = temazepam.

2.2. Instrumentation

The LC–MS system consisted of an Agilent 1100 Series HPLC system with an autosampler and an Agilent 1100 Series LC/MSD trap ion trap mass spectrometer (Bremen, Germany). The column

eluent was split 1:10, using an Accurate splitter (LC Packings, San Fransisco, CA, USA).

Eluent A was 0.1% formic acid with 5% (v/v) methanol and eluent B methanol with 5% water and 0.1% formic acid in gradient runs. The eluents were degassed with vacuum before and during use. The gradient used was 5–50% B in 0–0.5 min and 50–100% B in 0.5–2.5 min. The column was 50× 4.6 mm endcapped C_{18} reversed-phase Speedrod by Chromolith (Merck) and its temperature was 30 °C. The injection volume was 50 μl. The flow-rate was 2.5 ml/min, which was split 1:10 after column; 250 μl/min entering the mass spectrometer. For infusion experiments 100 μl Hamilton syringe (Bonaduz, Switzerland) and a micro pump from KDScientific, Model 100 (Boston, MA, USA) with the flow-rate of 4 μl/min was used in optimising the MS–MS parameters and for creating the library spectra.

The ionisation technique used was ESI operated in the positive ion mode. Operation parameters of the ESI ion source were as follows: drying gas temperature was 350 °C, drying gas flow 9.0 l/min, nebuliser gas pressure 241 kPa (35 p.s.i.), end plate voltage −3500 V, and end plate offset −500 V. Ion trap parameters were as follows: accumulation time was 10 ms, averages 1, rolling averaging off and ion charge control on. Nitrogen was used as drying and nebulising gas and was produced by Whatman (Haverhill, MA, USA) model 75–72 nitrogen generator. Helium (4.6, 99.996%) was used in the trap as damping and collision gas.

The autotune fragmentation amplitude in MS–MS mode was set to 1.0. The ion optics parameters were optimised for target ions. The time segments and corresponding target ions, scan range and ion optics parameters were: 0–0.40 min *no* target ions, 0.40–0.80 min *m/z* 240, 285, 286, scan *m/z* 50–300, skim1 21.5 V, capillary exit 90.0 V, 0.80–1.12 min *m/z* 136, 194, 240, 277, scan *m/z* 50–300, skim1 21.1 V, capillary exit 89.5 V, 1.12–1.65 min *m/z* 194, 233, 277, 324, 468, scan *m/z* 50–500, skim1 22.1 V, capillary exit 90.8 V, 1.65–2.5 min *m/z* 275, 289, 301, 329, scan *m/z* 50–340, skim1 22.1 V, capillary exit 90.8 V.

3. Results and discussion

The 14 compounds including acids, bases and neutrals, stable and labile compounds, as well as compounds with same nominal masses (Fig. 1) provide good heterogeneous series to test the suitability of LC–ESI–MS in forensic analysis. ESI was chosen instead of APCI, since ESI provides a gentler ionisation process than APCI allowing analysis of labile compounds. Positive ion mode was chosen, since it has been shown in earlier studies that with suitable mobile phase composition, it is possible to ionise efficiently, in addition to bases, neutrals such as steroids and acids such as flavonoids [25] and some pesticides [26].

All the compounds studied, basic as well as the neutral steroids and acidic phenobarbital, were efficiently ionised with the chosen eluent system. All spectra showed an abundant $[M+H]^+$, which was chosen for precursor ion for MS–MS analysis. In addition, the spectra of the neutral compounds, i.e. testosterone, nandrolone and metandienone, showed relatively intense sodium adduct ion, $[M+Na]^+$. Also a weak $[M+Na]^+$ ion was recorded for temazepam. Only the MS spectra of salbutamol, phenobarbital, amphetamine and 3,4-MDMA showed some fragment ions.

