II.7.8 Coumarin rodenticides

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Introduction

As coumarin rodenticides, warfarin, coumatetralyl, coumafuryl, coumachlor and bromadiolone are commercially available in Japan. The coumarin rodenticides do not show direct anticoagulant action causing bleeding, but inhibit the metabolic cycle of vitamin K; the inhibition causes the interference with protein biosynthesis of vitamin K-dependent coagulant factors (II, VII, IX and X factors) in the liver, which are very important for the blood coagulation system. The lowered coagulant factors cause the bleeding deaths of the rodents [1]. Warfarin, coumatetralyl or coumafuryl is not effective with single administration, but becomes effective by repeated intakes of a small amount of each poison for 4–5 days successively. Coumachlor and bromadiolone are much more potent and long-lasting rodenticides with long biological half-lives; they provoke poisoning signs and symptoms, which last for a long time, only with their single administration [2]. Such a potent rodenticide is called "super-warfarin".

Warfarin is also very popular as an oral anticoagulant drug for treatment and prevention of thromboembolism.

Although the analysis of coumarin rodenticides and anticoagulants is carried out largely by HPLC [3, 4], a GC/MS method for analysis of 4 rodenticides is presented in this chapter.

Reagents and their preparation

- Coumarin rodenticides can be obtained in the forms of crystals or powder. They are slightly water-soluble and almost stable under storage at room temperature [4].
- Standard compounds: warfarin and coumachlor can be purchased from Sigma (St. Louis, MO, USA); coumatetralyl and bromadiolone from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). A 100-mg aliquot each is dissolved in 100 mL methanol (1 mg/mL) as a stock solution. To use one of them as internal standard (IS), the above solution is diluted 10-fold with 50 % methanol aqueous solution (100 μg/mL). The above solutions should be stored at 4 °C under light-shading conditions.
- Mixed standard solution for calibration curves: 1-mL aliquots of the above 4 stock solutions (1 mg/mL) is mixed with 9 mL of 50 % methanol aqueous solution (final volume 10 mL, 100 μg/mL for each compound).
- Spiked serum solutions for the calibration curves [5]: 50-, 10-, 1- and 0.3-μL volumes of the above mixed standard solution (100 μg/mL) are spiked into 1-mL volume blank serum specimens (final concentration, 5, 1, 0.1 and 0.03 μg/mL, respectively).
- 30 % Methanol buffer solution: 70 mL of 0.1 M citrate buffer solution (pH 6.0) is mixed with 30 mL methanol.
- Extraction solvent: chloroform/isopropanol (9:1, v/v).
- Derivatization reagents: trimethylsilyldiazomethane (TMS-DAM, 10 %, v/v in hexane, GL Sciences, Tokyo, Japan), *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)

and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (both from Pierce, Rockford, IL, USA, and other manufacturers).
- Serum: pooled serum obtained from healthy subjects.

GC/MS conditions

GC column: a DB-5MS methylsilicone medium-bore capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, J & W Scientific, Folsom, CA, USA).

Conditions; GC/MS instrument: Shimadzu GCMS-QP5050A (Shimadzu Corp., Kyoto, Japan); column (oven) temperature: 210 °C (1 min, splitless) → 10 °C/min → 330 °C (3 min); injection temperature: 250 °C; carrier gas: He; flow rate: 0.9 mL/min (sampling time, 2 min); interface temperature: 250 °C; detector temperature: 250 °C; ion source: EI; electron energy: 70 eV.

Ions selected for quantitation: those shown in *Table 8.1*.

Procedure

i. A 0.5-mL volume of a specimen[a] is mixed with 1 mL of 0.1 M citrate buffer solution[b] (pH 6.0) and 20 μL IS solution[c].
ii. The solution is poured into an activated Oasis®HLB cartridges[d,e,f] (Waters, Milford, MA, USA).
iii. The cartridge is washed with 3 mL purified water and 3 mL of 30 % methanol buffer solution[g].

