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Fatal intoxications by acenocoumarol, phenprocoumon and warfarin: Method validation in blood using the total error approach☆

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ABSTRACT

A simple high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection has been developed and validated for simultaneous identification and quantification of three antivitamin K drugs (acenocoumarol, warfarin and phenprocoumon) in whole blood. The aim of this development was to propose an analytical technique adapted to the situations of forensic toxicology, i.e. intoxication with massive anticoagulant doses, when the usual coagulation tests could not be used. The blood sample, after spiked with prazepam as an internal standard (IS), was submitted to a liquid–liquid extraction (LLE) prior to HPLC analysis. A chromatographic separation was achieved on a C8 Symmetry column with a mobile phase consisting of an acetonitrile and phosphate buffer (pH 3.8) mixture in a gradient mode. Detection was carried out at a wavelength between 200 and 400 nm. This method has been validated with the concept of total error as decision criterion. Trueness ranged from 99.1% to 105.0% and precision was good with RSD between 1.3% and 6.7%. Consequently, this rapid and simple chromatographic technique is well adapted to focus intoxications with most important coumarinic drugs available on pharmaceutical market and is now routinely used in our laboratory for forensic "general unknown" screening.

1. Introduction

The coumarinic anticoagulants have been used for more than 50 years like antithrombotic drugs, and specially in the primary and secondary prophylaxis of thromboembolic disease. These drugs are very effective and very much used. The response to these drugs is based on an inhibition of the synthesis of vitamin K-dependent coagulation factors [1]. The great individual variability in response to coumarin anticoagulant is caused by many different factors, including the rate of absorption and metabolic transformation, diet, genetically determined resistance to the drugs and also drugs concentration in blood at steady state [1,2]. Consequently, administration of these products has to be made with respect of a narrow therapeutic index, is delicate and needs regular medical control to avoid risks of haemorrhage [1,2]. Indeed, the therapeutic overdose can be at the origin of particularly serious hemorrhagic accidents, causing death in case of late medical intervention [3].

In the group of 4-OH coumarins, the most frequently used anticoagulant is warfarin in the USA and the U.K., whereas acenocoumarol and phenprocoumon are the first-choice elsewhere. These products have nearly 100% oral bioavailability and a high degree of protein binding, approximately 99%. They are metabolized by hepatic cytochrom P-450 isoenzymes with production of inactive hydroxylated metabolites [1,4–5].

Coumarins inhibit hepatic synthesis of the vitamin K-dependent coagulation factors II, VII, IX and X as well as of the anticoagulant protein C and S. They inhibit the vitamin K epoxide reductase, preventing vitamin K from being reduced to its active form, cofactor in the synthesis of these clotting factors. The optimal therapeutic effect is observed only after 3–5 days of treatment, time necessary for the consumption of synthesized factors. The nature and the amount of antivitamines K introduced do not influence this latency time, but well the duration and the importance of the delayed anticoagulant action. The reversibility of the anticoagulant effect depends on the half-life of elimination of coumarins and on the speed of renewal of the coagulation factors [1,4–5].

Therapeutic monitoring of the effect of these drugs is usually done by measuring a coagulation parameter, the international normalized ratio (INR). The risk of haemorrhage increases significantly and linearly as soon as the parameter is higher than 3 [6]. However, several situations can impose direct identification and quantification of the anticoagulant agent, particularly in forensic cases [7]. Deaths can indeed be caused by excessive doses of antivitamins K, after volunteer consumption or not. The determination of the

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INR in a postmortem blood is not possible, and then only specific identification of anticoagulant drugs can confirm intoxication. All concentrations higher than the therapeutic index can be considered as potentially toxic, which was corresponding to 100 μg/L for acenocoumarol, 3600 μg/L for phenprocoumon and 3000 μg/L for warfarin [8].

The aim of this study was to develop a sensitive and specific method for the simultaneous determination of acenocoumarol, phenprocoumon and warfarin in postmortem blood by HPLC DAD. The method developed has been validated with the Enoval® program [9–11]. The limits of quantification were 40 μg/L for acenocoumarol and 400 μg/L for phenprocoumon and warfarin. Regression analysis of the calibration revealed good correlation ($R^2 > 0.995$) for all compounds. Accuracies were satisfactory, with CVs < 20%. The uncertainty of measurement was calculated by means of the concept of the total error.

This chromatographic technique allows the simultaneous research of oral anticoagulant drugs in forensic cases.