All the MS–MS spectra (Fig. 2) showed characteristic behaviour allowing unambiguous identification of the compounds. One characteristic product ion was chosen for quantitative analysis (Table 1). Fragmentations of amphetamine and 3,4-MDMA were similar than presented earlier [27]. Buprenorphine produced *m/z* 414 by loss of three membered ring $CH_2CHCH_2CH_2$ and *m/z* 396 by further loss of water [28]. Protonated clenbuterol fragmented by loss of water (*m/z* 259) followed by the loss of isobutene (*m/z* 203) as presented earlier [29,30]. Similarly, fragmentation of salbutamol occurs first by water loss (*m/z* 222) and further loss of isobutene produced ion *m/z* 166. The loss of water from *m/z* 166 produced ion *m/z* 148 [31]. The monitored product ion of LSD, *m/z* 223, was formed by loss of diethylamide and the loss of CH_3NCH_2 produced *m/z* 281 [32]. Fragmentation of the steroids studied [23,33–35] as well as morphine [36] produced the same main product ions than presented earlier. The monitored product ion *m/z* 177 of protonated phenobarbital is formed by loss of 56 mass units from heterocyclic ring by unknown mechanism. Psilocybine showed similar fragments as presented by Bogusz et al. [37]. After triple MS experiments

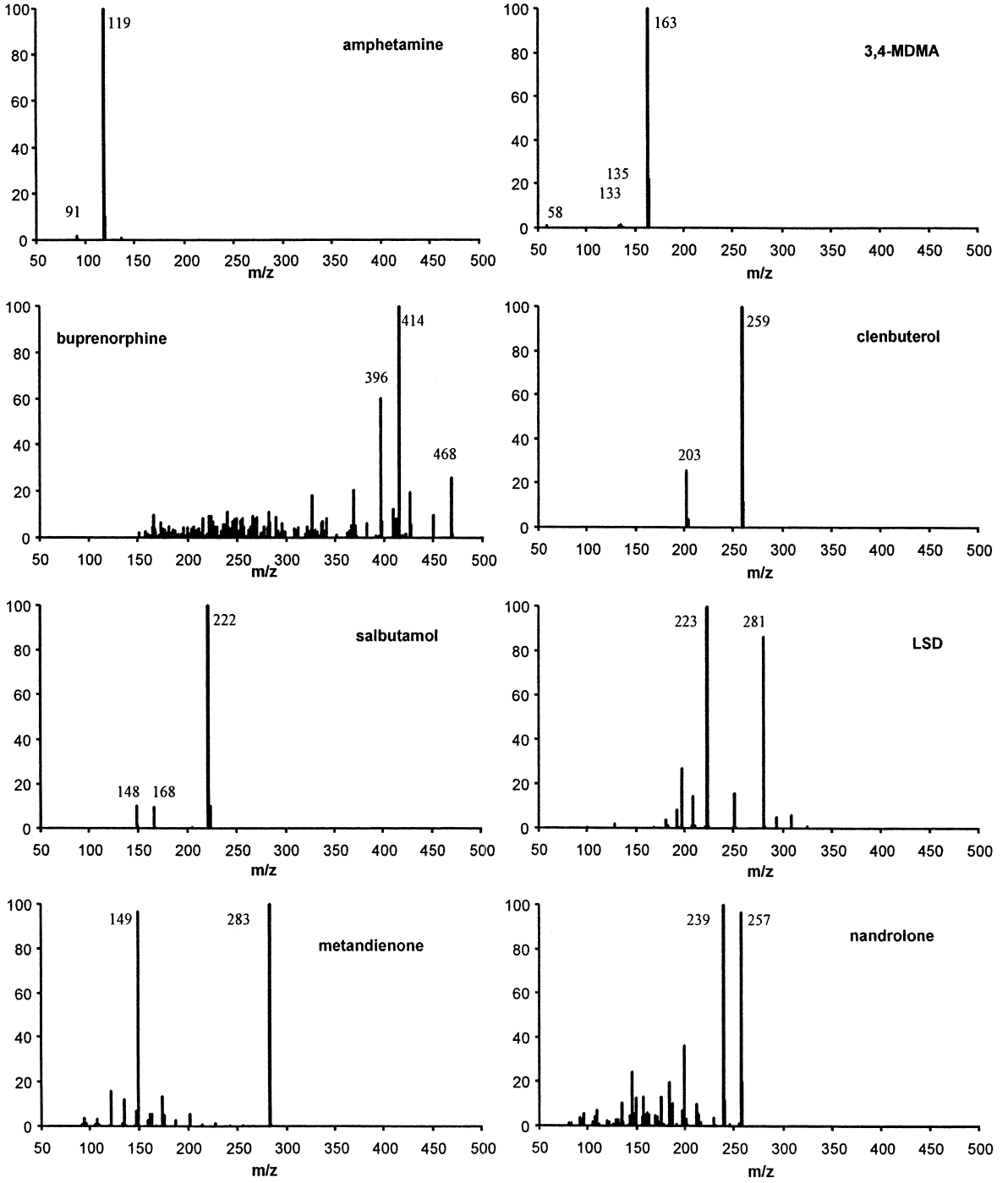

Fig. 2. The MS–MS spectra of amphetamine, 3,4-MDMA, buprenorphine, clenbuterol, salbutamol, LSD, metandienone, nandrolone, stanozolol, testosterone; morphine, phenobarbital, psilocybine, and temazepam. *y*-Axis: relative abundance.

