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Headspace solid-phase microextraction with 1-pyrenyldiazomethane on-fibre derivatisation for analysis of fluoroacetic acid in biological samples

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Abstract

A new and in part automated headspace solid-phase microextraction method for quantitative determination of the highly toxic rodenticide fluoroacetic acid (FAA) in serum and other biological samples has been developed. FAA and deuterated acetic acid (internal standard) were extracted from acidified samples by a StableFlex divinylbenzene–Carboxen on polydimethylsiloxane fibre. The acids were derivatised on the fibre in-situ with 1-pyrenyldiazomethane and detected using gas chromatography–mass spectrometry with electron impact ionisation and selected ion monitoring. The calibration curve for FAA in serum was linear over the range from 0.02 to 5 μg/ml, with limits of detection and quantification of 0.02 and 0.07 μg/ml, respectively. The method was also tested with spiked whole blood, urine, stomach contents and kidney samples. It was sufficiently reliable, reproducible and sensitive for use in routine forensic toxicology applications. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Derivatisation, GC; Fluoroacetic acid; 1-Pyrenyldiazomethane

1. Introduction

Fluoroacetic acid (FAA) is an extremely toxic and malicious poison [1]. It is the toxic substance found in the South African plant *Dichalpetalum cymosum* [2]. The sodium salt of FAA has been synthetically produced and is used as a rodenticide (known commercially as compound 1080) in several countries [3]. It has also been used as a poison for feral animals in some parts of the USA, Australia and New Zealand [4]. Its toxic effect is caused by inhibition of aconitase activity in the Krebs cycle. Since FAA is highly water soluble, tasteless and colourless in solution and has a latency period between ingestion and the appearance of toxic symptoms of at least 1 h, it can easily be surreptitiously administered or accidentally ingested. For these reasons the use of FAA is restricted in many countries. Nevertheless, it is still available from old product stocks and can easily be prepared from simple chemicals [5]. Therefore, forensic toxicology laboratories need a convenient and sensitive method for the routine analysis of FAA in serum, whole blood and other biological samples.

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The lethal dose of FAA has been estimated at 2–10 mg/kg body mass [1]. No data concerning blood or serum levels after poisoning with FAA in man are available. However, concentrations in liver and kidney (2.5–17 μg/g) of poisoned animals and tissues (58–76 μg/g) and urine (368 μg/ml) in one human fatality [1] indicate that in cases of severe FAA intoxication serum levels should be above 1 μg/ml.

Due to its physicochemical properties (poor UV absorbance, high water solubility, low volatility from aqueous solutions) FAA is not found by the usual systematic toxicological screening methods and must be detected by special techniques. These include gas chromatography (GC) with electron-capture or mass spectrometric (MS) detection after liquid–liquid extraction and derivatisation with diazomethane [6], pentafluorobenzylbromide [7–9], or benzyldimethylphenylammonium hydroxide [10] and headspace (HS) GC–MS from dried samples after ethanolic esterification [11].

Solid-phase microextraction (SPME) was introduced over a decade ago by Arthur and Pawliszyn [12] as a rapid solvent-free extraction technique for the analysis of volatile and semi-volatile compounds from a variety of matrices. The method uses a modified syringe assembly which houses a short fused-silica micro-fibre externally coated with a stationary phase. A range of phases is available for different applications [13–15]. The technique involves either the equilibrium or non-equilibrium partitioning of analytes between the stationary phase and sample, followed by desorption of the analytes in the hot injector of a GC system. SPME can be operated in two modes, either HS sampling or with immersion directly into the sample. The range of analytes that can be extracted by the technique can be extended by the use of various derivatisation procedures performed either before or during extraction or in the injection port of the GC [13].