Table 8.1
Molecular formulae and mass spectral ions for coumarin rodenticides (anticoagulants)

Common name (IUPAC)	Molecular formula	M.W.	DI*	Principal mass spectral ions (*m/z*)			DP**
				ME derivative	TMS derivative	TBDMS derivative	
Warfarin (3-(α-acetonylbenzyl)-4- hydroxycoumarin)	$C_{19}H_{16}O_4$	308.4	265 103 131	279 322 91	337 193 380	261 379 423	131 103 145
Coumatetralyl (3-[1-(2-furyl)-3-oxobutyl]-4- hydroxycoumarin)	$C_{19}H_{16}O_3$	292.4	292 121 188	306 175 202	364 260 245	407 349 321	–
Coumachlor (3-[1-(4-chlorophenyl)-3- oxobutyl]-4-hydroxycoumarin	$C_{19}H_{15}ClO_4$	342.8	299 121 43	313 356 125	371 414 373	261 413 458	165 137 180
Bromadiolone (3-[3-(4′-bromobiphenyl-4-yl)- 3-hydroxy-1-phenylpropyl]-4- hydroxycoumarin)	$C_{30}H_{23}BrO_4$	527.4	–	–	–	–	158 173 143

* DI: mass spectra of underivatized compounds by the direct inlet method.
** DP: mass spectra of underivatized compounds measured by GC/MS.

iv. A target compound and IS are eluted with 4 mL of chloroform/isopropanol (9:1, v/v)[h].
v. A small amount of upper aqueous layer is removed with a Pasteur pipette. An appropriate amount of anhydrous Na_2SO_4[i] is added to the lower organic phase and mixed well. After settlement of the mixture, clear organic phase is transferred to a glass vial with a screw cap and evaporated to dryness under a stream of nitrogen[j].
vi. A 50-μL volume of TMS-DAM is added to the residue, capped, vortex-mixed for 15 s and heated at 60 °C for 30 min on a heat block or in a water bath for methyl derivatization[k].
vii. After cooling to room temperature, the solution is evaporated to dryness under a stream of nitrogen; the residue is dissolved in 50 μL ethyl acetate.
viii. A 1-μL aliquot of it is injected into GC/MS for measurements in the selected ion mode (SIM)[l].

Assessment and some comments on the method

Warfarin absorbed into a human body is metabolized almost entirely; it is excreted into urine in the forms of 7-hydroxywarfarin, 6-hydroxywarfarin and warfarin alcohol. For analysis of such metabolites in urine, the details of the procedures were reported by de Vries et al. [6] and Maurer et al. [7].

