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Simultaneous quantification of carbamate insecticides in human plasma by liquid chromatography/tandem mass spectrometry

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ABSTRACT

Carbofuran (CFN), carbosulfan (CSN) and fenobucarb (FBC) are carbamate pesticides that are widely used in gardening and agriculture for the control of insects. Human poisoning due to occupational or self-poisoning exposures is also reported, so assays are required to quantify the plasma concentration of these insecticides. An LC–MS/MS method was developed and validated for the simultaneous quantification of these three carbamate insecticides in the plasma of patients with acute intentional self-poisoning. Plasma samples were pretreated by acetonitrile for protein precipitation. Chromatography was carried out on a Luna C18(2) analytical column with gradient elution using a mobile phase containing acetonitrile and water with 10 mM ammonium acetate. Mass spectrometric analysis was performed by an Applied Biosystems MDS Sciex API 2000 triple quadrupole mass spectrometer coupled with electrospray ionization (ESI) source in the positive ion mode. The total run time was 7 min. The assay was validated over a concentration range from 10 to 1000 ng/ml for CSN and FBC and 20–2000 ng/ml for CFN. The precision and accuracy for both intra- and inter-day determination of all analytes were acceptable (<15%). No significant matrix effect was observed. Stability of compounds was established for short term bench and autosampler storage as well as freeze/thaw cycles. The method was effectively applied to 270 clinical samples from patients with a history of acute intentional carbamate self-poisoning.
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1. Introduction

Carbamate insecticides are widely used in gardening and agriculture due to their broad spectrum of activity, relatively short environmental persistence, and relatively low mammalian toxicity [1]. However they are potentially toxic to humans, particularly in the context of intentional self-poisoning. Carbamate insecticides reversibly inhibit acetylcholinesterase, thereby preventing the catabolism of acetylcholine, and inducing characteristic cholinergic manifestations of poisoning, similar to that observed from organophosphorus pesticide poisoning [2].

Three of the more commonly used carbamate insecticides are carbofuran (CFN), carbosulfan (CSN) and fenobucarb (FBC). Their structures are shown in Fig. 1. Exposures may occur accidentally, with occupational use, or as a result of intentional self-poisoning. For example, in Sri Lanka, these are the most common carbamate insecticides presenting to hospitals following acute poisoning; in the case of carbosulfan, the case fatality for such exposures exceeds 10% [3]. Data regarding the toxicokinetics and concentration–response relationship of carbamate insecticides in humans following acute poisoning are extremely limited. This information may assist in understanding the time course of the clinical manifestations of acute poisoning and inform management decisions.

A review of the literature noted that liquid chromatography/tandem mass spectrometry (LC–MS/MS) has been used for the quantification of carbamate insecticides and their environmental degradation products in non-biological samples (e.g. water, fruit, vegetable) [4–12]. Recently, a LC–MS/MS method was reported to determine pesticides (including CFN) residues in serum samples of agriculture workers [13]. Liquid–liquid extraction method was used for sample preparations and long running time was required.