SPME has been used to analyse biological samples for a range of drugs and their metabolites, volatile anaesthetic gases, solvents and various endogenous metabolites [16–18]. Most published procedures use manual SPME methods which can be labour intensive in the clinical or toxicology laboratory setting where high sample throughput is essential. This paper describes a novel, automated procedure for the quantitative determination of FAA using HS-SPME with on-fibre 1-pyrenyldiazomethane (PDAM) derivatisation. The method has been applied to various biological specimens of forensic interest. PDAM was selected as it requires mild derivatisation conditions, has low volatility, is relatively stable and forms stable pyrenylmethyl ester reaction products. Previously it has been used with HS-SPME for measurement of short-chain fatty acids in sewage sludge [19], milk [20] and faeces [21].

2. Experimental

2.1. Biological samples

Samples of blood or serum and urine were obtained from the authors or with consent from colleagues. The specimens were either analysed fresh or frozen immediately and stored at −20 °C. Post-mortem blood, stomach contents and kidney samples were collected from cadavers being investigated at the Institute of Legal Medicine of the University Hospital Charité. Only biological samples spiked with FAA were investigated since there were no cases available of intoxication with this poison.

2.2. Reagents and materials

FAA-sodium salt was purchased from Riedel-de Haën (Seelze, Germany), deuterated sodium acetate (D_3C-COONa) from Sigma (Deisenhofen, Germany) and PDAM from Molecular Probes (Leiden, The Netherlands). All chemicals were used without further purification. PDAM is light sensitive and should be considered hazardous and handled with care. All other analytical grade reagents were obtained from Merck (Darmstadt, Germany). Water was doubled distilled.

StableFlex divinylbenzene (DVB)–Carboxen on polydimethylsiloxane (1 cm long, 50/30 μm thick) SPME fibres and SPME fibre syringe holders were from Supelco (Deisenhofen, Germany). Headspace vials (10 ml), silicon/PTFE seals and steel caps were from CS-Chromatography Service (Langerwehe, Germany). For homogenisation of stomach contents

and tissue samples an Ultra Turrax homogeniser (Janke and Kunkel, Heitersheim, Germany) and a Sonorex ultrasonic bath (Bandelin Electronic, Berlin, Germany) were used.

2.3. Instrumentation

A GC system (Model 6890) linked to bench-top quadrupole MS instrument (Model 5973) (Agilent, Waldbronn, Germany) fitted with a narrow bore (0.75 mm I.D.) injection liner (Supelco) was used for all analyses. The GC column was a HP-5 MS fused-silica capillary column (30 m×0.25 mm I.D., film thickness 0.25 μm) from Agilent. Helium was used as the carrier gas at a flow-rate of 1.0 ml/min. The GC–MS system was linked to a multipurpose sampler (Model MPS 2) (Gerstel, Mühlheim an der Ruhr, Germany) which allowed for accurate control of sample vial temperature and agitation conditions. This sampler could be programmed to automatically perform all of the HS-SPME extraction, on-fibre derivatisation and GC injection port desorption steps.

2.4. Sample pretreatment

A 200-μl volume of serum, 200 ng D_3-acetic acid (as internal standard, 20 μl of a 10 μg/ml aqueous solution), 1.2 ml of 1 *M* H_2SO_4 and 0.7 g Na_2SO_4 were added to a 10 ml HS vial. The vials were quickly closed with silicon/PTFE septa, sealed with steel caps and placed into the multipurpose sampler. Whole blood samples were prepared using the same procedure. Urine or homogenised stomach contents were treated in the same way, however, as higher levels of FAA were likely in these types of specimen in cases of intoxication only 20 μl of sample was analyzed.

As an example of tissue, samples of kidney were investigated. A 1-g amount of kidney, 1 ml 0.9% NaCl (aqueous) and 1 μg D_3-acetic acid (100 μl of 10 μg/ml aqueous stock solution) were added to a Ultra Turrax mixer and fully homogenised. Afterwards 3 ml of distilled water was added and the homogenate placed in an ultrasonic bath for 15 min. A 200-μl volume of the resulting mixture was then treated as described for serum.