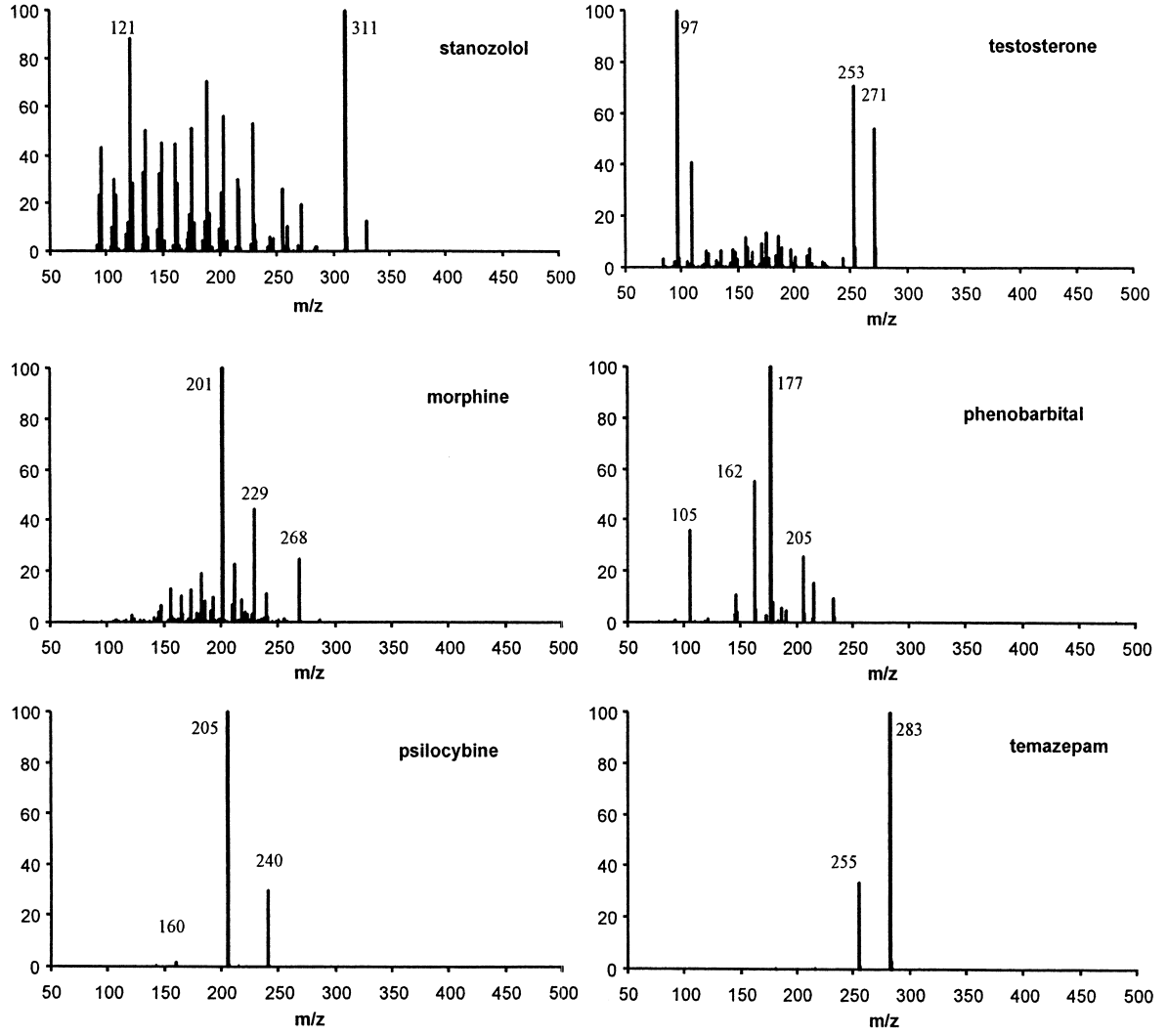

Fig. 2. (*continued*)

done with psilocybine the mechanism is proposed to be initial cleavage of diethylamine group (m/z 240) followed by water loss (m/z 222). The other pathway produced m/z 205 (psilocin) by dephosphorylation followed by cleavage of diethylamine group (m/z 160). Similarly to the earlier work, temazepam produced two abundant product ions m/z 283 and m/z 255 formed by loss of water and losses of water and carbon dioxide, respectively [38].

The use of monolithic column, gradient elution and MS–MS allowed the analysis of all the 14 compounds within 5 min including stabilisation time for the next injection. The gradient elution is necessary, since the polarity of the compounds varied from non-polar steroids to ionic psilocybine. The capacity factors k with the LC method used varied between 2.1 and 9.7 ($t_0 = 0.212$ min, calculated from $t_0 = V_m/F$) being acceptable (Table 1). This means that salts and other possible compounds eluting without retention are separated from the compounds studied and the suppression in ESI is minimised. The relative standard deviation of the retention times was 0.45–4.8% indicating good reproducibility of LC system (Table 1). Although many of the compounds

Table 1
