Determination of Sodium Monofluoroacetate (1080) in Biological Samples as its 4-Bromomethyl-7-Methoxycoumarin Derivative by RP-HPLC

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Abstract

A high-performance liquid chromatography (HPLC) method with fluorescence detection is described for the determination of sodium monofluoroacetate (MFA-Na) in biological samples. 4-Bromomethyl-7-methoxycoumarin is used as a derivatization reagent and reacted with MFA-Na to form 7-methoxy-4-methylenecoumarin monofluoroacetate for HPLC analysis. Chromatographic separation is performed on a Hewlett Packard RP-18 column using methanol–water (60:40, v/v) as the mobile phase. A fluorescent detector is employed with the excitation and emission wavelengths as 319 nm and 390 nm, respectively. The novel method yields a good linear relationship when the concentration of MFA-Na is within 1 and 500 *n*mol/mL (r = 0.9996). The detection limit is 50 *p*mol/mL. The established method is applied to determine MFA-Na in biological samples. The recovery rates of MFA-Na are between 81% and 88%, and the relative standard deviations are less than 5%. The method shows good sensitivity and selectivity for the determination of MFA-Na in biological samples.

Introduction

The chemical name for 1080 is sodium monofluoroacetate (MFA-Na), commonly known as 1080. The compound was first used as a rodenticide in the USA in the 1940s, and it has been used as a pest control in New Zealand since the 1950s. MFA-Na is the only poison registered for aerial control of possums (1). It is extremely toxic; with the LD_{50} in different species varied between 6–100 µg/kg for canine and murine animals and 714 µg/kg for human being (2).

There have been many cases of poisoning reported for fluoroacetate, with its sodium salt widely used as a rodenticide. Due to the potential toxicity of MFA-Na to the non-target animals, the possibility of secondary poisoning, and the investigation of the use of the substance as a poison in a crime, a sensitive method is required to characterize this compound in biological samples.

MFA-Na is strong, polar, water soluble, and non-volatile. To make the analysis possible, techniques such as capillary zone electrophoresis (3), ion-chromatographic (4), and ^{19}F NMR (5) have been used to detect this compound with poor detection limits. Prior derivatization of MFA-Na for gas chromatography (GC)–electron capture detection (6) or GC–mass spectrometry (7) has been used. GC–ECD determination was the most sensitive method employed by far. However, the complicated chromatogram and the poor selective detector make it difficult to differentiate the peak of MFA-Na derivative from the other impurity.

High-performance liquid chromatography (HPLC), one of the most effective separation tools, has been used for the analysis of a wide variety of samples including fatty acids. As UV–vis spectroscopy provides usually less sensitivity and selectivity than fluorescence detector; the latter technique is preferred as a detection procedure. MFA-Na itself has no fluorescence. Therefore, pre-chromatographic derivatizations are necessary for HPLC separation with fluorescent detection (8). 4-Bromomethyl-7-methoxycoumarin (BrMMC) is a common fluorescence-labeling reagent, which is used for the determination of compounds possessing a carboxylic group (9,10) and provides good sensitivity.

The aim of the present paper was to develop a sensitive HPLC analytical method with fluorescence detection for MFA-Na determination in biological samples. It was targeted to obtain better resolution and selectivity than those achieved by the GC methods and HPLC–UV–vis detection (11).

Experiment

Chemicals and reagents

MFA-Na (content > 98%) was obtained from the Public Security Bureau of Guangdong Province (China). BrMMC and tetrabutylammonium bromide were purchased from

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Sigma–Aldrich (St Louis, MO). Methanol was of HPLC grade, and all other chemicals were of analytical grade. All HPLC buffers solutions were filtered through 0.45-µm Millipore filters prior to use. The stock solution of MFA-Na at the concentration of 100 *n*mol/mL was prepared by dissolving 0.1 mg MFA-Na in 10 mL distilled water. BrMMC with the concentration of 100 *n*mol/mL and 5% of tetrabutylammonium bromide were prepared in acetonitrile separately. All solutions were stored in refrigerator before use.