2.5. HS-SPME extraction, derivatisation and analysis (definitive procedure)

All SPME fibres were pre-conditioned by inserting them into the GC injector according to the manufacturer's instructions. A solution (2.5 mg/ml) of PDAM derivatisation reagent was prepared in *n*-hexane and kept in the dark. The solution was stored at −20 °C and was stable for several weeks. The HS-SPME extraction and on-fibre derivatisation procedure was based on Pan and co-workers [19,20], however in this case all the steps were performed automatically by the multipurpose sampler. After optimisation the following conditions were used:

—Preheating and incubation of the sample: 15 min at 90 °C in the heating station of the sampler, agitated at 250 rpm.

—Loading of the fibre with PDAM solution: 15 min immersion at room temperature in the washing station of the sampler.

—Coupled HS adsorption and on-fibre derivatisation: 30 min at 90 °C, in the heating station of the sampler, agitated at 150 rpm.

—Desorption: 10 min at 250 °C in the GC–MS injection port, split valve closed for 3 min.

The GC–MS system was operated under the following conditions: solvent delay 9 min; injector 250 °C; interface transfer line 280 °C; ion source 230 °C; quadrupole 106 °C; oven temperature program 100 °C (2 min) then 20 °C/min to 300 °C (2 min). The MS system was operated in the single ion monitoring (SIM) mode for FAA (m/z 292 and 215) and D_3-acetic acid (m/z 277 and 215). The detector signals were collected, integrated and recorded using a G1701BA Chem-Station (Agilent). With these conditions no carry over of the two acids was found between runs.

3. Results and discussion

3.1. Retention times, mass spectra and choice of derivatising reagent

Deuterated FAA is not available commercially. Therefore, D_3-acetic acid was selected as internal standard as it does not occur naturally in biological samples and other constituents in the matrices did

not cause an interference with the SIM chromatograms of its PDAM ester. The GC conditions used produced good peak shapes for both acids with D_3-acetic acid eluting at 9.7 min and FAA at 10.0 min. The electron impact mass spectra of the 1-pyrenylmethyl esters of FAA and D_3-acetic acid are shown in Fig. 1. Both spectra were simple and gave relatively intense molecular ions at *m/z* 292 and *m/z* 277, respectively, which were suitable for SIM. The base peak at *m/z* 215 arose from the cleavage of the bond between the pyrenylmethyl group and the fatty acid methyl mono-ester [19–21]. A typical SIM chromatogram from a serum sample at these three *m/z* values is shown in Fig. 2. Chromatograms at *m/z* 277 and *m/z* 292 show only the internal standard and FAA, whereas the trace at *m/z* 215 displays many other peaks which are probably caused by other derivatised fatty acids in the serum matrix. For identification of FAA in biological samples the area ratio of the peaks *m/z* 292 and *m/z* 215 (A_{292}/A_{215}) must be in the range 0.35 to 0.45. For quantitative determinations only the peak areas of the molecular ions of FAA and D_3-acetic acid were used.

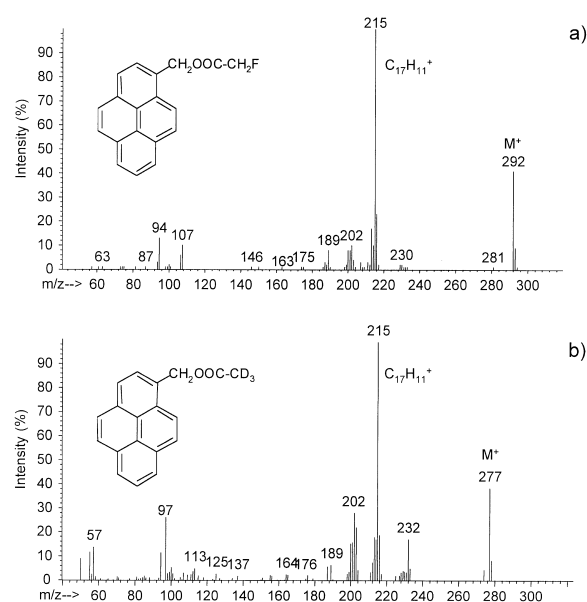

Fig. 1. Electron impact (70 eV) mass spectra of the 1-pyrenylmethyl esters of (a) fluoroacetic acid and (b) D_3-acetic acid.