The compounds, precursor ion (protonated molecule), precursor used in quantitation (MRM), fragmentation voltages, retention times with standard deviations (SD), retention and resolution parameters, linearities and within-day reproducibilities

Compound	MS		LC–MS					
	$[M+H]^+$ (MRM ion)	Fragment amplitude (library)[a]	t_r (min)	SD (t_r)	k	LOD (μg/ml)	r	RSD (%) ($n=15$)
Amphetamine	136 (119)	0.65	1.04	0.015	3.9	0.05	0.996	25.9
3,4-MDMA	194 (163)	0.7	1.07	0.015	4.1	0.03	0.995	11.2
Buprenorphine	468 (414)	1.0	1.39	0.018	5.5	0.032	0.998	20.1
Clenbuterol	277 (259)	0.65	1.15	0.012	4.5	0.03	0.998	13.4
Salbutamol	240 (222)	0.5	0.91	0.008	3.3	0.01	0.995	7.8
LSD	324 (223)	0.85	1.24	0.009	4.8	0.01	0.999	16.4
Methandienone	301 (149)	0.65	2.02	0.009	8.5	0.03	0.995	12.0
Nandrolone	275 (239)	0.7	1.98	0.009	8.3	0.012	0.993	10.0
Stanozolol	329 (121)	1.1	2.28	0.018	9.7	0.05	0.996	16.9
Testosterone	289 (97)	0.7	2.09	0.017	8.8	0.03	0.998	11.4
Morphine	286 (201)	0.85	0.68	0.027	2.1	0.05	0.999	14.4
Phenobarbital	233 (177)	0.65	1.37	0.020	5.4	0.05	0.997	28.8
Psilocybine	285 (205)	0.7	0.64	0.031	2.1	0.05	0.997	12.5
Temazepam	301 (255)	0.65	1.82	0.009	7.6	0.03	0.997	11.3

[a] Manually tuned amplitudes where the abundance of $[M+H]^+$ (precursor ion) is 10–15%.

are not fully separated, as shown in Fig. 3, the use of MS–MS allows unambiguous identification of the compounds.