2. Experimental

2.1. Reagents and chemicals

Acenocoumarol (ACN) was kindly provided by Novartis Pharma (Vilvoorde, Belgium). Phenprocoumon (PPC) was generously supplied by Roche (Bruxelles, Belgium), Warfarin (WF) by Therabel Pharma (Bruxelles, Belgium) and Prazepam (PZ) obtained by Certa (Bruxelles, Belgium). These products were obtained as certified reference compounds. Acetonitrile, methanol (MeOH), ethyl acetate and diethyl ether were purchased from Biosolve (Valkenswaard, The Netherlands), hydrogen chloride and sodium dihydrogenophosphate from Merck (Darmstadt, Germany). All reagents were at least of analytical grade. HPLC-grade water was produced by a laboratory MilliQ system (Millipore, Bruxelles, Belgium). Pooled blank blood was purchased from the blood bank of the Hospital.

2.2. Chromatographic system

The development and validation work was carried out on a chromatographic system consisting of a Waters Alliance 2695 Separation Module, equipped with a quaternary, low-pressure mixing pump, a degassing line and a thermostated autosampler, connected with a Waters 2996 photodiode array detector (Zellik, Belgium). The Empower® software (Waters) was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms). Baselines were visually inspected and were manually adjusted when necessary.

HPLC separation was performed at 25 °C using a Symmetry® C8 analytical column (250 mm × 4.6 mm i.d.) packed with 5 μm diameter particles (Waters), equipped with a guard column (20 mm × 4.6 mm) containing identical packing material. The autosampler was programmed with an injection volume of 40 μL, a carousel temperature of 4 °C and a run time of 35 min.

The mobile phase consisted of acetonitrile (A) and a phosphate buffer (B). It was delivered at 1 mL/min, with the following step-wise gradient elution program: 0–1.0 min, 13% A; 1.0–9.0 min, 13–35% A; 9.0–28.0 min, 35–80% A; 28.0–30.0 min, decrease from 80 to 13% A; 30.0–35.0 min, column equilibration with 13% A. The UV–vis spectra were obtained in the range 200–400 nm.

2.3. Solutions preparation

Phosphate buffer solution was prepared by dissolving 12 g of natrium dihydrogenophosphate solution in two litters of ultrapure water. The pH was then adjusted to 3.8 with phosphoric acid. All

Table 1
Method validation.

	ACN		PPC	WF
Response function				
	Linear reg.		Linear reg.	Linear reg.
Trueness				
40 μg/mL	1.96[a]	400 μg/mL	1.13[a]	−0.33[a]
60 μg/mL	−0.87[a]	600 μg/mL	−0.21[a]	−0.61[a]
100 μg/mL	5.04[a]	1000 μg/mL	0.298[a]	1.76[a]
250 μg/mL	4.83[a]	2500 μg/mL	1.39[a]	0.91[a]
500 μg/mL	0.50[a]	5000 μg/mL	3.08[a]	4.77[a]
Precision (repeatability/intermediaire precision)				
40 μg/mL	4.94/5.80[b]	400 μg/mL	3.54/3.57[b]	5.04/6.72[b]
60 μg/mL	2.73/4.60[b]	600 μg/mL	3.20/3.20[b]	2.46/4.86[b]
100 μg/mL	2.29/3.52[b]	1000 μg/mL	3.15/3.15[b]	2.30/2.85[b]
250 μg/mL	1.31/3.54[b]	2500 μg/mL	1.70/2.53[b]	3.28/3.28[b]
500 μg/mL	3.40/5.34[b]	5000 μg/mL	3.58/5.56[b]	3.19/3.83[b]
Accuracy (β-expectation tolerance)				
40 μg/mL	[−10.30; 14.22][c]	400 μg/mL	[−5.94; 8.19][c]	[−15.51; 14.84][c]
60 μg/mL	[−12.52; 10.79][c]	600 μg/mL	[−6.51; 6.09][c]	[−13.79; 12.57][c]
100 μg/mL	[−3.52; 13.59][c]	1000 μg/mL	[−5.91; 6.50][c]	[−4.46; 7.96][c]
250 μg/mL	[−5.69; 15.35][c]	2500 μg/mL	[−4.67; 7.44][c]	[−5.56; 7.38][c]
500 μg/mL	[−12.63; 13.64][c]	5000 μg/mL	[−10.51; 16.67][c]	[−3.42; 12.95][c]
Linearity				
Range (μg/mL)	[40, 500]	Range (μg/mL)	[400, 5000]	[452, 5000]
Slope	1.007	Slope	1.033	1,051
Intercept	2.684	Intercept	−24.32	−41.25
R^2	0.9953	R^2	0.9959	0,9974
LLOQ (μg/mL)	40	LLOQ (μg/mL)	400	400

[a] Relative bias (%).
[b] (R.S.D. %).
[c] Limits (%).

prepared solutions were filtered through a 0.45-μm HV filter (Millipore) prior to use.