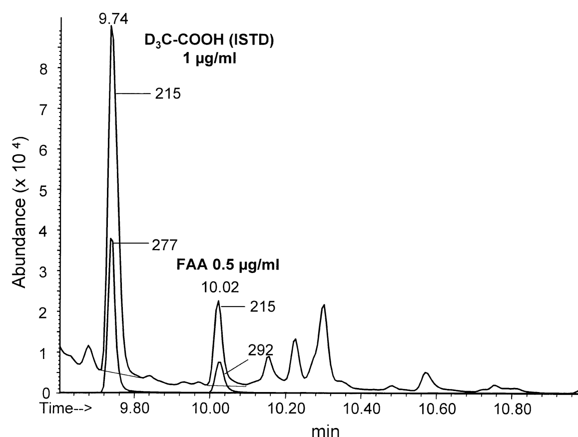

Fig. 2. Single ion monitoring chromatograms at *m/z* 215, *m/z* 277 and *m/z* 292 obtained from 200 μl of serum spiked with 0.5 μg/ml FAA and 1 μg/ml of deuterated acetic acid as internal standard. HS-SPME conditions: fibre loaded with PDAM for 15 min, adsorption and in-fibre derivatisation 30 min at 90 °C, fibre desorbed for 10 min at 250 °C in the GC–MS injection port, split valve closed for 3 min.

During the course of the method development two silylating compounds, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (a trimethylsilyl derivatisation reagent) and *N*-*tert*.-butyldimethyl-*N*-methyltrifluoroacetamide (a *tert*.-butyldimethylsilyl derivatisation reagent) were also investigated as potential on-fibre derivatisation reagents. However, these proved to be unsuitable as derivatised FAA and D_3-acetic acid both eluted within the derivatisation reagent peak on the chromatogram.

3.2. Optimisation of the HS-SPME conditions in serum

The StableFlex DVB–Carboxen on polydimethylsiloxane fibre was initially chosen since according to the manufacturer, it is particularly suitable for the extraction of low molecular mass polar compounds. Optimisation of the HS-SPME conditions was performed with serum samples spiked with 1.0 μg/ml FAA and D_3-acetic acid. All measurements were performed in duplicate with a good agreement between both results always obtained. The mean values are given in the figures. As FAA is a

relatively strong (pK_a=2.6) and hydrophilic acid with a low volatility (boiling point 165 °C), highly acidic (pH<1), combined with salting out (excess of Na_2SO_4) extraction conditions were used. The effect of adsorption and derivatisation temperature is shown in Fig. 3a. The peak area of FAA increased over the entire temperature range tested, whereas the peak area of the internal standard has its optimum value between 70 and 80 °C and then decreased. This decrease of the peak area of the internal standard can probably be explained by its lower boiling point in comparison to FAA and other acids present in the serum sample. At high temperature the limited amount of PDAM on the fibre is consumed to a higher extent by these competing acids leading to a decrease in the yield of the D_3-acetic acid derivative. For routine measurements an incubation temperature of 90 °C was used to maximize the signal for FAA. Fig. 3b shows the effect of incubation time at 90 °C on peak areas for the two derivatised acids. The peak area of D_3-acetic acid was almost independent of the time. FAA showed an increase in signal from 10 to 20 min and then levelled off. For routine measurements an incubation time of 30 min was employed.

