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Short communication

The determination of trifluoroacetic acid in rat milk samples by ^{19}F-NMR spectroscopy and capillary gas chromatography

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1. Introduction

Hydrochlorofluorocarbon-123 (HCFC-123) is considered as a substitute for ozone-depleting halons and chlorinated fluorocarbons. Because of the expected widespread use of HCFC-123, it is necessary to study its toxicity. Important aspects of the toxicological evaluation are the identification and the quantification of the metabolites in different biological matrices. The major metabolite of HCFC-123 is trifluoroacetic acid (TFA) [1] and several analytical methods (GC, GC-MS, NMR) have been developed to determine TFA in blood [2,3], urine [2,4–6] and amniotic fluid [7]. Furthermore, TFA, covalently bound to proteins, was identified in rat liver [1], kidney [8] and other organs [3].

For the investigation of reproductive toxic effects, it is important to determine metabolites in the matrix milk. Therefore, the aim of these investigations was to develop two independent analytical methods for the determination of TFA in rat milk and to compare the analytical results.

2. Experimental

2.1. Chemicals

TFA ($>99.9\%$), *t*-butyl methyl ether ($>99.8\%$), benzyl alcohol ($>99\%$) and acetic anhydride ($>99.5\%$) were purchased from Fluka (Basel, Switzerland) and TFA-glysine from Bachem (Basel, Switzerland). Sodium chloride (extra pure), anhydrous sodium sulphate ($>99\%$), ethanol ($>99.8\%$), sodium hydroxide p.a. and acetic acid (100%) were from Merck (Darmstadt, Germany), trifluoroacetic anhydride ($99+\%$), sodium nitrite ($97+\%$), sodium methoxide (95%), anhydrous diethyl ether (99%) and deuterium oxide (99.9% atom D) were from Aldrich (Steinheim, Germany), anhydrous sodium carbonate ($>99\%$) and benzyl amine (99%) were from Rie del-de-Haen (Seelze, Germany),

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dichloromethane (glass distilled grade) was from Rathburn (Walkerburn, Scotland), methanol (Baker analysed) from Baker (Deventer, Holland) and SIGMACOTE from Sigma (St. Louis, MO). Water was obtained from a Milli-Q-purification system (Millipore, Eschborn, Germany).

2.2. Rat milk samples

Study samples have been obtained from an inhalation study with HCFC-123 in lactating Sprague–Dawley rats. Pregnant and/or lactating dams were exposed to the test substance (1000 ppm) by whole body inhalation for 6 h per day from day 6 to 19 p.c. and from day 5 to 21 p.p. Milk was collected after separation of the litters from the dams for at least 6 h immediately after exposure on days 10 and 18 p.p. More details are given elsewhere [9]. For calibration and recovery experiments milk samples of untreated dams were used. All samples were stored at −20°C until analysis.

2.3. Analysis of TFA

2.3.1. Method A (^{19}F-NMR quantitation after saponification)

An aliquot of the rat milk sample (0.3 or 0.5 ml) was pipetted into a polypropylene microcentrifuge tube and diluted with an equal volume of 10 M $NaOH/D_2O$. After a period of 30 min, during which denaturation of the milk sample occurred, the precipitation was separated by centrifugation (10 000 rpm for 10 min at 10°C). The alkaline supernatant was then transferred to a silanized NMR tube (5 mm). Samples prepared with 0.3 ml rat milk were further diluted (1:1) with D_2O.

Calibration standards were prepared by the addition of defined volumes (30–100 μl) of accurately prepared dilutions of TFA in water to control rat milk which was further treated as described above.

^{19}F-NMR spectra were recorded on Bruker AM and DPX 300 spectrometers at 282.41 and 282.38 MHz, respectively. Both spectrometers were equipped with QNP probe heads.

Aquisition parameters used were as follows: 6024 Hz spectral width, 8192 data points, 5 μs pulse width (~ 30° pulse), 0.68 s acquisition time (without delay between pulses) and 3000 scans. Chemical shifts are reported relative to the external reference standard $CFCl_3$ ($\delta = 0.00$ ppm) in ppm. Processing parameters: LB = 1.0 Hz, sodium trifluoroacetate: $\delta = -75.3$ ppm, TFA: $\delta = -75.6$ ppm. Spectra were processed on an NEC Image 466 PC using the Bruker WINNMR software. The equation for the linear calibration was: $y = 1460.5x + 125.75$.

2.3.2. Method B (^{19}F-NMR quantitation without previous saponification)

Equal volumes (0.4 ml) of the rat milk sample and D_2O were pipetted into a silanized NMR tube (5 mm). The sample was then homogenized by shaking the sealed NMR tube.