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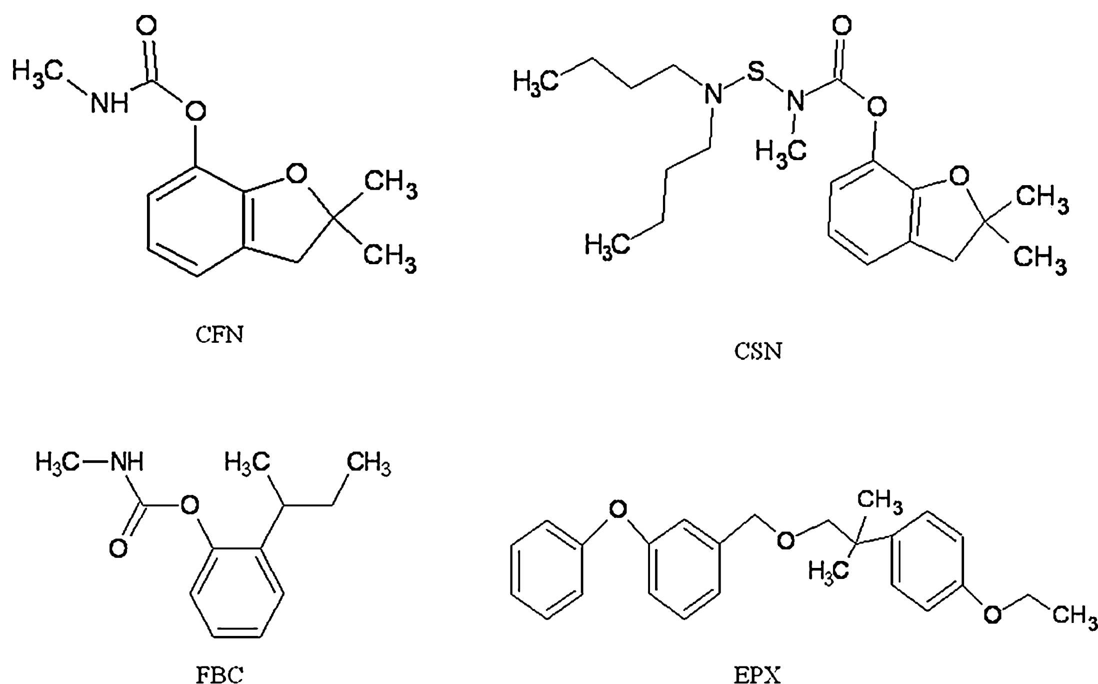

Fig. 1. Chemical structures of carbofuran (CFN), carbosulfan (CSN), fenobucarb (FBC) and the internal standard (IS) etofenprox (EPX).

In this paper, we developed and validated an LC–MS/MS method for simultaneous measurement of CFN, CSN and FBC in human plasma provided by patients presenting to hospital with a history of acute carbamate self-poisoning with simple protein precipitation method and a shorter running time.

2. Materials and methods

2.1. Materials and reagents

Carbofuran, carbosulfan, fenobucarb and etofenprox were purchased from Sigma–Aldrich Corporation (St Louis, MO, USA) (Fig. 1). All solvents were HPLC grade and were obtained from Merck (Kilsyth, VIC, Australia). Blank human plasma was obtained from Australian Red Cross.

2.2. Equipment

The analysis was performed using a MDS Sciex API2000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Applied Biosystems Inc., Foster City, CA, USA) and a divert valve. The HPLC system consisted of a Shimadzu SLC-10A VP system controller with two LC-10AD pumps and a SIL-20AC-HT autosampler.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was performed on a Luna C18(2) analytic column (50 mm × 2.0 mm, 5 μm, Phenomenex, Torrance, CA, USA) protected by a C18 guard cartridge (4 mm × 3 mm, Phenomenex, Torrance, CA, USA). Gradient elution was used with mobile phase consisting of 10 mM ammonium acetate in water (solvent A) and acetonitrile:water in the ratio of 95:5 (v/v) (solvent B). The elution gradient was 0% B to 100% B (0–2 min), 100% B (2–6 min), and 100% B to 50% B (6–7 min) at a flow rate of 0.25 ml/min. The mobile phase solvents were filtered through a 0.22-μm DURAPORE® membrane filter (Millipore, Bedford, MA, USA) before use.

The detection was made with electrospray ionization operating at positive ion mode and the tandem spectrometer was operated in the multiple reactions monitoring (MRM) mode. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solution at the rate of 1 μg/ml using a Harvard infusion pump. The optimized parameters were as follows: turbo ion spray temperature, 400 °C; ion spray voltage, 5000 V; declustering potential (DP), 20 V (CSN), 36 V (FBC), 40 V (CFN) and 52 V (EPX); enhanced potential (EP), 10 V (CSN, CFN, EPX) and 12 V (FBC); collision energy (CE), 20 V (CSN and CFN), 21 V (FBC), and 31 V (EPX); collision cell entrance potential (CEP), 10 V (CSN), 15 V (EPX), 16 V (CFN) and 19 V (FBC); collision cell exit potential (CXP), 3 V (FBC) and 15 V (CSN, CFN and EPX). The ion transition chosen for MRM were m/z 221.8 → 164.9 for CFN, 381.2 → 160 for CSN, 208 → 95 for FBC, and 359 → 183 for internal standard (IS) EPX. The AppliedBiosystems Analyst version 1.4.2 software was used to control the LC–MS/MS system and to collect and analyse the data.

