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Systematic toxicological analysis: computer-assisted identification of poisons in biological materials

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Abstract

A new software was developed to improve the chances for identification of a ''general unknown'' in complex biological materials. To achieve this goal, the total ion current chromatogram was simplified by filtering the acquired mass spectra via an automated subtraction procedure, which removed mass spectra originating from the sample matrix, as well as interfering substances from the extraction procedure. It could be shown that this tool emphasizes mass spectra of exceptional compounds, and therefore provides the forensic toxicologist with further evidence—even in cases where mass spectral data of the unknown compound are not available in ''standard'' spectral libraries.

Keywords: Systematic toxicological analysis; Computer-assisted identification of poisons

1. Introduction

An important objective of forensic toxicological analysis is the identification of initially undetermined substances in biological material—summarized as ''general unknown analysis''.

Finding a poison that is a priori unknown in a case of a suspected intoxication is a difficult task, because more than 7500 drugs or pesticides are presently available on the market worldwide [1]. To develop a method for systematic toxicological analysis, sophisticated extraction procedures are needed to isolate analytes from the aqueous matrix, since suitable sample preparation is the most important prerequisite for the identification of poisons by gas chromatography–mass spectrometry (GC–MS) in biosamples [2].

Specimens that have to be investigated range from body fluids to tissue samples of an often altered (decomposed) nature, resulting in the presence of putrefactive compounds [3]. Because—especially with advanced putrefaction—body fluids like urine and blood are not always available, tissue samples gain increased importance. This led Scheurer and Moore in 1992 to the conclusion that ''without doubt, the future of drug extractions in forensic toxicology lies with the rapid and efficient extraction of human tissues, especially brain and liver'' [4].

To meet the qualitative and quantitative variable composition of matrices in forensic cases, an automated clean-up procedure for the wide range of

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biological material, including tissues rich in lipoid components like brain, liver or putrefied blood, was developed and showed high recovery and reproducibility [5].

To develop a fully automated method for systematic toxicological analysis based on GC–MS, development must go beyond sophisticated sample preparation to focus also on the extraction of relevant analytical information (mass spectra of analytes) from the chemical noise [6].

To perform a proper systematic toxicological analysis, all peaks in a run by GC–MS analysis have to be searched against ''standard'' spectral libraries [1]. Because large numbers of spectra (~2600) are obtained by every GC–MS run, this is a very time-consuming task. Therefore, a method for retrieving target compounds via reconstructed mass chromatograms was proposed by Maurer and is now widely employed in forensic laboratories [2]. In this procedure, target ions have to be defined, which limits its applicability for the ''general unknown'' screening.

The final reference to ''standard'' spectral libraries can only identify those compounds for which corresponding mass spectra are available—which led Polettini to the conclusion that none of the methods described in his review about hyphenated chromatographic and spectroscopic techniques would be able to identify unknown substances whose reference data are not included in the searched database [7].

In order to find clues in these difficult cases as well, a new approach for systematic toxicological analysis was investigated by introducing ''sample-inherent mass spectra libraries''.

2. Instrumentation

High-purity helium (99.999%, Messer, Austria) was the carrier gas used for gas chromatography.

Analyses were performed on a Hewlett-Packard HP 6890 gas chromatograph with an HP 6890 autosampler connected to a HP 5973 MSD (Hewlett-Packard, Palo Alto, CA). In the gas chromatograph, an MDN-5S-column (15 m×0.25 mm, 0.25-μm film thickness, Supelco) was installed with a constant flow of helium at 1 ml/min. The following temperature program was used, with a total running time of 20 min: the initial column temperature was set to 100 °C for 2 min, increased to 200 °C by 25 °C/min and held at 300 °C after a ramp of 20 °C/min. Splitless injections were performed with the injection-port temperature set to 280 °C. The transfer line of the instrument was adjusted to 300 °C. Full-scan EI spectra were recorded from m/z 50 to 550 with three scans per second.

3. Material studied, methods and techniques

Postmortem blood and tissue samples were extracted by a semi-automated solid-phase extraction procedure on an ASPEC XL (Gilson, Villiers-le-Bel, France), controlled by a personal computer with ASPEC XL-software Ver. 4.00 (Abimed, Langenfeld, Germany), published earlier by our workgroup [5].

The standard solution to calibrate the retention index calculation contained a mixture of the *n*-alkanes C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, C_{30}, and C_{32} in methanol, and 100 ng per compound were injected per GC–MS run.

To calculate the retention time variations the standard solution was measured on three successive days in duplicate, in pure solution and spiked to three different extracts from liver samples.