ACN, PPC, WF and PZ powders were separately dissolved in methanol to obtain stock solutions at 2 g/L. Then the solutions were mixed and appropriately diluted in methanol to obtain two pools of working solutions: the first solution at 250 mg/L for PPC and WF and 25 mg/L for ACN, and the second at 25 mg/L for PPC and WF and 2,5 mg/L for ACN, whereas the I.S., Prazepam, was diluted in methanol at 10 mg/L. These standard solutions were stored at +4 °C.

Blood calibration standards at 25, 50, 100, 200, 400 and 800 μg/mL for ACN, and at 250, 500, 1000, 2000, 4000 and 8000 μg/mL for PPC and WF were prepared by adding appropriate volumes of the respective stock solutions to blank blood. Blood validation standards were prepared at 40, 60, 100, 250 and 500 μg/mL for ACN, and at 400, 600, 1000, 2500 and 5000 μg/mL for PPC and WF.

2.4. Sample preparation

To 1 mL of whole blood were added 100 μL of a 10 mg/L IS solution, 1 mL of 0.2 M HCl solution and 8 mL of the extraction solvent,diethyl ether and ethyl acetate (50/50, v/v), in a 10-mL borosilicate tube. The tubes were shaken for 15 min then centrifuged at 3000 rpm for 10 min. The organic phase was transferred into a 10-mL borosilicate tube and evaporated to dryness at 30 °C under a gentle stream of nitrogen. Dry extracts were re-dissolved in 100 μL of a mixture of phosphate buffer–acetonitrile (95:5, v/v). The samples were transferred into vials, and 40 μL were injected into the LC–DAD.

3. Results and discussion

3.1. Method validation

A statistical approach based on the total error measurements, including both bias (trueness) and standard deviation (precision), was applied to validate the method. The tolerance interval used is called the "β-expectation tolerance interval", defined as an interval in which it is expected that at least a define proportion of future results (β) will lay inside. It can be used as a predictive tool which can guarantee that at least a proportion β (e.g. 0.95 or 95%) of future results will be included in the set acceptance limits. With such a validation strategy, the developed method has been accepted by the laboratory for forensic identification of coumarin overdoses.

The method has been validated with the Enoval program (Arlenda, Liège, Belgium). In order to validate the method, calibration curves were tested in duplicate, one set at the beginning and the second at the end of the HPLC run, on three separate occasions. Calibration curves were obtained by plotting ratios of analyte peak area divided by internal standard peak area versus the analyte concentrations in spiked samples. Validation standards were analyzed in triplicates at five concentration levels (at 40, 60, 100, 250 and 500 μg/mL for ACN, and at 400, 600, 1000, 2500 and 5000 μg/mL for PPC and WF), on three different days too.

The concept of total error calculated with the Enoval program has been used to evaluate the validation parameters (regression model, trueness, precision, accuracy profile, LLOQ, etc.). The acceptance limits were fixed at ±20%, which is acceptable for forensic cases definitively.

3.2. Response function

Since it was acceptable, the simple linear regression was selected for validation, with consideration of the easibility. The concentration of the unknown sample was then calculated using a simple linear regression analysis model.

3.3. Trueness

Trueness gives information on systematic error. It refers to the closeness of agreement between the exact concentration in certified material and the obtained mean of results. The trueness was expressed in terms of relative bias (%) for each concentration level of the five validation standards for ACN, PPC and WF (Table 1). Trueness was acceptable for the three analyzed anticoagulants, since the bias did not exceed the value of ±15%.