Synthesis of 7-methoxy-4-methylenecoumarin monofluoroacetate

A mixture of BrMMC (269.1 mg, 1 mmol) and MFA-Na (400.0 mg, 4 mmol) equilibrated with tetrabutylammonium bromide was stirred in 25 mL of acetonitrile at 80°C in the dark for 1.5 h. The excessive amount of MFA-Na was removed by filtration, and the solvent was evaporated under reduced pressure. The residue was washed with 20 mL of water, dried, and recrystallized in hexane before use as standard for the fluorescence spectra study. The fluorescence intensity was measured on a RF-5301PC fluorescence spectrometer (Shimadzu, Japan) to record the excitation wavelength of 319 nm and emission wavelength of 390 nm, respectively. The fluorescence quantum yield was determined at 298 K as described by Bella et al. (12) using quinine sulfate as a standard (φ_f = 0.545 in 0.1M H_2SO_4).

HPLC apparatus and chromatographic condition

All measurements were carried out on an Agilent 1100 HPLC (HP, Palo Alto, CA) instrument equipped with programmable fluorescence (FL) detector. All chromatographic separations were performed on a Hewlett Packard RP-18 column (200 mm × 4.6 mm, 5 µm) with the column temperature set at 26°C. An aliquot (10 µL) of the sample was injected into the HPLC system. The mobile phase was consisted of methanol–water (60:40, v/v), and the flow-rate was 1.0 mL/min. Fluorescence was monitored at λex/λem = 319/390 nm. The Agilient ChemStation software was used for the instrument control and data analysis.

Sample preparation and derivatization

The serum sample was diluted five-fold with distilled water prior to extraction; other samples including meet, rice, or liver were homogenized by a blender. To a 100 µL diluted serum sample or 0.5 g homogenized samples, 0.5 mL acetone–water (4:1, v/v) was added. It was extracted by ultrasonication for 5 min and centrifuged at 4000 rpm for 5 min and the extract collected. The extraction process was repeated 3 times, and the combined extract was evaporated to approximately 0.5 mL with nitrogen steam in 30°C water bath. 2 mol/L HCl was added to the 0.5 mL residuum to adjust the pH to approximately 2.0. After vortexing for 5 min, the mixed sample was extracted with 0.5 mL ethyl acetate and centrifuged at 4000 rpm 10 min three times. The organic layer was transferred to a flask and evaporated to dryness in 35°C bath under nitrogen after adding 10 µL of 10% Triethanolamine, and then the sample was ready for the derivatization procedure.

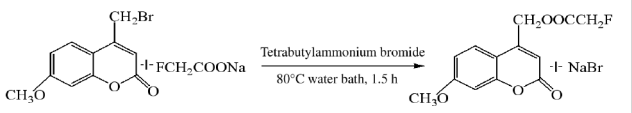

Figure 1. Derivatization of MFA-Na with BrMMC to form MMC-MFA.

BrMMC solution (100 *n*mol/mL) and 5% tetrabutylammonium bromide with 0.5 mL acetonitrile were added to the sample. The mixture was shaken and allowed to stand for 90 min in bath at 80°C in the dark. The reaction is shown in Figure 1. The derivative solution was diluted to 1 mL and ready for HPLC analysis. Triplicate injections for each sample were made. The concentration of MFA-Na was calculated from the calibration curve.

Preparation of calibration curves

Rat serum without MFA-Na was diluted five-fold with distilled water and used as blank. Blank samples (100 µL) were spiked with various amounts of MFA-Na, with the final MFA-Na concentrations of 1.0, 5, 10, 50, 100, and 500 nmol/mL, respectively. Sample preparations were repeated as described in the Sample preparation and derivatization section. The linear calibration graph was plotted by peak area of MMC-MFA versus the concentration of MFA-Na. The slope, intercept, and correlation coefficient (r) were calculated using Microsoft Excel software. The detection limit was defined as the amount that could be detected with a signal-to-noise ratio of 3.