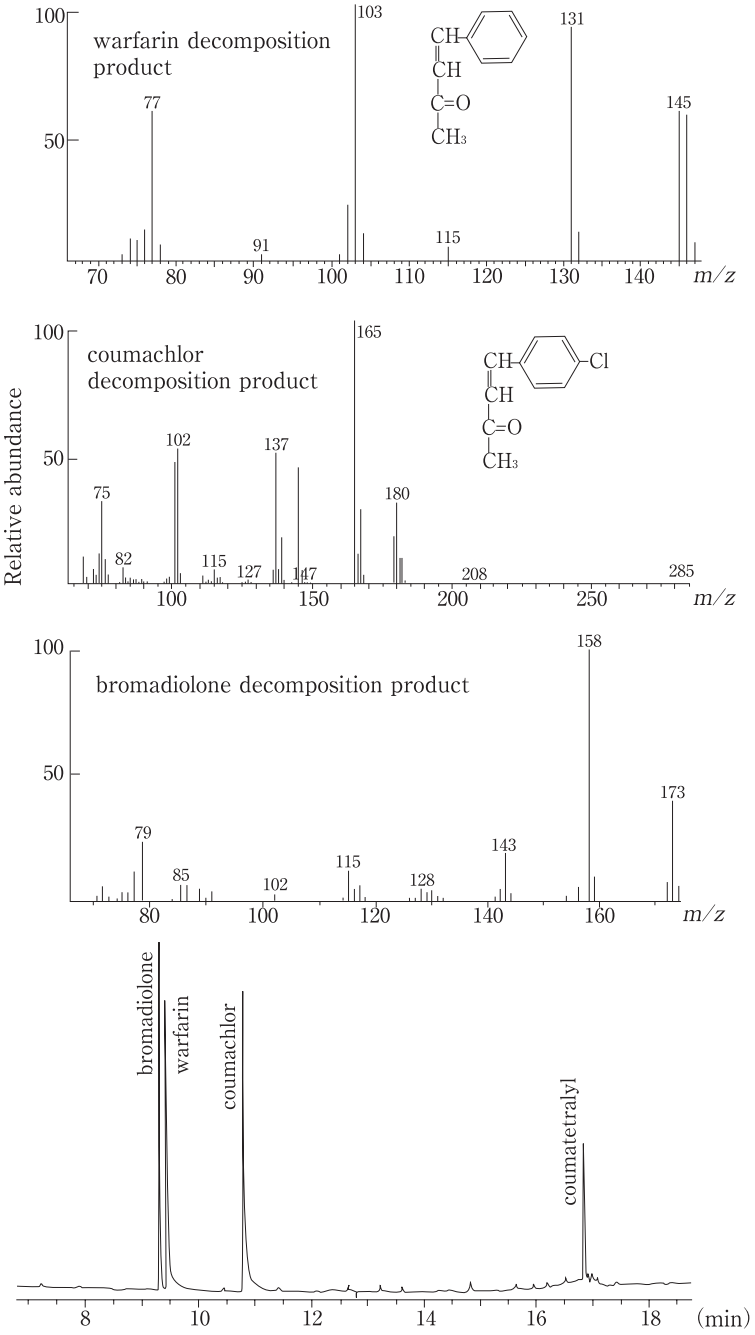
As an elution solvent for the solid-phase extraction cartridge, dichloromethane or chloroform/isopropanol (9:1, v/v) was best to get good recovery rates of the 4 coumarin rodenticides; they gave the rates of 92–97 %. A centrifugal freeze dryer can be used in place of the nitrogen stream, because it is useful for rapid evaporation without decomposition.

The functional group of the coumarin rodenticides is -OH. Because they are nonvolatile and highly adsorptive, derivatization is required for their GC and GC/MS analysis [8–10]. Among the 4 compounds tested, only coumatetralyl can be detected without any derivatization; other 3 compounds are immediately decomposed by heat of injection chamber, resulting in the detection of decomposition products. For warfarin and coumachlor, their derivatization is essential. Both compounds can be methylated with TMS-DAM [11], trimethylsilylated with BSTFA[m] [9,10,12] and *tert*-butyldimethylsilyl (TBDMS)-derivatized with MTBSTFA[n] [9]. For bromadiolone, however, it is difficult to detect the compound by GC (/MS) after any derivatization (*Table 8.1*). Since bromadiolone is highly toxic, the author dared to detect its decomposition product (*Figure 8.1*).

For rapid screening analysis of drugs and poisons at the spot of medical treatments, the analysis without derivatization seems more common. Therefore, the results obtained from GC/MS analysis of warfarin, coumachlor and bromadiolone without derivatization are shown in *Figure 8.1*. The mass spectra of warfarin, coumatetralyl and coumachlor after different derivatizations are shown in *Figures. 8.2–8.4*. The respective principal ions are summarized in *Table 8.1*. The identities of the underivatized compounds and their derivatized forms were confirmed by GC/MS in the chemical ionization mode. *Figure 8.5* shows TIC and SIM chromatograms for some coumarin rodenticides; the SIM chromatogram was also obtained from serum of a patient being treated with warfarin.