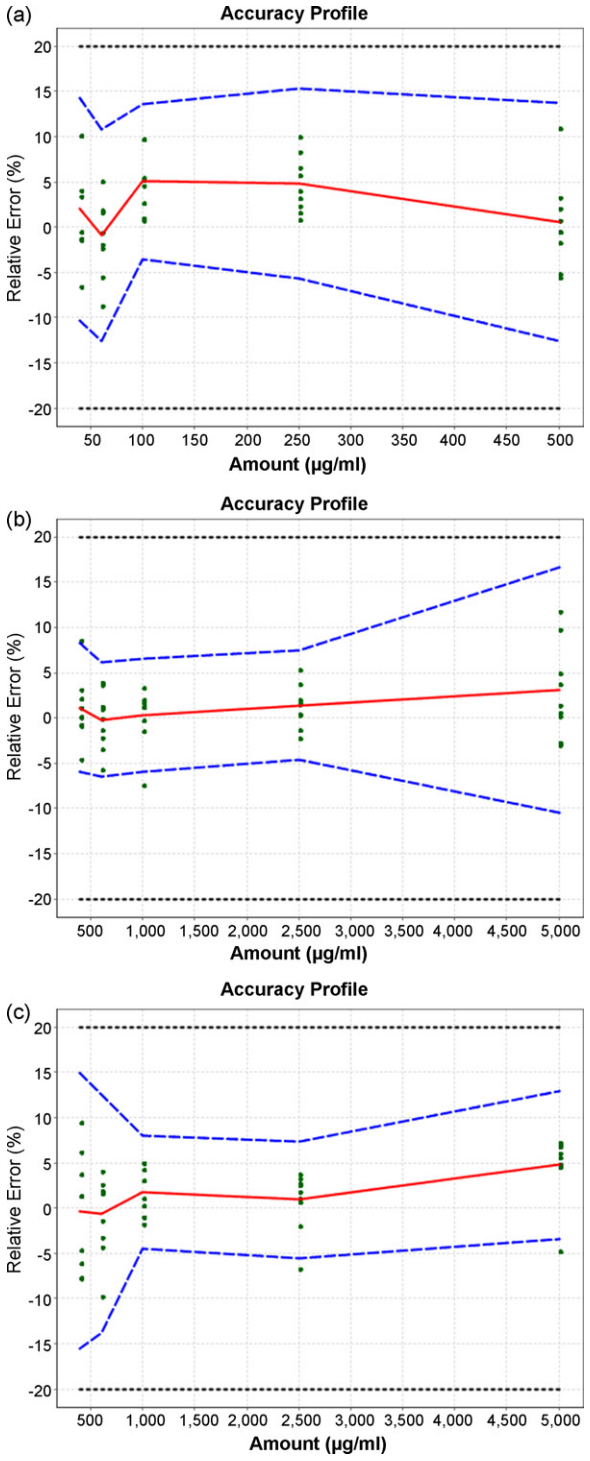

Fig. 1. Accuracy profiles for ACN (a), PPC (b) and WF (c) using a linear regression model. Relative bias (- - -), acceptance limits (. . .), β-expectation tolerance limits (– – –), relative back-calculated concentrations (·).

QC samples have been prepared by spiking whole blood samples.

3.4. Precision

Precision gives information about random errors and was evaluated at two levels: repeatability and intermediate precision. The precision of the developed method, expressed in terms of relative standard deviation (R.S.D.) values, was determined for the different antivitamins K. The R.S.D. values were relatively low, about