Assay precision and precision

Quality control (QC) samples were prepared by spiking various amounts of MFA-Na to the rat serum blank or rice blank with final concentrations ranging from low (0.5 µg/mL or 0.5 µg/g), middle (5 µg/mL or 5 µg/g), to high (10 µg/mL or 10 µg/g). The absolute recoveries of different MFA-Na concentration were determined. The intra-day precision was determined by analyzing each QC sample (n = 3) on the same day. The inter-day precision was determined by analyzing each QC sample over a period of 5 days. The relative standard deviation (RSD) was used as an index of precision.

Results and Discussion

Chromatographic separation

The fluorescence properties of MMC-MFA

The absorption spectra of MMC-MFA show an absorption maximum at 319 nm with molar extinction coefficients of 21500 mol/L/cm. At the excitation wavelength 319 nm, the emission wavelength of 390 nm was confirmed. The quantum efficiency, based on quinine sulfate as a reference, was 0.622. Results showed MMC-MFA with high quantum efficiency provide sensitive fluorescent detection.

HPLC separation condition

The derivative product MMC-MFA was separated and detected by RP-HPLC equipped with fluorescent detector. MFA-Na solution (5 µL) (100 *n*mol/mL) with BrMMC solution (20 µL) (100 *n*mol/mL) were reacted as derivatization standard. Figure 2 shows the separation result of MMC-MFA [retention time (t_R) = 4.511 min], BrMMC (t_R = 10.246 min), and outgrowth (t_R =

3.405, 3.813 min) of the derivatization reaction products.

Because biological sample are complex, the influence of the existing fatty acid, such as formic acid, acetic acid, and propionic acid with their BrMMC esters were investigated. With the chromatographic condition described in the HPLC apparatus and chromatographic condition section, MMC-MFA (t_R= 4.438 min), BrMMC (t_R = 10.259 min), formic acid ester (t_R = 3.916 min), acetic acid ester (t_R = 5.534 min), and propionic acid ester (t_R = 6.533 min) were well separated as shown in Figure 3. The alcoholysis product and hydrolysate of BrMMC had the retention times of: t_R = 3.410 min and t_R = 11.45 min, respectively. The result indicated that the existence of fatty acids had no influence on chromatographic separation.

Optimum of derivatization conditions

As previously reported, the derivatization process consisted first of isolation of MFA-Na from the samples, followed by derivatization with BrMMC in acetonitrile with tetrabutylammonium bromide as catalyst to obtain the final fluorescent product. Attempts were made to improve the reaction condition of the derivatization in factors of stuff ratio, derivatization temperature, and reaction time.

First, the effects of BrMMC concentrations on the derivatization reaction were studied. To 5 nmol of MFA-Na, 1, 2, 3, 4, and 5 times of BrMMC were added. Derivatization reaction and HPLC analysis were carried out according to the methods described in the Experimental section. The result showed that the yield reached maximum at the ratio of 1:3 (MFA-Na vs. BrMMC) and reached a plateau, with increasing amounts of BrMMC. Therefore, the minimum amount of BrMMC required is three times of the concentration of MFA-Na. However, in order to keep safe excess amounts of labeling agent, a ratio of 1:4 is used in the study.

The effects of temperature were also studied. Samples with a MFA-Na versus BrMMC ratio of 1:4 were heated at the temperatures of 60°C, 70°C, 80°C, and 90°C for 90 min. The result indicated that the reaction temperature of the derivatization was preferred at 80°C.

The reaction time was then optimized too. At the reaction temperature of 80°C and the MFA-Na versus BrMMC ratios of 1:4, the maximum yield of MMC-MFA was reached after 70 min. The yield remained constant from 70 to 120 min. A reaction time of 90 min was used in this study.

Linearity, detection limits, precision and accuracy

A calibration curve for MMC-MFA was prepared as described in the Preparation of calibration curves section. A good linear relationship, ranging from 1 to 500 nmol/mL was obtained, with the equation of $y = 118.04x - 88.03$, when y was the peak area of MMC-MFA and x was MFA-Na concentrations (nmol/mL). The correlation coefficient (r) was 0.9996. The detection limit was 50 pmol/mL.