For the preparation of the calibration standards, defined volumes (50–100 μl) of accurately prepared dilutions of TFA in water were added to control rat milk and the samples were then diluted with D_2O as described above. ^{19}F-NMR spectra were recorded as described in Section 2.3.1. The equation for the linear calibration was $y = 1413x + 305.82$

2.3.3. Method C (GC determination)

Added to 0.5 ml rat milk were 2.5 ml of semiconcentrated hydrochloric acid (3.3 M). After storing the sample for 1 h at 4°C, the precipitation was separated by centrifugation (9000 rpm for 30 min). The whey was transferred quantitatively to a second centrifuge tube and after adding 0.5 g sodium chloride the sample was extracted 6x with 1 ml t-butyl methyl ether, where phase separation was achieved by centrifugation (9000 rpm for 15 min). The combined etheral extracts were dried over sodium sulphate and after filtration treated with 0.3 ml of an etheral solution of phenyldiazomethane (ca. 0.8 M) which was prepared according to the method decribed by Klamm et al. [10]. This solution was stored overnight in a tightly sealed tube at room temperature and then analysed by GC/ECD.

For calibration, trifluoroacetic acid benzyl ester was synthesized from trifluoroacetic anhydride

and benzyl alcohol according to Karashima et al. [11]. Purity of the benzyl ester was checked by GC/ECD as well as ^{1}H-NMR and the content was determined by ^{1}H-NMR.

Analysis was performed using a Hewlett–Packard Model 5890 series II plus gas chromatograph equipped with autosampler, split–splitless injector and electron capture detector. The chromatographic column was a DB-FFAP fused silica column (30 m × 0.32 mm i.d., 0.25 μm film thickness) from J&W.

Chromatographic conditions—carrier gas: helium; temperature: oven 40°C (0 min), 40–90°C (3°C min^{-1}), 90–250°C (30°C min^{-1}), 250°C (10 min); detector: 280°C; injector: 250°C.

3. Results and discussion

The ^{19}F-NMR spectroscopy provides a specific method for the identification and quantification of TFA in biological matrices.

Study samples, which were prepared according to method A, show in their ^{19}F-NMR spectra only the signal of sodium trifluoroacetate at $\delta = -75.3$ ppm (Fig. 1a). On the other hand, when an aliquote of the same sample was prepared according to method B, besides the large TFA signal at $\delta = -76.6$ ppm a second very small signal appeared 0.2 ppm upfield from the TFA signal, indicating an additional fluoro-containing compound (Fig. 1b).

Today, the long-term stability of modern NMR spectrometers is sufficiently high to apply the method of external standard calibration for the quantification of compounds. In our investigation, calibration curves were obtained by integrating the ^{19}F-NMR signals of the spiked rat milk samples and plotting the peak areas against the known concentration of TFA in the standard samples. Then, the peak areas of the corresponding ^{19}F-NMR signal in the study samples were interpolated against these calibration curves to calculate the TFA concentrations present. In this way, recovery experiments were not necessary.

Slightly different calibration curves were obtained with methods A and B, but both were linear over the concentration range investigated, coefficients of correlation were >0.999 in all cases. The limit of detection which, in the NMR spectroscopy depends among other things on the field strength as well as on the number of accumulated scans (see Section 2), was 5 μg ml^{-1}.

For the GC determination of TFA in rat milk, a more extensive sample pretreatment was necessary which included extraction and derivatization steps. The extraction from the whey (after denaturation of the milk samples with semiconcentrated