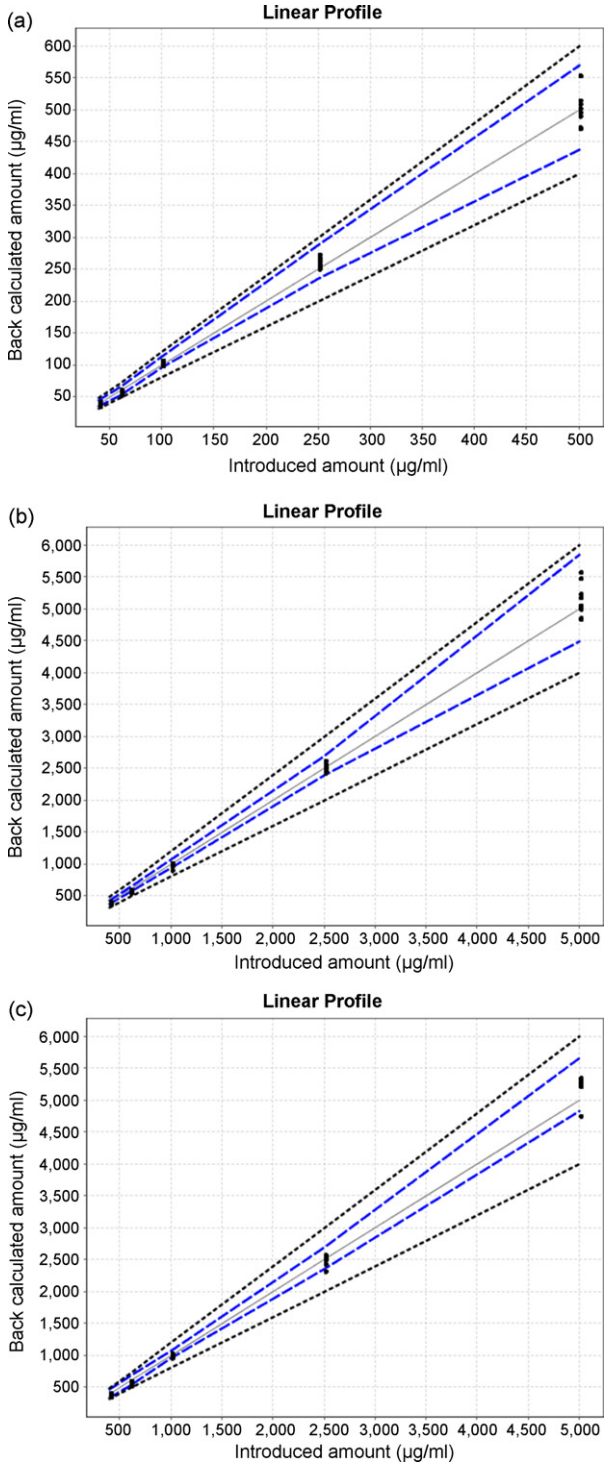

Fig. 2. Linearity profiles for ACN (a), PPC (b) and WF (c).

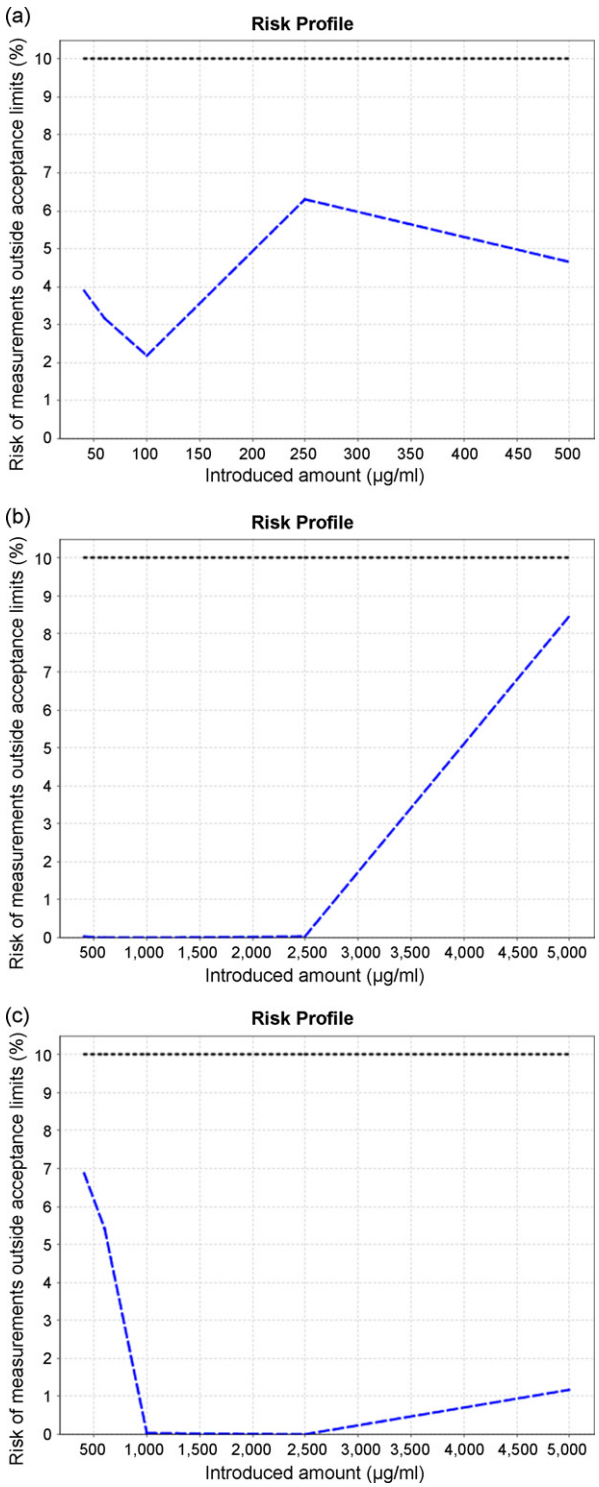

Fig. 3. Risk profiles for ACN (a), PPC (b) and WF (c) obtained by concentration level. The maximum tolerated risk is set at 10 %.