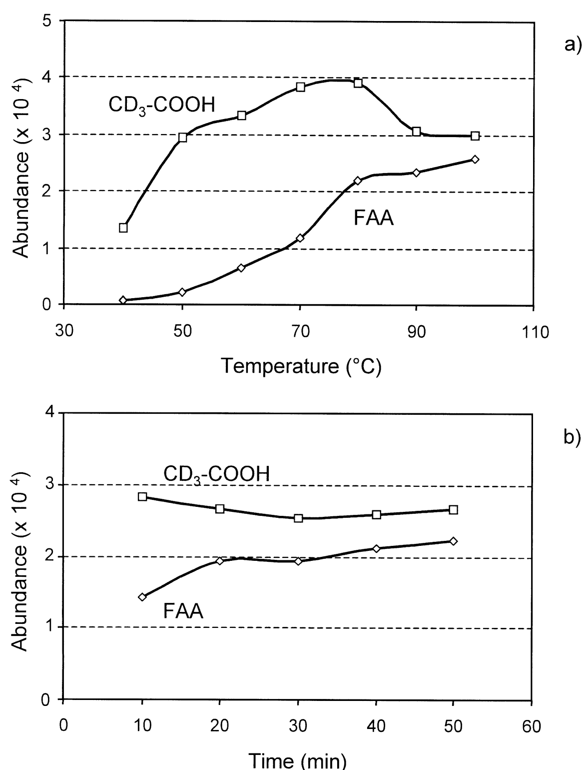

Fig. 3. Effect of the (a) extraction temperature with fibre exposure time of 30 min and (b) time at headspace sampling at temperature of 90 °C. A 200-μl volume of serum was spiked with 1 μg/ml of FAA and D_3-acetic acid and analysed using the conditions as described in Fig. 2.

Under normal HS-SPME conditions the concentration of analyte on the fibre is thermodynamically controlled by the distribution constants between the phases and a plateau is reached when the equilibrium is attained. However, in this case the acids are irreversibly transformed into esters on the fibre. Peak areas should increase with adsorption time as long as no depletion of the analyte in solution or derivatisation agent in the fibre occurs. Due to the hydrophilicity and high boiling points of both acids a depletion of the analyte concentration in the matrix solution is not expected under these experimental conditions. Therefore, the nearly constant peak areas found after incubation times of 20 min can probably be explained by depletion of the PDAM derivatisation agent on the fibre. In addition to FAA and D_3-acetic acid other fatty acids from the matrix are present in the HS and these will also rapidly react with PDAM and eventually exhaust the available supply of reagent. This may also lead to a change in calibration, if urine or tissue samples, with a clearly higher excess of other fatty acids are investigated instead of serum or whole blood (see below).

Fig. 4a shows that increasing fibre immersion times in the *n*-hexane–PDAM solution from 5 to 15 min had only a small effect on the resultant peak areas for both FAA and D_3-acetic acid. The adsorption equilibrium was almost complete after 5 min, however for routine measurements 15-min immersions were used. Increasing serum volume (20 to 500 μl) with the same amounts of 1 *M* H_2SO_4 and Na_2SO_4 in the vial did not lead to an increase in the peak areas, as calculated from the FAA concentration increase and from the changed phase ratio between the sample and HS volume (Fig. 4b). An explanation could be that with increasing sample volume, the PDAM reagent is more rapidly used up through competition from other fatty acids in the matrix. Since this effect is different for FAA and D_3-acetic acid it is important to use a small sample volume and to always use the same sample volume

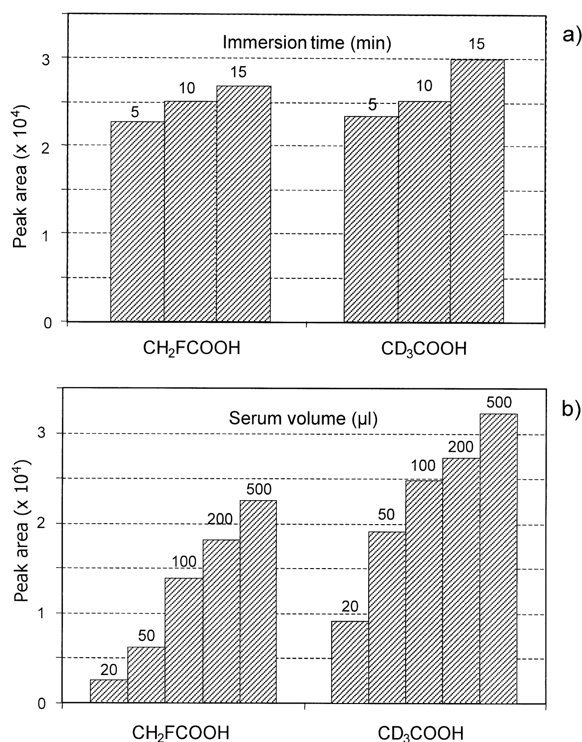

Fig. 4. Effect of the (a) fibre impregnation (2.5 mg/ml *n*-hexane–PDAM solution) time using 200 µl serum spiked with 1 µg/ml of FAA and D_3-acetic acid (b) volume of serum (spiked with 1 µg/ml of FAA and D_3-acetic acid) used on the GC–MS peak area (*m/z* 292 and 277 ion, respectively) response for FAA and D_3-acetic acid. Other experimental conditions as described in Fig. 2.