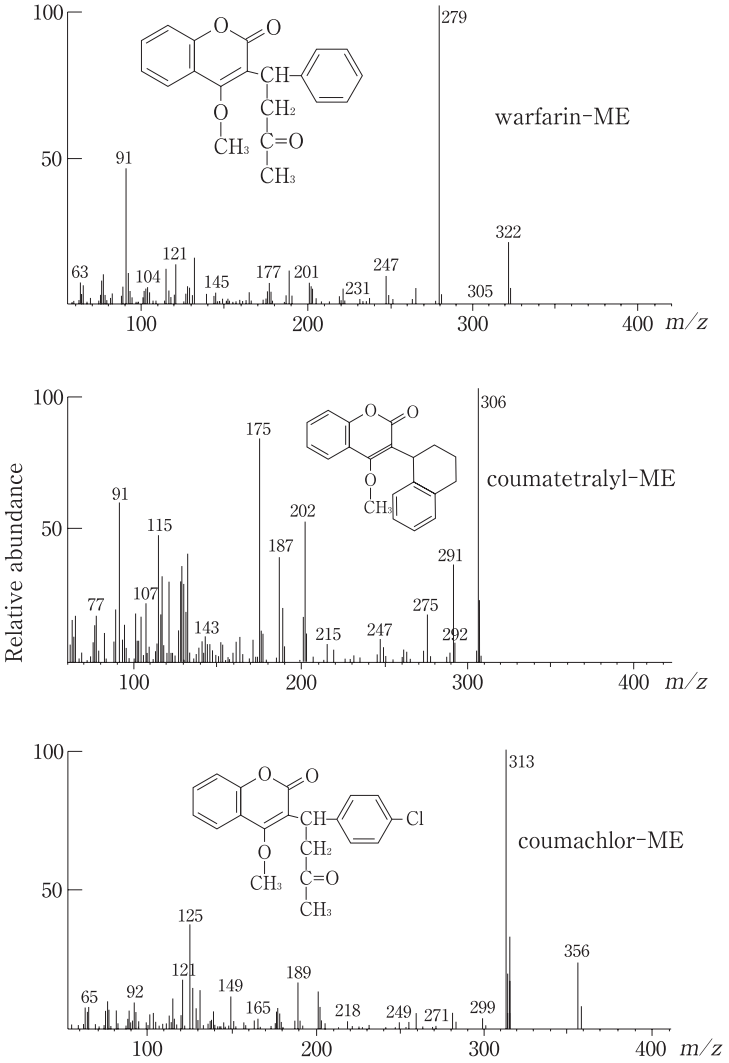
The quantitative ranges in the SIM mode for coumarin rodenticides in sera after methyl derivatization were: 10–2,000 ng/mL for warfarin, 5–2,000 ng/mL for coumatetralyl and 10–5,000 ng/mL for coumachlor; that for a decomposition product of bromadiolone in serum without derivatization, 30–5,000 ng/mL. The detection limits were 20, 10, 20 and 30 ng/mL for