5.8%, 5.6% and 6.7%, respectively for ACN, PPC and WF. These results illustrate the good precision of the developed method.

3.5. Accuracy and LLOQ

The accuracy profiles for each drug are based on the total error of the measurements, i.e. systematic and random errors. Accuracy refers to the closeness of agreement between the test result and the accepted reference value, called the conventionally true value

Table 2
Estimates of the measurement uncertainties related to acenocoumarol, phenprocoumon and warfarin.

Analyte	Concentration (μg/L)	Uncertainty of the bias (μg/L)	Uncertainty (μg/L)	Expanded uncertainty (μg/L)	Relative expanded uncertainty (%)
Acenocoumarol	40	0.96	2.51	5.02	12.55
	60	1.39	3.09	6.18	10.30
	100	1.72	3.92	7.83	7.83
	250	4.87	10.10	20.20	8.08
	500	13.18	29.79	59.58	11.92
Phenprocoumon	400	4.86	15.10	30.19	7.55
	600	6.40	20.23	40.46	6.74
	1000	10.50	33.20	66.40	6.64
	2500	30.54	70.27	140.5	5.62
	5000	136.6	309.7	619.5	12.39
Warfarin	400	12.25	29.53	59.05	14.76
	600	15.34	32.96	65.92	10.99
	1000	12.36	31.04	62.09	6.21
	2500	27.37	86.55	173.1	6.92
	5000	80.84	207.7	415.4	8.31

too. Acceptance limits were settled at 20% for each concentration level. The method can be considered as accurate for each concentration included in the tested range. Limit of quantification has been determined at 40 μg/L for ACN, at 400 μg/L for PPC and for WF (Fig. 1).

3.6. Linearity

The linearity of an analytical method is the ability within a definite range to obtain results directly proportional to the concentration (quantity) of the analyte in the sample. For the different anticoagulants, the model of regression selected was the linear regression model. Indeed, as presented in Table 1, these models can be considered as linear with slope values obtained for the different anticoagulants always between 0.85 and 1.15, the optimal range [12,13]. The linearity of the model of regression selected was also demonstrated using the absolute β-expectation tolerance limits, with acceptance limit at 20% expressed in the concentration units. The method was considered as valid within the range for which the dashed curves are within the dotted acceptance limits (Fig. 2).

3.7. Risk assessment

By using the β-expectation tolerance intervals obtained with the previously selected regression models, risk of having future measurement falling outside the specified acceptance limits was assessed. The maximum risk tolerated was set at 10%. Fig. 3 shows the risk profiles for each studied anticoagulants. In all cases the risk did not exceed 10%.

3.8. Uncertainty assessment

Uncertainty of measurements has been evaluated by the same validation study. In fact, as long as the experimental design used for the validation is representative of the sources of variability that will be encountered during routine analysis, this uncertainty estimate is relevant for the results obtained in the laboratory having validated the analytical procedure. As shown in Table 2, the relative expanded uncertainty of each anticoagulant irrespective of the concentration levels did not exceed 20%.

4. Conclusions

The validation method developed for the identification and quantification of acenocoumarol, warfarin and phenprocoumon with the concept of total error for decision criterion gave us complete satisfaction. Indeed, the aim of this development was to propose an analytical technique adapted to the situations of forensic toxicology, i.e. intoxication with massive anticoagulant doses. Analysis of these three anticoagulant drugs have been made in one simple injection with a satisfactory analytical quality since the calculated total error does not exceed 20%. Analytical method is linear from 40 to 500 μg/L for ACN, and from 400 to 5000 μg/L for PPC and WF. These blood concentrations include toxic level for ACN (100 μg/L). For PPC and WF intoxications, blood samples need to be diluted in order to be quantified.

The uncertainty study revealed a maximum 20% of error, which is very satisfying for forensic cases. Consequently, simultaneous research, and, if necessary, quantification, of coumarinic drugs available on pharmaceutical market can be performed easily by forensic toxicological laboratories.

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