in calibrations and analysis. In these investigations 200 µl of serum was used.

3.3. Linearity, limits of detection (LODs), limits of quantification (LOQs) and reproducibility

Calibration was performed with 200 µl of serum at seven FAA concentrations (0.02–5 µg/ml) under the optimised conditions with 200 ng of D_3-acetic acid internal standard added. The calibration curve was linear (R^2=0.9991) over this range. LOD and LOQ were estimated according to the German Industrial Norm (DIN 32645) as threefold and 11-fold the deviation of the baseline noise, respectively. Under these conditions the LOD was 0.02 µg/ml and the LOQ 0.07 µg/ml for FAA. These LOD and LOQ values were confirmed by measurement of spiked serum samples at concentrations between 0.02 and 0.1 µg/ml. The method was therefore sufficiently sensitive for the detection of serum FAA in cases of intoxication.

Reproducibility was determined using the same spiked (1.0 µg/ml FAA) serum sample measured 16 times within 1 day. This gave a standard deviation of 10,6%. In another series of experiments 10 different spiked (1.0 µg/ml FAA) serum samples were measured within 4 days. The following results were obtained: mean concentration 0.95 µg/ml, standard deviation 0.14 µg/ml (15%).

3.4. Application to whole blood and other biological matrices

The HS-SPME method for serum was applied to other biological samples (vital and post-mortem whole blood, urine, stomach contents and kidney tissue). Each of these matrices was spiked with different (1 to 100 µg/ml) FAA concentrations before analysis. Whole blood samples were analysed in the same way as for serum. For tissue the method was changed with regard to the amount of sample used and additional preparation steps included (see Section 2.4). Similar LOQs for FAA were found for all these matrices and these were comparable to the serum value. The SIM chromatograms obtained from a spiked postmortem whole blood sample is shown in Fig. 5.

During analysis of urine, stomach contents and kidney a matrix interference occurred in some samples which affected the peak areas of FAA and the

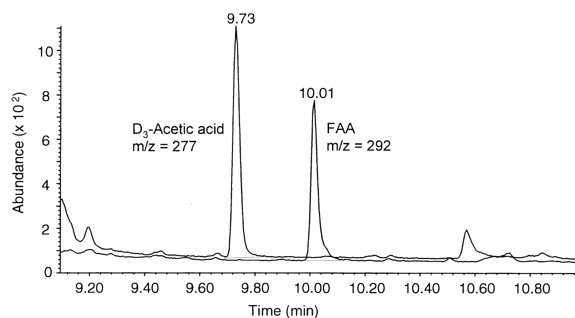

Fig. 5. Single ion monitoring chromatograms (*m/z* 277 and *m/z* 292) for 200 µl postmortem whole blood sample spiked with 1 µg/ml FAA. HS-SPME conditions as described in Fig. 2.

internal standard leading to errors in quantification. For these specimens it maybe preferable to perform quantification by standard addition methods.

4. Conclusion

HS-SPME combined with on-fibre PDAM derivatisation is a rapid, sensitive and reproducible method for analysis of FAA in a variety of biological samples encountered in toxicological investigations. By using the recently available multipurpose MPS 2 sampler we have automatically performed all of the HS-SPME derivatisation, extraction and desorption steps. This new approach has many advantages over the existing techniques for measurement of FAA which involve a number of discrete and labour intensive steps. We have demonstrated that automated HS-SPME affords great potential; it should find a number of novel applications in clinical and forensic laboratories in future.

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