

LC/MS/MS Analysis of Anticoagulant Rodenticides in Hair

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Introduction

Hair testing has been established as a routine method in drug monitoring. Hair analysis provides information concerning multiple abuse or long-term exposure to drugs and/or medications. The number of substances which are detectable in hair has increased enormously during the past years. However, not much work has been published concerning the analysis of hair for coumarin derivatives such as the common anticoagulant phenprocoumon and rodenticides of the warfarin or superwarfarin type, which are used to kill rats, mice and other rodent pests. Encouraged by a forensic investigation in a case of domestic violence, there was a need to develop an analytical method for the detection of anticoagulant residues in hair. This should help to answer the question, if a person had been incorporating anticoagulants over a longer period of time. For the analysis of coumarin derivatives in serum, an HPLC method, using UV and fluorescence detection, is described in the literature¹. Since hair is a critical matrix, which is known to produce a lot of interfering signals and a large background in UV detection, tandem mass spectrometry seems to be preferable as detection method because of its higher sensitivity and selectivity.

Experimental

Sample Preparation

Hair samples were extracted with methanol under sonication. First, the hair was washed for 5 min each with water (5 mL), acetone (5 mL) and hexane (5 mL). After drying, the hair was cut into small pieces of about 1 mm which were placed in a polypropylene vial. A 50 mg sample of hair was used for analysis and 5 mL of methanol was added. The closed vial was sonicated for 4 h at 50°C. The methanol was evaporated and the residue was dissolved in 1 mL phosphate buffer (0.05 M, pH 6). After addition of 0.5 mL of citrate-phosphate buffer (pH 5.5; mixture of 0.1 M citric acid solution and 0.2 M disodium hydrogen phosphate solution, 2:3, v/v), the anticoagulants were extracted by vortexing with 3 mL chloroform-acetone (1:1, v/v) for 1 minute. The mixture was centrifuged for phase separation. The lower organic layer was separated and the solvent was evaporated under a slight stream of nitrogen at 40°C. The residue was reconstituted in the mobile phase.

Experimental

LC/MS/MS Method

The LC/MS/MS consisted of an Agilent 1200 Rapid Resolution Liquid Chromatograph and an Agilent 6410A Triple Quadrupole Mass Spectrometer. Due to the chemical structure of the rodenticides, different ionization sources (API-ES, APCI and APPI) were coupled to the mass spectrometer to determine the optimal sensitivity and selectivity for the five analytes. Determination of the different MRM transitions for all analytes was carried out by flow injection analysis of standards at concentration levels around 0.5 ppm using MassHunter Optimizer, an automated MRM method development software. The chromatographic separation of the coumarin derivatives was carried out on a Zorbax CN column (50*2.1mm, 1.8 µm). The mobile phase consisted of 5 mM Ammonium acetate in water and methanol (flow 0.4 mL/min). The total analysis time, including conditioning of the column, was set to 13 min. The parameters of the complete LC/MS/MS method, including the MRM transitions, are summarized in Table 1a and b.

Table 1a: LC/MS/MS method parameters

Column:	Agilent Zorbax CN RR-HT (50*2,1 mm, 1.8 µm)		
Column temp:	60°C		
Mobile phase:	A: 5mMol Ammoniumacetate in water B: Methanol		
Flow rate:	0.4 ml/ min		
Gradient:	15 % B at 0 min; 15 % B at 1.0 min 40 % B at 3.0min; 90% B at 6.0 min 90 % B at 7.7 min; 15% at 7.8 min		
Stop time:	12 min	Post time:	3 min
Ini Vol:	5 ul		
Source:	Standard ESI Source		
Time Seg#			
1	0.0 min	ESI pos. To MS	Delta EMV 400V
2	3.5 min	ESI neg. To MS	Delta EMV 600V
3	8.5 min	ESI pos. To Waste	Delta EMV 400V

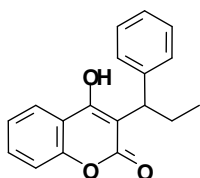
Source Parameters:
Gas Temp: 320°C Gas Flow 8l/min
Nebulizer: 50 psi Capillary:4000 V

Rodenticide	Polarity	Prec Ion [m/z]	Frag(V)	CE1(V)	Prod Ion 1 [m/z]	CE2(V)	Prod Ion 2 [m/z]
Phenprocoumon	POS	281	110	12	203	28	175
Warfarin	POS	309	100	12	163	16	251
Bromadiolon	NEG	527	175	40	250	40	181
Bromdifacoum	NEG	523	180	44	135	52	81
Difenacoum	NEG	443	180	36	293	36	135

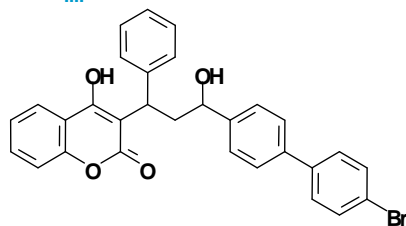
Table 1b: Data Acquisition parameters for the optimized MRM transitions