GC–MS data files recorded in the ChemStation software (Agilent Technologies, Waldbronn, Germany) were transformed to text-files by ''macros'', and afterwards imported to the new software FORGE (Forensic GC–MS Data Exploration).

FORGE—developed under MATLAB (Version 6.0.0.88 Release 12, The MathWorks)—enabled the creation and use of ''sample-inherent mass spectra libraries'' and GC–MS exploration using forward- and target-search supporting custom libraries.

The following calculation experiments were performed in FORGE on a Pentium 4 computer with 1024 MB RAM using the operating system Windows 2000.

3.1. Retention index

The standard solution containing a mixture of *n*-alkanes was used to automatically calibrate the retention index calculation from the retention time. Linear, quadratic and cubic regression models were

evaluated. After applying statistical tests the quadratic model was chosen for further calculations.

3.2. Baseline correction

Baseline correction was performed on all files for each mass chromatogram separately (from m/z 50 to 550). The baseline was adjusted automatically, connecting local minima and simultaneously minimizing the slope of connecting lines and number of points used, within intervals of up to 50 scans between points.

3.3. Creating ''sample-inherent mass spectra libraries''

''Sample-inherent mass spectra libraries'' contain all sample-inherent mass spectra originating from the sample matrix and interfering substances from the extraction procedure. To create ''sample-inherent mass spectra libraries'', first characteristic mass spectra had to be selected automatically from ''negative'' cases and were then stored together with the Retention Index in a library file. A characteristic mass spectrum was identified by two positive slopes followed by two negative slopes in a mass chromatogram. At least two mass chromatograms had to show a peak, and the minimum intensity had to be 0.2% of the highest peak in the chromatogram.

3.4. Subtraction of ''sample-inherent mass spectra''

To ''simplify'' the total ion current chromatogram after a GC–MS screening, the intensity of each mass spectrum in the ''sample-inherent mass spectra library'' was adjusted to the corresponding mass spectrum of the ''positive'' case included within the allowed retention time window: ± 0.1 min. The library spectrum was fitted into the chromatogram in such a way that at least 90% of the total intensity of all ions, and additionally all masses with an intensity of more than 12% of the base peak of the library spectrum were contained in the scan of the ''positive case''.

These ''sample-inherent mass spectra'' were subtracted from the total ion chromatogram within a range of ± 5 scans around the maximum fit, and the total ion chromatogram was recalculated to reflect the subtraction.

4. Results and discussion

To obtain evidence of the presence of a poison in a general unknown case, even when mass spectral data of the unknown compound are not available in the ''standard'' spectral libraries, a new approach for systematic toxicological analysis, which introduces ''sample-inherent mass spectra libraries'', was investigated.

A prerequisite for this new approach, which was developed in our laboratory is a sample preparation method that provides reproducible extraction results and therefore similar extraction profiles for each matrix [5].

Based on this method, a first attempt for a procedure to filter the total number of acquired mass spectra in general unknown cases was introduced at the TIAFT meeting in Helsinki [8]: after a GC–MS screening, the total ion current chromatogram was simplified by an automated, computer-assisted subtraction procedure, and ''suspicious'' substances could be marked in the reduced chromatogram. This procedure made use of ''macros'' within the ChemStation software (Agilent Technologies), where possibilities to generate and apply libraries are limited.

Therefore the software FORGE (Forensic GC–MS Data Exploration), which contains a number of new algorithms for the evaluation of complex mass spectrometric data, was recently developed.

This new software ''simplifies'' the total ion current chromatogram after a GC–MS screening by comparing the obtained mass spectra to a ''mass spectral library from negative cases'' received from standard extracts of samples where no toxic compounds could be detected and the case history gave no indication of intoxication. This ''mass spectral library from negative cases'' is a collection of mass spectra representing impurities from the matrix and the sample preparation procedure and therefore is referred to as a ''sample-inherent mass spectra library''. Mass spectra from a ''positive'' sample (suspected intoxication) that are not included in the ''sample-inherent mass spectra library'' are suspi-