Figure 8.1



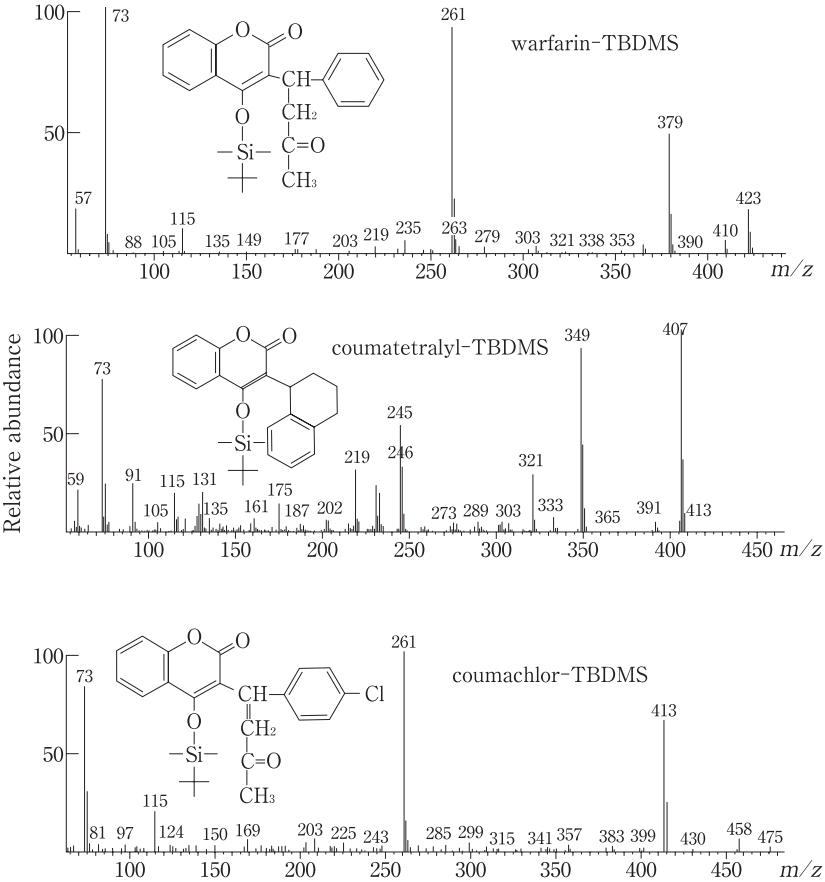
TIC (bottom panel) and mass spectra obtained by GC/MS for coumarin rodenticides (anticoagulants) without any derivatization. The concentration of each rodenticide in the mixture solution was 2 µg/mL. For GC/MS conditions, see text. Column (oven) temperature: 50 °C→20 °C/min→330 °C.

Figure 8.2



Mass spectra of methyl derivatives of 3 coumarin rodenticides (anticoagulants). The concentration of each rodenticide was 2 µg/mL. For GC/MS conditions, see text.

Figure 8.3



Mass spectra of TBDMS derivatives of 3 coumarin rodenticides (anticoagulants). The concentration of each compound and GC/MS conditions are the same as specified in *Figure 8.2*.

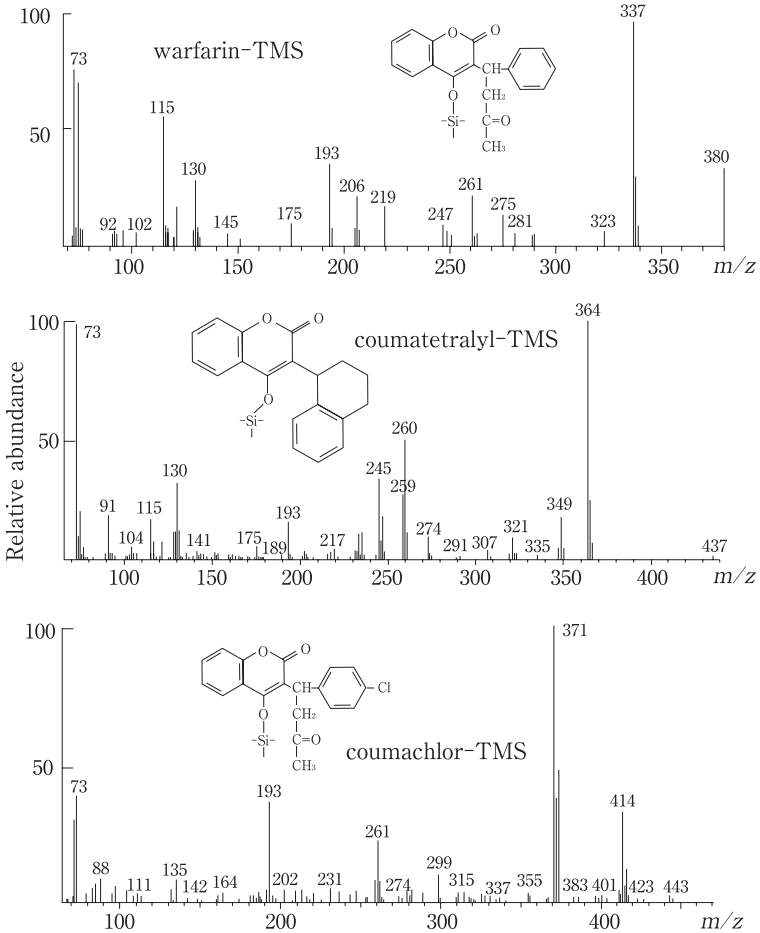
warfarin, coumatetralyl, coumachlor and bromadiolone, respectively. There are no interfering impurity peaks due to blood overlapping the test peaks in the SIM chromatograms.