Rodenticides

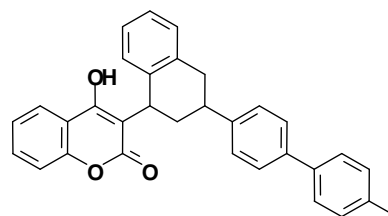
Phenprocumon
(M_{mi} : 280.1 Da)



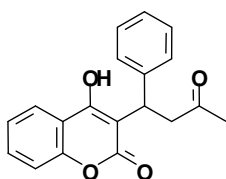
Bromadiolon
(M_{mi} : 526.1 Da)



Difenacoum
(M_{mi} : 444.2 Da)



Warfarin
(M_{mi} : 308.1 Da)



Brodifacoum
(M_{mi} : 522.1 Da)

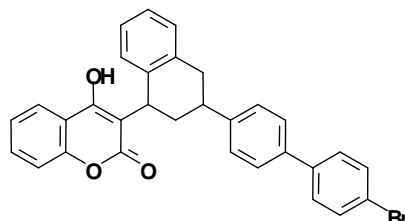


Figure 1: Structures of investigated rodenticides

Results and Discussion

Due to the chemical structure of the rodenticides, three different ionization techniques (API-ES, APCI and dopant-APPI) were investigated to achieve optimal sensitivity and selectivity for the five rodenticides. A comparison of the sensitivity values (normalized S/N results for a 50 ppb rodenticide standard) is shown in **Figure 2**. APPI and ESI, both performed in positive polarity, are the optimum ionization modes for phenprocumon and warfarin, whereas difenacoum and brodifacoum produce the best results in negative APCI and ESI mode. Bromadiolon has a unique position; it only ionizes in negative mode, using APCI and ESI. Negative APPI showed poor results for all five compounds. For routine analysis, a standard electrospray source was chosen.

Using an LC/MS/MS method which started in positive mode and switched to negative polarity after 3.5 min resulted in the best response (**Figure 3**) for all five compounds. After 8.5 min another time segment was added, in which the MS diverter valve was switched to waste.

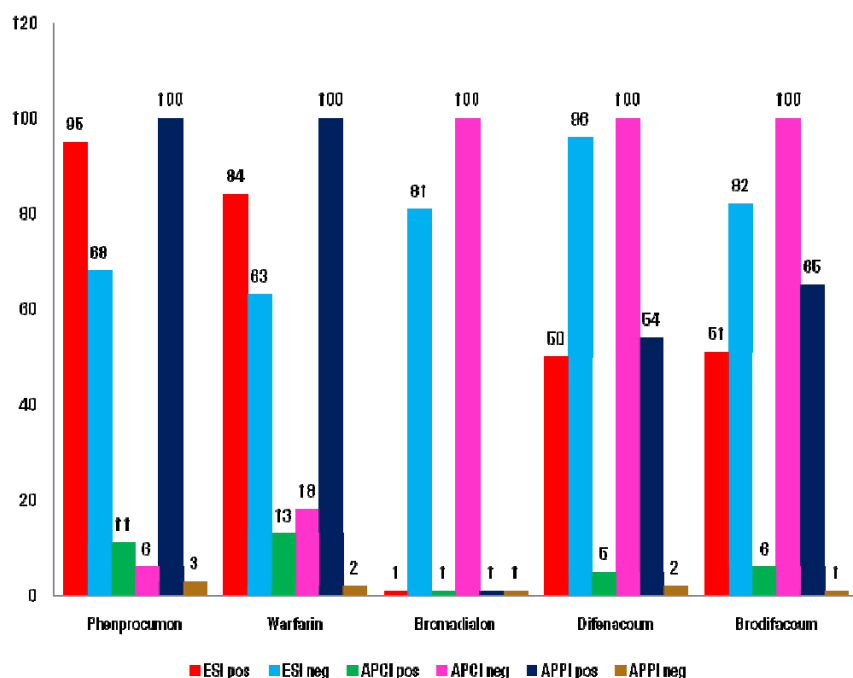


Figure 2: Comparison of different ionization techniques (S/N values for the rodenticides normalized to 100%)