2.4. Preparation of stock and working solutions

Primary stock solutions of CFN, CSN and FBC were prepared separately by dissolving each compound in acetate buffer (pH = 6) to yield a concentration of 0.25 mg/ml, respectively. The combined working standard solutions were prepared by combining the aliquots of each primary solution and serially diluting with acetate buffer.

The stock solution of IS (EPX) was prepared in acetonitrile at the concentration of 0.5 mg/ml. The working solution of IS then was prepared by diluting an aliquot of stock solution with acetate buffer to achieve the concentration of 5 μg/ml. All stock solutions were kept at −20 °C until use, while the working solutions were kept at 4 °C and discarded after 30 days.

2.5. Preparation of calibration standards and quality control (QC) samples

The calibration human plasma standards were prepared by spiking 20 μl of the working standard solution into a pool of

human blank plasma (100 μl) to achieve the concentration range 10–1000 ng/ml for CSN and FBC and 20–2000 ng/ml for CFN. Quality control (QC) samples at three concentration levels (30, 400 and 800 ng/ml for CSN and FBC; 60, 800 and 1600 ng/ml for CFN) were prepared by spiking the appropriate working standard solutions into human blank plasma (100 μl).

2.6. Sample preparation

An aliquot of 100 μl plasma sample was mixed with 20 μl IS working solution and 20 μl acetate buffer. For all samples, 200 μl of acetonitrile was added for protein precipitation. After vortex-mixing for 10 s and centrifuging at 1000 × g for 10 min, the supernatant was transferred into HPLC vial and 10 μl was injected to the instrument.

2.7. Method validation

2.7.1. Selectivity and matrix effect

Six lots of blank human plasma from different sources were screened for potential endogenous interferences at the retention time of the analytes. Matrix effect was evaluated by comparing peak areas of all analytes in spike-after preparation samples with the corresponding peak areas in neat solution. Two different concentrations were evaluated by analyzing five samples at each level.

2.7.2. Linearity and lower limit of quantification (LLOQ)

The linearity of the method was determined by analysing seven calibration standard samples at concentrations from 10 to 1000 ng/ml for CSN and FBC, and from 20 to 2000 ng/ml for CFN. The acceptable tolerance for accuracy and precision was 20% for LLOQ and 15% for other standard points [14]. The calibration curve was constructed by least squares linear regression of the peak area ratios of each analyte to IS obtained against the corresponding concentrations using a weighting factor of $1/\chi^2$ (reversed square of the concentration) [15]. The LLOQ was defined as the lowest concentration in the calibration curve with acceptable precision and accuracy.

2.7.3. Accuracy and precision

The intra-day accuracy and precision was evaluated by analysing 5 replicates of QCs at three concentration levels using a freshly prepared calibration curve on the given day. Additional QC samples were also analysed on five different days in order to assess inter-day accuracy and precision. Precision was represented by percent relative standard deviations (RSD%) while accuracy was obtained by expressing the mean of the measured concentrations as a percentage of the nominal concentration.

2.7.4. Stability test

The post-preparative stability was determined by injecting preparations of processed samples for up to 24 h (in the autosampler at 4 °C) after the initial injection. Short-term stability in the matrix during 3 h (bench storage) was determined at ambient temperature (24 ± 3 °C) at concentrations of QC samples. The stability was also tested after three freeze/thaw cycles using the concentrations of QC samples. The samples were stored at −80 °C between freeze/thaw cycles, and then they were thawed by allowing them to stand at room temperature for approximately 30 min. The samples were then returned to freezer for 24 h.

2.8. Application

This method was applied to quantify plasma concentrations of CSN, CFN and FBC in 270 plasma samples provided by patients presenting to hospitals in Sri Lanka with a history of acute pesticide poisoning. This study was coordinated by the South Asian Clinical Toxicology Research Collaboration (SACTRC; www.sactrc.org).