Suitability of the library including the MS–MS spectra and retention times was tested in the identification of the compounds studied with 476 standard

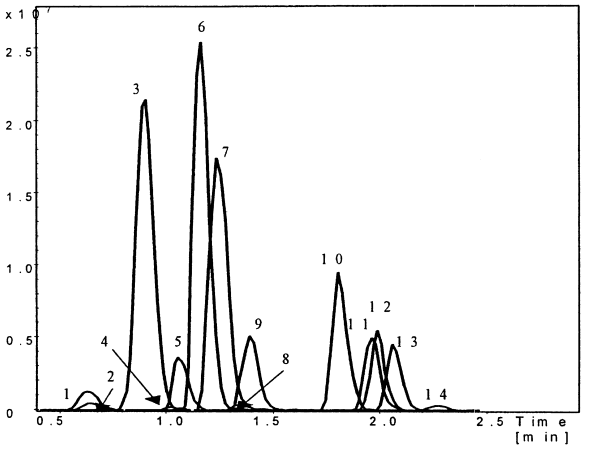

Fig. 3. One point smoothed extracted ion chromatograms of the target (MRM) ions. Compounds are: (1)=psilocybine, (2)=morphine, (3)=salbutamol, (4)=amphetamine, (5)=3,4-MDMA, (6)=clenbuterol, (7)=LSD, (8)=phenobarbital, (9)=buprenorphine, (10)=temazepam, (11)=nandrolone, (12)=metandienone, (13)=testosterone, (14)=stanozolol. The concentration for each substance is 12–15 μg/ml. *y*-Axis: intensity.

sample injections with concentrations varying between 0.1 and 40 μg/ml. The search algorithm compares the unknown spectrum to the spectra in the library (Fit) and the spectra in the library to the unknown (Rfit) from which it generates a value (Purity). The maximum purity value is 1000 indicating perfect correlation between measured and library spectra. The purity values were clearly better (640–900) for concentrations above 0.3 μg/ml than below (354–868). This was mainly due to increased background interferences and decreased repeatability of relative abundances of the product ions at concentrations below 0.3 μg/ml. The results indicated that the purity values better than 600 provided reliable identification of the compounds studied. The results also showed the necessity of including retention time to the library search. For the compounds producing several abundant product ions (morphine, buprenorphine, testosterone, stanozolol), it was not so critical as for the compounds producing one very intense product ion with few weak other ions (amphetamine, 3,4-MDMA). By combining retention time and MS–MS spectra information in the library search the reliability of the identification can be increased significantly. All the compounds were found within a very narrow retention time window (±2.5 s) due to good reproducibility of the LC. Although the identi-

fication can be carried out reliably with the library, the visual evaluation of the MS–MS spectra has to be done to confirm the positives.

The library matches were also evaluated with 50 authentic samples and pharmaceutical preparations concentrations varying between 0.2 μg/ml and 3.75 mg/ml. The reliability of the identification was compared to the results obtained with conventional methods (GC, GC–MS, TLC and HPLC). The samples were dissolved in small amount of methanol, diluted with deionised water and filtrated to autosampler vials. The compounds identified with the conventional methods were all identified unambiguously by the developed LC–MS–MS method by using a library search. The correct compound in all the samples was the first one in the MS–MS library hit list and found within ±3% retention time window. Fig. 4 shows an example for the identification of phenobarbital from a tablet by comparison of the recorded spectrum against the library spectrum. It is worth to note that morphine and stanozolol showed some tailing obviously due to secondary interactions with the silica, even though endcapped C_{18} monolithic column was used. However, the column used did not show any memory effect even after the very high concentrations injected.