Results and Discussion

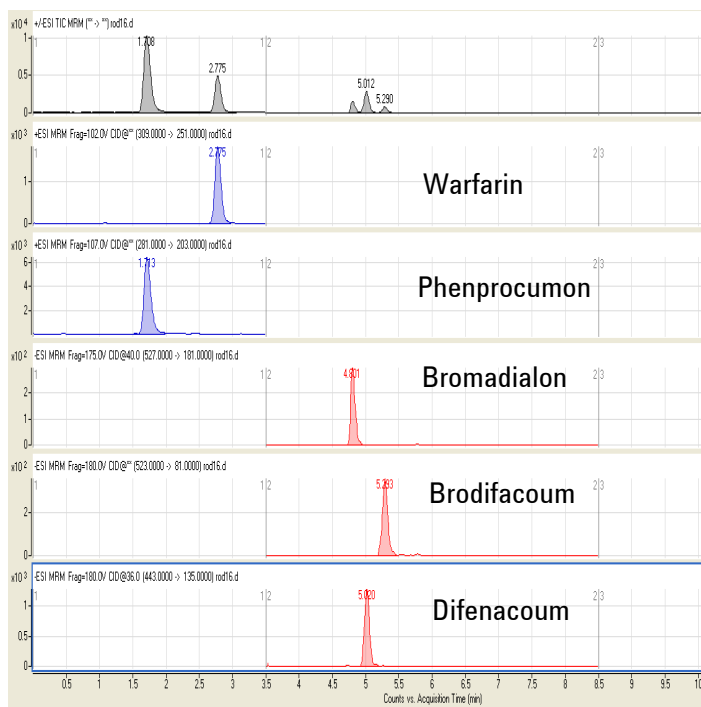


Figure 3: TIC and EICs for the five rodenticides (10 ppb standard in MeOH/water)

The defined calibration range for the anticoagulants in matrix was 0.5-500 ng/ml. A seven-level calibration with three replicate injections for each compound was measured. The calibrations were linear over the range tested and the correlation coefficients were > 0.99 for all five rodenticides (Figure 4).

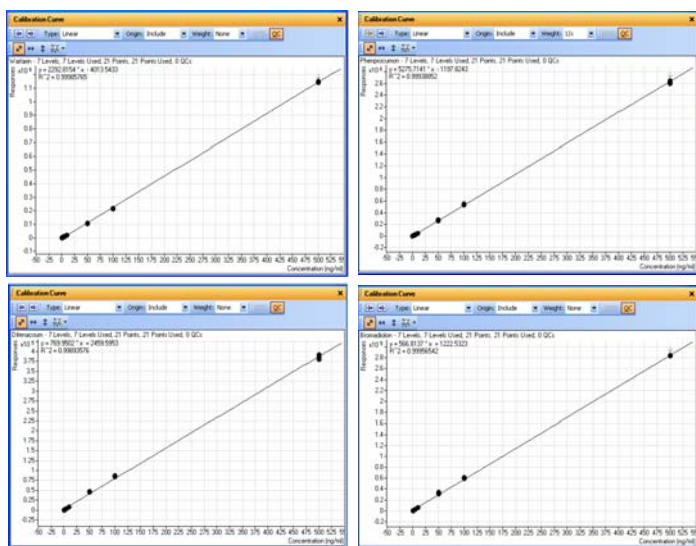


Figure 4: Calibration curves of four rodenticides (calibration range: 0.5-500 ppb)

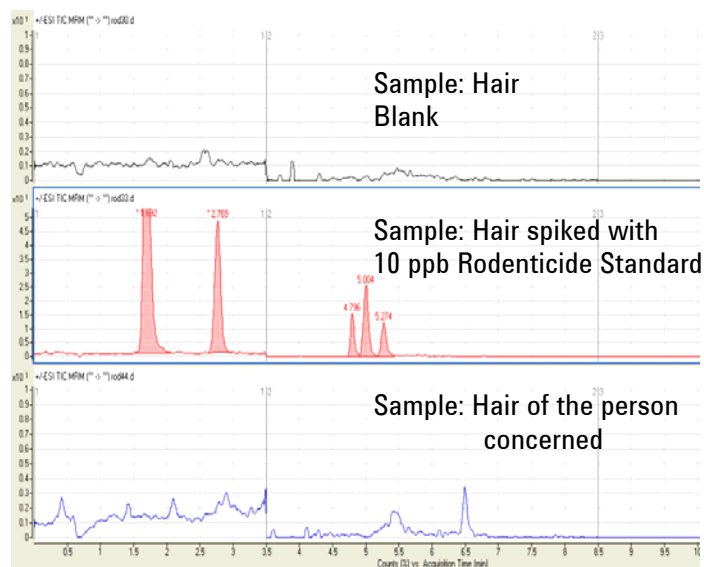


Figure 5: Results of the forensic investigation

An intra-assay (within-day reproducibility) and inter-assay precision (day-to-day reproducibility) test to validate the LC/MS/MS method will be carried out in the near future. In the case of the forensic investigation of domestic violence, the calibration data of the rodenticides in matrix were compared with the analysis data of a hair sample of the person concerned and a hair blank (Figure 5). The comparison clearly showed, that there is no evidence for incorporation of the anticoagulants over a longer period of time.

Conclusions

- Different ionization techniques can be used for the analysis of the anticoagulant rodenticides. The use of a standard ESI source allows the detection of all analytes with optimal response while changing the polarity on a time-based scale.
- In the calibration range (0.5-500 ppb) all compounds (in matrix) show a linear response with correlation coefficients >0.99.
- In the case of a forensic investigation of domestic violence, which was the reason for developing a LC/MS/MS method for anticoagulant rodenticides, there was no evidence for incorporation of the rodenticides over a longer period of time.

¹ E.Kuijpers, J. den Hartigh, J.Savekoul and F. De Wolff Journal of Analytical Toxicology, Vol 19, Nov./Dec. 1995