Therapeutic and toxic concentrations of warfarin

The poisoning symptoms by warfarin do not appear shortly after its administration, but appear 12–48 h after and last for 48–75 h [13]. The symptom most frequently observed is bleeding; necrosis of skin tissues was occasionally reported [1]. Nakahata et al. [13] reported that doses of warfarin to be required for controlling the blood coagulation system differed greatly (about 14-fold) among different patients. Also for poisoning symptoms, great variations are expected among individuals.

Since there is no relationship between blood warfarin concentration and bleeding [1], coagulation tests such as prothrombin time test (PT) and thrombo test (TT) are required for the diagnosis of coumarin anticoagulant poisoning, for the assessment of its severity and for ob-

Figure 8.4



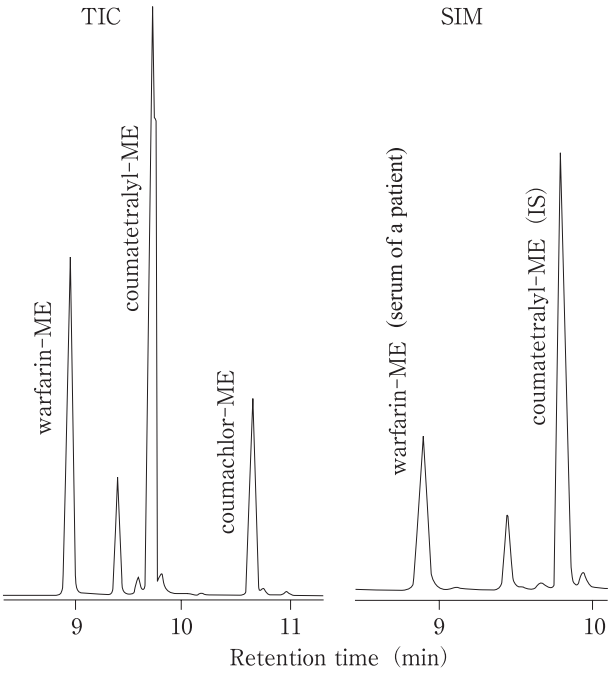
Mass spectra of TMS derivatives of 3 coumarin rodenticides. The concentration of each compound and GC/MS conditions are the same as specified in ***Figure 8.2*****.**

servation of the process [14]. It depends on the backgrounds of patients; but when the International Normalized Ratio (INR) exceeds its therapeutic range (2.0–3.0), there is a high risk of bleeding. Especially for the second-generation anticoagulant rodenticides effective for long times (super-warfarins), the long-time follow-up of coagulation ability is necessary, because they remain in the body for a period longer than that with the first-generation rodenticides, causing the elongation of the period for hemorrhage.

Warfarin metabolites are excreted into urine and feces (*via* bile); about one third of a total warfarin administered is excreted into urine as its metabolites. Warfarin is not excreted in the unchanged form, but excreted in the metabolite forms. When warfarin is administered orally, 99% of the dose is excreted within 6 days.