Table 1
Matrix effect results of CFN, CSN and FBC.

Analyte	Nominal concentration (ng/ml)	Matrix effect (n = 5)	Mean ± SD (n = 10)
CFN	40	97.8%	103
	1000	110%	± 9.1%
CSN	20	105%	103
	500	102%	± 10.5%
FBC	20	94.9%	101
	500	110%	± 12.1%

3. Results and discussion

3.1. LC–MS/MS optimization and sample preparation

During the development of the method, CSN was found to be unstable due to its hydrolysis to CFN. Therefore, we tried different solvents to prepare the stock solution of analytes. Acetate buffer with pH = 6 was found to be able to stabilize CSN and was used in this assay for stock solution preparation. Different chromatographic conditions were investigated to optimize sensitivity, peak shape and separation. The use of neutral volatile salts, ammonium acetate as mobile phase was found to be preferable to analyte separation and ionization than the use of acid (e.g. formic acid). Gradient elution was applied in the assay. Simple protein precipitation procedure with acetonitrile was found to be suitable for sample preparation without effect on separation and ionization of all analytes.

Based on the chemical structures of the analytes, electrospray ionization operated at positive ion mode was used for LC–MS/MS analysis to provide optimum sensitivity and selectivity. Protonated forms of each analyte $[M+H]^+$ were found to be dominant ions in the Q1 scan, and were used as the precursor ions to obtain Q3 product ion spectra. The optimized MRM transitions (precursor ion $m/z \rightarrow$ product ion m/z) are as follows: 221.8 → 164.9 for CFN, 381.2 → 160 for CSN, 208 → 95 for FBC, and 359→183 for internal standard (IS) EPX.

Under these LC-/MS/MS condition, the retention times of CFN, CSN, FBC and IS were 1.69, 4.58, 2.36, and 4.32 min, respectively. Representative LC–MS/MS chromatograms were shown in Fig. 2.

3.2. Method validations

3.2.1. Selectivity and matrix effect

Six different lots of blank human plasma were checked for any false positive MS responses. No interferences from endogenous plasma substances were observed and a good separation of the analytes was achieved using the described LC–MS/MS conditions.

As shown in Table 1, no obvious matrix effects were found for all the analytes as the results ranged from 101% to 103% which was within the acceptable limit.

3.2.2. Linearity and LLOQ

Calibration curves in spiked human plasma were linear over the range of 10–1000 ng/ml for CSN and FBC, and 20–2000 ng/ml for CFN, respectively. The linearity of standard curves (r^2) for all analytes were greater than 0.99 using $1/\chi^2$ weighting. For each point of calibration standards, the back calculated concentrations from the equation of calibration curves were within ±15% deviation. The calibration curve had a reliable reproducibility across the calibration range. The LLOQs were found to be 10 ng/ml for CSN and