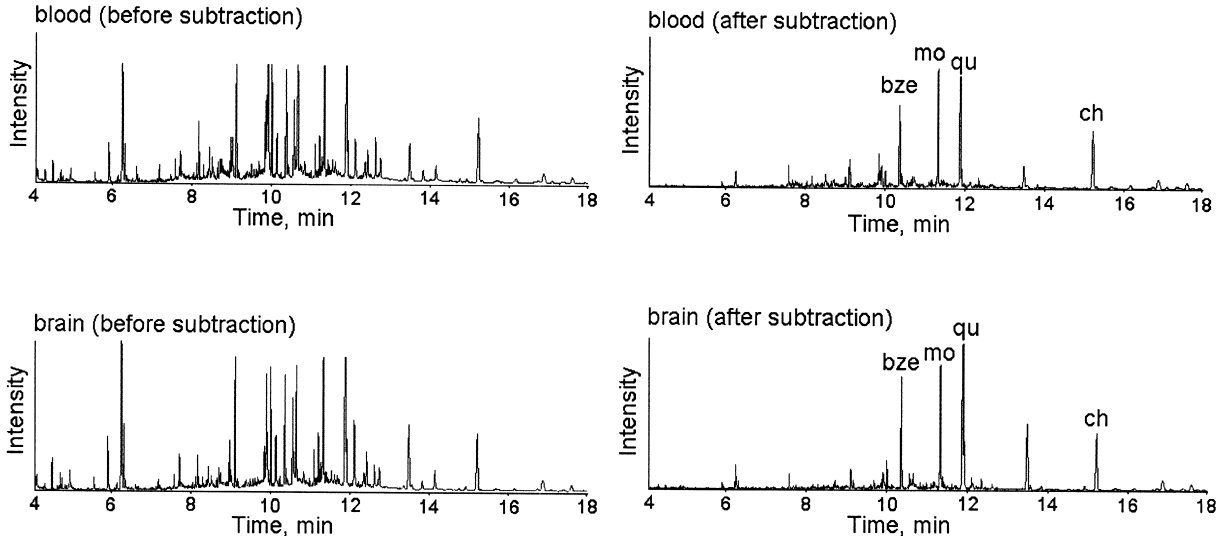

Fig. 1. Silylated extracts from postmortem samples of blood and brain from a case of drug overdose, before and after subtraction of the ''sample-inherent mass spectra library''. Suspicious peaks could be identified as benzoylecgonine (bze), morphine (mo), quinine (qu) and cholesterol (ch).

cious and therefore are highlighted in the reconstructed chromatogram and are a target for further investigation—independent of any entry in ''standard'' libraries.

The pilot study presented in Helsinki clearly showed that retention time must be considered for the creation of ''sample-inherent mass spectral libraries'' and for the subtraction procedure.

For the automatic determination of retention index windows, a mixture of 10 *n*-alkanes was used and each *n*-alkane was identified by its mass spectrum in a forward search procedure. Afterwards, the retention index calculation was calibrated from the retention times at the maximum fit and a quadratic model was used to calculate the regression curve.

For each *n*-alkane of the mixture, retention time variations were evaluated and found to be very small, with ± 0.01 min corresponding to ± 1 mass spectrum between days.

Three ''negative'' blood and brain samples (no indication of intoxication) were extracted, and GC–MS was performed on each extract. After baseline correction, a ''sample-inherent mass spectra library'' was created as described in the materials, methods and techniques section.

Then subtraction of the ''sample-inherent mass spectra'' from a ''positive'' blood and brain extract (intoxication with drugs) was performed. The intensity of each mass spectrum in the ''sample-inherent mass spectra library'' was adjusted to the corresponding mass spectrum of the ''positive'' case, and these ''sample-inherent mass spectra'' with sufficient fit were subtracted. Since suspicious peaks in the chromatogram of the ''positive'' case were not considered for subtraction, they could be highlighted after recalculation of the total ion chromatogram.

The results are demonstrated in Fig. 1: complex silylated extracts from postmortem samples of blood and brain from a case with drug overdose are shown before and after subtraction of the ''sample-inherent mass spectra library''.

After subtraction of the chemical noise from the matrix and the extraction procedure, suspicious peaks were highlighted and could be identified as benzoylecgonine (bze), morphine (mo), quinine (qu; I.S.) and cholesterol (ch).

5. Conclusion

The results of our investigation clearly show that the newly developed software FORGE greatly facilitates computer-assisted identification of poisons in

biological materials in systematic toxicological analyses.

The presented tool promises a substantial reduction of mass spectrometric data in the screening process of ''general unknown'' cases. Moreover, it renders the forensic toxicologist's finding of ''no poisons detected'' evidence-based, provided that a representative number of ''negative'' cases are included in the ''sample-inherent mass spectra library''.

Because this approach is independent of currently utilized separation, derivatization, and ionization techniques, it could also be useful for processing other kinds of mass spectrometric data—like CI, electro spray and APCI spectra—as long as the applied analytical procedure provides reproducible results.

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