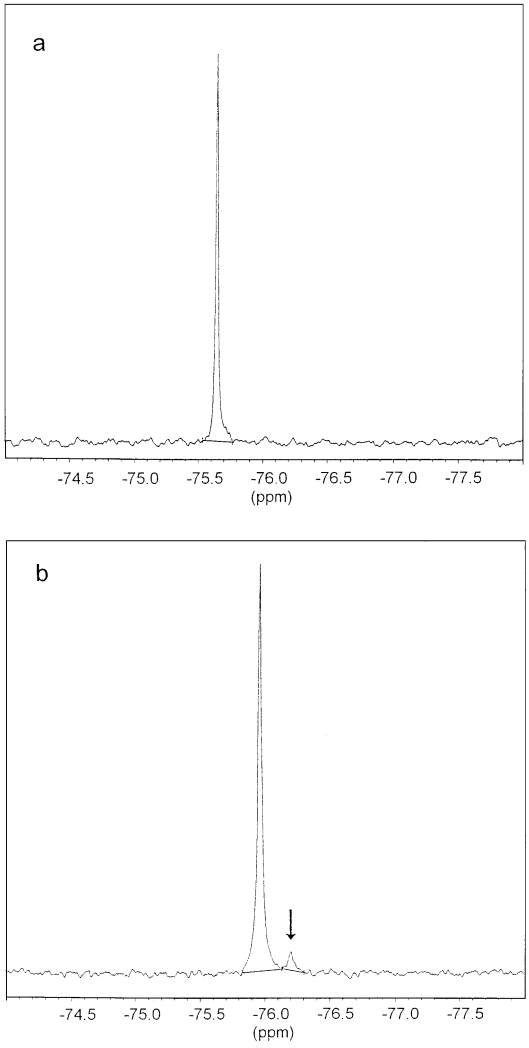

Fig. 1. ^{19}F-NMR spectra of study sample 611: (a) sample prepared according to method A; (b) sample prepared according to method B.

Table 1
Recoveries of trifluoroacetic acid (TFA) in rat milk according to method C

Spiked compound	Spiking level (μg ml^{-1})	Recovery (%)	CV[a,b] (%)
TFA	38.8	97.4	2.9
	3.8	97.7	9.9
TFA-Gly	—	2.0	—[c]

[a] CV, coefficient of variation.
[b] Three determinations.
[c] Single determination.

hydrochloric acid) was carried out with *t*-butyl methyl ether using the salting-out effect. The extracted TFA was then derivatized with phenyldiazomethane to the corresponding benzyl ester according to the method of Karashima [11]. For the calibration, trifluoroacetic acid benzyl ester, which is not commercially available, was synthesized. The detection limit of the GC-ECD method, as set by the blank level, was 0.5 μg ml^{-1}. Overall recoveries of the TFA were determined at two different concentration levels after spiking rat milk samples. In addition, a recovery experiment was performed after spiking rat milk with TFA-glysine in order to investigate to what extent TFA, covalently bound to peptides, is hydrolysed under these conditions. Results are given in Table 1.

The concentrations of TFA in the study samples as measured by ^{19}F-NMR (methods A and B) are reported in Table 2. The values are unexpectedly high and range from 29.9 to 65.0 μg ml^{-1}, while no detectable concentrations were found in the milk samples of untreated dams. It is obvious that the NMR values of samples prepared according to method A (with the exception of sample 613) are higher (105–123%) than those determined with GC and method C. On the other hand, TFA values of samples prepared according to method B (601, 604, 606, 611) are lower and agree better with those determined by GC.

This may be explained by the fact that the additional fluoro-containing metabolite (Fig. 1b, marked with an arrow) is saponified under the conditions of method A and that the released TFA is determined together with the TFA originally existing in the untreated sample. No further investigations were carried out concerning the structure elucidation of this minor metabolite but it is remarkable that otherwise identified metabolites of HCFC-123 have very similar ^{19}F-chemical shifts. For instance, Harris et al. detected an N^{ξ}-trifluoroacetylated lysine adduct in livers of rats exposed to HCFC-123 [1]. This adduct resonates in the ^{19}F-NMR spectrum 0.2 ppm upfield from the TFA-signal. Furthermore, Urban and Dekant identified *N*-trifluoro-acetyl-2-aminoethanol as a minor metabolite of HCFC-123 in rat urine [4]. Again, the signal of this compound appeared slightly upfield (0.2 ppm) from the TFA-signal. Both metabolites have the same type of structure (CF_3CONHR).

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Table 2
Concentration of trifluoroacetic acid (TFA) in rat milk samples

Sample	NMR (μg ml^{-1})		GC (μg ml^{-1})
	Method A	Method B	Method C
601	60.4	53.4	52.3
602	63.0	—	51.2
603	38.4[a]	—	—
604	50.0	40.0	40.6
605	59.6[a]	—	—
606	62.6	46.8	51.5
608	51.8	—	45.5
609	35.0	—	29.9
611	65.0	59.6	61.9
613	35.6[a]	—	38.2
508[b]	n.d.[c]	n.d.[d]	
509[b]	n.d.[c]	n.d.[d]	
510[b]	n.d.[c]	n.d.[d]	
511[b]	n.d.[c]	—	
513[b]	n.d.[c]	—	

n.d., not detectable.
[a] 0.3 ml rat milk was used for sample preparation.
[b] Blank control milk samples.
[c] Detection limit 5 μg ml^{-1}.
[d] Detection limit 0.5 μg ml^{-1}.

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