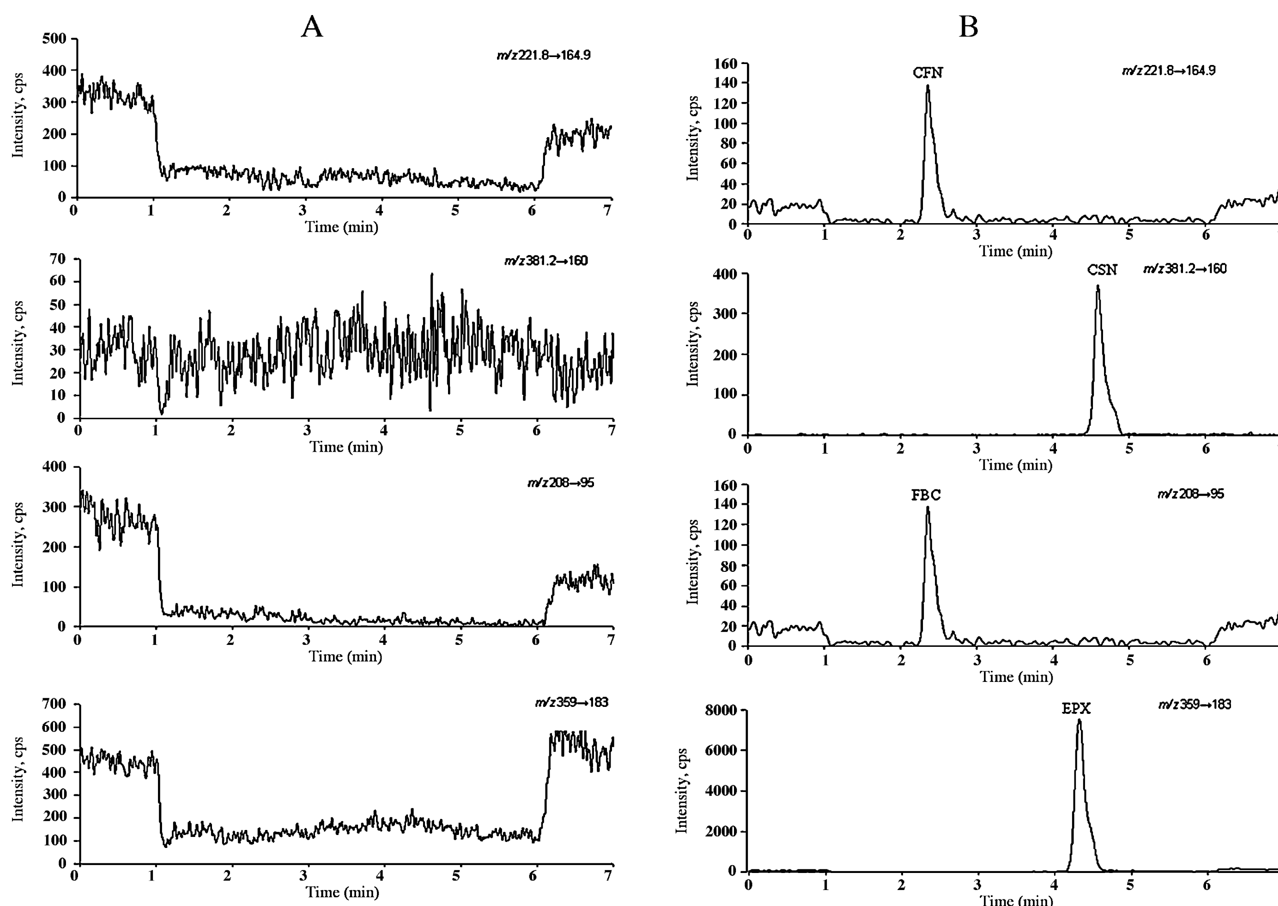

Fig. 2. Representative extracted ion chromatogram (XIC) of (A) blank plasma; and (B) calibration standard samples at LLOQ level.

Table 2
LLOQ for the analysis of CFN, CSN and FBC in human plasma ($n = 5$).

Analyte	Nominal concentration (ng/ml)	Measured concentration (ng/ml)					Mean accuracy	%RSD
CFN	20	20.0	19.0	18.6	20.6	21.1	99.3%	5.3%
CSN	10	10.3	10.2	9.93	9.75	10.3	101%	2.4%
FBC	10	10.5	10.0	9.22	10.5	10.2	101%	5.2%

Table 3
Intra-day accuracy (% of nominal concentration) and precision (%RSD) of CFN, CSN and FBC in human plasma ($n = 5$).

Analyte	Nominal concentration (ng/ml)	Mean accuracy	%RSD
CFN	60	97.4%	6.17%
	800	96.3%	3.21%
	1600	94.4%	7.26%
CSN	30	104%	7.42%
	400	111%	1.18%
	800	94.7%	3.24%
FBC	30	108%	6.83%
	400	102%	10.2%
	800	92.4%	4.44%

Table 4
Inter-day accuracy (% of nominal concentration) and precision (%RSD) of CFN, CSN and FBC in human plasma ($n = 5$).