Repeatability of the method was expressed as RSD (n = 3). Results are shown in Table I. For all measurements, RSD values were found to be lower than 5% for the standards ranging from low (0.5 µg/mL or 0.5 µg/g), middle (5 µg/mL or 5 µg/g), to high (10 µg/mL or 10 µg/g) data on accuracy, which is expressed as the recoveries obtained were between 81% and 88%. The results for intra- and inter-day precision are shown in Table I. The intra- and inter-day RSD (n = 3) were less than 6%.

Application on sample analysis

Samples of serum and food from a poisoned human body in a real toxicology criminal case were taken for examination. Samples were extracted and derivatized as described in the Sample preparation and derivatization section. The chro-

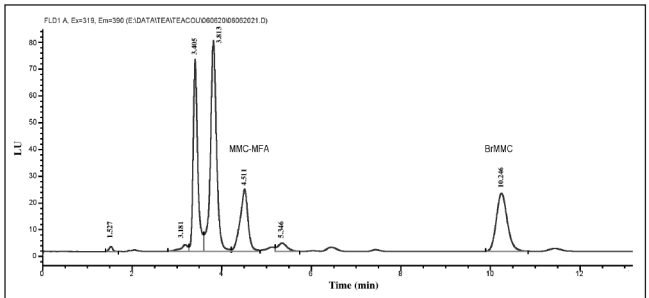

Figure 2. Chromatogram for the derivatization sample of standard.

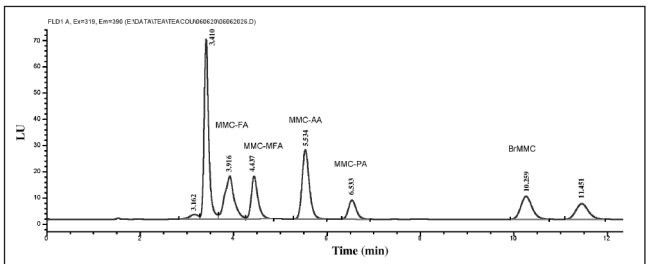

Figure 3. HPLC Chromatogram of MFA-Na and some organic acids as their derivative of BrMMC.

Table I. Result of Accuracy and Precision Data of Different MFA-Na Quality Concentration in Samples (n = 3)

	Blood		Rice		Meat		Intra-day
Samples Inter-day	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	RSD (%)
Low QC (0.5 µg/mL or 0.5 µg/g)	81.4	4.9	82.3	3.9	87.3	2.1	4.2
Middle QC (5 µg/mL or 5 µg/g)	83.2	3.8	86.3	2.4	88.6	1.3	4.7
High QC (10 µg/mL or 10 µg/g)	81.9	4.4	83.7	4.2	86.4	1.9	5.7

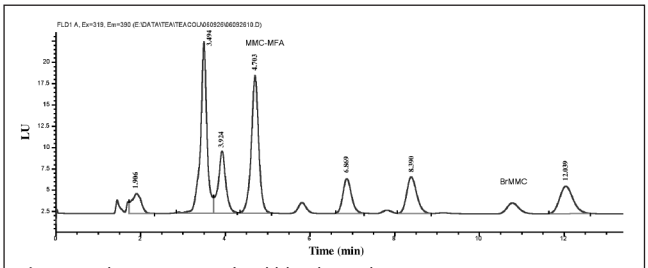

Figure 4. Chromatogram of real blood sample.

matograms were more complex than that of standards only (Figure 2). However, the resolution of the MMC-MFA is not impaired, and the peak area ratios can be calculated without difficulty. The chromatogram obtained are shown in Figure 4.

Conclusion

The proposed HPLC method based on precolumn flurescence derivatization appears to be suitable for the rapid and sensitive determination of MFA-Na in biological samples. Separation of MFA-Na by HCl hydrolysis and liquid–liquid extraction is adopted. MFA-Na reacted with BMMC to form MMC-MFA for determination, which offers better selectivity and sensitivity. The method also shows good precision and reproducibility, which has been applied to determine the MFA-Na in spiked biological samples and in the tissues from the toxicology criminal case with satisfactory results.

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