When a single small dose of warfarin is administered by mistake, there is no need for treatments. Even for the intake of a large amount of warfarin or for repeated intakes, the oral or

Figure 8.5



TIC for the 3 standard coumarin rodenticides (anticoagulants) and SIM chromatogram for the serum extract of a patient undergoing the warfarin therapy after methyl derivatization. For the TIC, each compound at 2 μg/mL was used.

intravenous administration of vitamin K is very effective for recovery; the PT values become normal in about 24 h.

Warfarin is used for prevention of thrombosis after the operations of cardiac valve replacement and of the coronary bypass conduit construction. The decision of its proper doses is made by monitoring coagulation ability using PT and TT. However, during such therapies, fatalities due to hemorrhage by the action of various deuteropathic factors were reported [15].

The blood warfarin concentrations in seven patients taking warfarin as a therapeutic drug were 191–800 ng/mL. The therapeutic blood warfarin concentrations were reported to be 0.3–10 μg/mL in literature; toxic ones not less than 10 μg/mL [16, 17].

Notes

a) When a specimen is serum, the ratio of warfarin bound with serum proteins is very high; the free warfarin not bound with them is only about 1 % [1, 13].
b) A viscous specimen, such as serum, should be diluted with an equal volume or 2 volumes of the buffer solution to get better trapping efficiency.
c) As IS, one of the coumarin anticoagulants other than a target compound is chosen. For analysis of warfarin, coumatetralyl is good as IS.

d) An Oasis®HLB Plus cartridge is activated by passing 3 mL methanol and 3 mL purified water through it.
e) It can be replaced by a Sep-Pak C_{18} cartridge (Waters). The drying of the cartridge or the inclusion of air does not affect the recovery rate for the Oasis®HLB cartridge, but lowers the rate for the Sep-Pak C_{18} cartridge.
f) The flow rate of the sample solution through the cartridge should not be faster than 2 mL/min.
g) The same syringe should be used for washing the cartridge, because the residual specimen solution inside the syringe should be completely poured into the cartridge.
h) The elution should be made at a flow rate not faster than 2 mL/min. After elution, the small amount of upper aqueous layer should be immediately removed with a Pasteur pipette, because water-soluble coumatetralyl and bromadiolone may easily transfer into the aqueous phase. Upon elution with chloroform/isopropanol, the use of a plastic disposable syringe causes its melting; a glass syringe should be used for solutions containing chloroform.
i) Anhydrous Na_2SO_4 is used for removing water dissolved in the organic solvent.
j) The drying up should be made completely. When a trace amount of water remains, the derivatization is not successful, and the derivatized product is easily hydrolyzed [9].
k) Upon GC/MS analysis of warfarin, coumachlor and bromadiolone, they are decomposed by heat of the injection chamber and detected as heat-decomposition products. Therefore, derivatization is recommendable for warfarin and coumachlor.
l) Bromadiolone cannot be derivatized by any method. It had to be measured using its heat-decomposition product.
m) The residue is dissolved in 20 μL of well-dried *N*,*N*-dimethylformamide and 50 μL BSTFA, capped, vortex-mixed for 15 s and heated at 90 °C for 45 min for TMS derivatization. After cooling to room temperature, a 1-μL aliquot of it is injected into GC/MS for measurements in the SIM mode. It should be noted that the derivative is easily decomposed and thus should be measured soon after derivatization.
n) The residue is dissolved in 20 μL of well-dried *N*,*N*-dimethylformamide and 50 μL MTBSTFA, capped, vortex-mixed for 15 s and heated at 60 °C for 20 min in a water bath. After cooling to room temperature, a 1-μL of it is injected into GC/MS for measurements in the SIM mode. *N*,*N*-Dimethylformamide is used for dissolution of a refractory target compound in derivatization reagent solution and for enhancement of the reactivity.

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