Analyte	Nominal concentration (ng/ml)	Mean accuracy	%RSD
CFN	60	105%	9.60%
	800	103%	10.1%
	1600	97.0%	11.3%
CSN	30	95.9%	8.04%
	400	107%	6.19%
	800	104%	8.22%
FBC	30	108%	7.14%
	400	98.3%	6.89%
	800	104%	6.61%

FBC, 20 ng/ml for CFN, respectively with acceptable accuracy and precision (Table 2).

3.2.3. Accuracy and precision

Five replicates of QC samples at three concentrations (low, medium and high) were processed on the same day and also on five separate days of analysis to determine intra- and inter-day accuracy and precision. As shown in Tables 3 and 4, values of precision were within 12% for all analytes and those for accuracy ranged from 94.4% to 105% for CFN, 94.7% to 111% for CSN, and 92.4% to 108% for FBC, respectively.

3.2.4. Stability

As shown in Table 5, CFN, CSN and FBC plasma samples were stable for up to 3 h in room temperature, for 24 h after sample preparation in the autosampler and after three freeze/thaw cycles as the relative deviation were within ±15% for all analytes at different concentrations.

Table 5
Stabilities of CFN, CSN and FBC in human plasma quality control samples ($n = 3$).

Analyte	Nominal concentration (ng/ml)	% loss/gain in stability study		
		Post-preparative	Freeze–thaw	Short term
	60	–	+12.3%	+12.3%
CFN	800	−4.04%	+11.6%	+5.91%
	1600	–	+13.1%	+6.29%
	30	–	−11.4%	−6.06%
CSN	400	−2.96%	−13.5%	−14.9%
	800	–	−5.47%	−12.2%
	30	–	+5.45%	−1.13%
FBC	400	−5.11%	+10.2%	+8.20%
	800	–	+9.54%	+13.0%

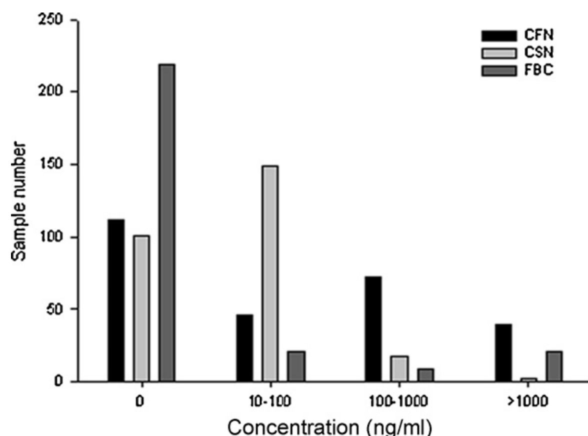

Fig. 3. Concentration distribution of CFN, CSN and FBC in the tested clinical samples.

3.3. Application to clinical study

The developed assay was successfully applied to simultaneously measure CFN, CSN and FBC levels in 270 plasma samples from patients with a history of acute carbamate self-poisoning. All three compounds were detected in 30 samples suggesting a mixed exposure. None of these carbamates were detected in 39 samples suggesting a negligible exposure; however, those patients who were symptomatic were probably exposed to another pesticide (e.g. organophosphorus compound). Of the remaining samples, two analytes were measured in 91 samples and only a single analyte was present in 110 samples. CFN was detected in 158 samples at a concentration ranging from 20.7 to 62,200 ng/ml. CSN was detected in 170 samples at a concentration ranging from 10 to 5840 ng/ml. FBC was only detected in 53 samples at a concentration ranging from 11.4 to 198,000 ng/ml. The concentration distribution of these analytes in 270 tested plasma samples is shown in Fig. 3.

Overall, these results indicated that our method detected and quantified the concentration of CFN, CSN and FBC simultaneously in patients' plasma samples both accurately and quickly. Further, we have identified the range of plasma concentrations observed in patients with intentional self-poisoning, and the potential for multiple coingestants rather than a single exposure in such patients.

4. Conclusions

In summary, we have developed a rapid, accurate and robust LC–MS/MS method for simultaneous quantification of CFN, CSN and FBC in human plasma. We used simple protein precipitation method for sample preparation that provides a format for clinical studies of pesticide poisoning. We have successfully applied this method for analysis of 270 clinical samples.

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