The emphasis in forensic samples is often in qualitative analysis. However, the evaluation of a quantitative method for the compounds studied is of great interest. Since the chemical and physical properties of the compounds studied varied a lot, an external standard method was used in quantitation. The method was evaluated by determining linearity, repeatability, and limits of detection ($S/N \geq 3$). The correlation coefficients (r) were between 0.993 and 0.999, within the concentration range of 0.1–30 μg/ml ($n=6$) indicating good linearity of the method. The linearity of LSD, nandrolone and buprenorphine started to deteriorate at concentrations above 40 μg/ml. This was obviously due to saturation of the surface of the electrically charged droplets in ESI described earlier by Kostiainen and Bruins [39]. Within-day reproducibility of the method was evaluated at three concentration levels (3.0, 10 and 30 μg/ml; five injections each). The relative standard deviations (RSDs) were typically below ±20%, but the RSDs for phenobarbital (±28.8%) and amphetamine (±25.9%) were clearly worse than for the other compounds. However, repeatability of the method is acceptable for forensic analysis purposes, although it suffers from the limited number of data points due to co-eluting compounds and narrow peaks being only 2–7 s wide. With current settings, one data point is collected in about 400 ms for one parent ion [i.e. accumulation time (up to 10 ms) + the scan time (30.4 ms (for m/z 50–500) + isolation time (290 ms) + fragmentation time (40 ms) + scan time (30.4 ms)]. If more than one compound must be analysed within same cycle, it means that the number of data points is decreased and may not be enough for reproducible integration of the peaks. With the ion trap used in this work, the time needed for one data point cannot be decreased. Limits of detection were typically between 10 and 50 ng/ml, which is sufficient in a forensic analysis. This sensitivity was achieved also for neutral steroids and acidic phenobarbital. The whole evaluation process was carried out with two monolithic columns from different batches. The results did not show any significant differences.

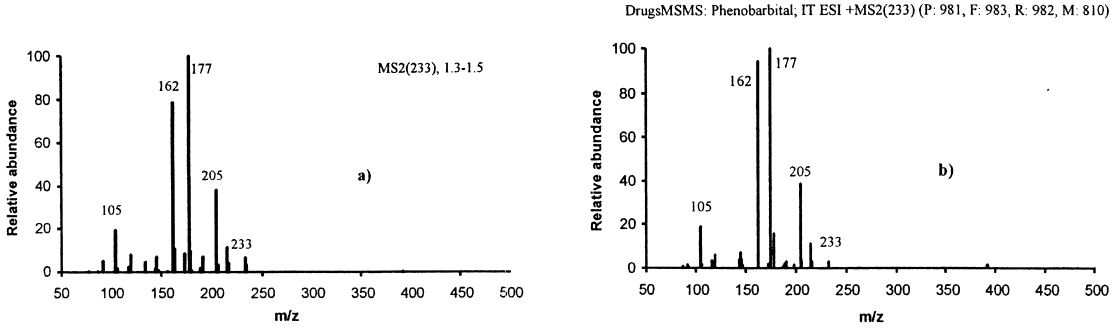

Fig. 4. The comparison between (a) measured MS–MS and (b) library spectrum of phenobarbital.

4. Conclusions

The method developed and validated was shown to be applicable for identification, confirmation and quantitation of different forensically interesting compounds. The results showed that very different kind of forensic compounds, bases, acids and neutrals can be ionised by positive ion ESI allowing analysis of several compounds within one run. The use of monolithic column together with MS–MS detection provided significantly shorter analysis times than with the methods normally used in forensic laboratories. A laboratory library including MS–MS spectra and retention times provides fast and unambiguous identification of the compounds. The quantitative method was acceptable for forensic purposes, although it suffered from a lack of data points due to a relatively long time (400 ms) required to collect one data point with the ion trap used. However, this is not a problem, e.g. with triple stage quadrupoles.

Acknowledgements

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