Biomarkers, March–April 2007; 12(2): 145–154

informa
healthcare

Using dried blood spots stored on filter paper to measure cholinesterase activity in wild avian species

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Abstract

Birds of prey that are poisoned by cholinesterase inhibitors (e.g. organophosphate and carbamate insecticides) are often cared for at animal shelters, rehabilitation centres and wildlife diagnostic facilities. Plasma cholinesterase (ChE) activity is a recognized method of assessing exposure to these insecticides, but standard blood-handling protocols are difficult to follow in non-laboratory settings. The primary objective of this study was to expand upon a method for storing human blood on filter paper without the need for complicated equipment or refrigeration, and to test its utility for measurement of ChE activity in avian blood. ChE activity from whole blood, plasma, and dried blood spots was analysed from 169 wild birds and comparisons made among sample types. ChE activity measured in whole blood haemolysates and dried blood spots were significantly correlated ($r = 0.74$, $p < 0.001$), as was ChE activity measured in plasma and dried blood spots ($r = 0.68$, $p < 0.001$). This study demonstrated that monitoring pesticide exposure in birds could be conducted using elementary blood sampling, preserving and shipping techniques.

Keywords: *Avian, cholinesterase, dry blood spot, filter paper, pesticides*

(Received 14 February 2006; accepted 6 July 2006)

Introduction

It has been shown that even though most cholinesterase-inhibiting pesticides are relatively short lived in the environment, birds of prey are repeatedly exposed through a variety of different routes such as the ingestion of exposed prey and dermal contact (Mineau et al. 1999). As such, an increasing number of birds of prey are sublethally poisoned by cholinesterase inhibitors (e.g. organophosphate (OP) and carbamate insecticides) and are being cared for at various animal shelters, rehabilitation centres and wildlife diagnostic facilities. Measurement of plasma cholinesterase (ChE) activity is a recognized method of non-destructively assessing the exposure of wild birds exposed to these insecticides (Mineau & Tucker 2002a,b).

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500600907788

Normal sampling procedure for avian ChE activity determination requires the centrifugation of blood and the isolation and quick storage of the plasma fraction at ultra-low temperature (e.g. −80°C). Ultra-low freezing is especially critical in order to prevent reactivation of enzyme activity through spontaneous hydrolysis when carbamate insecticides are involved. When blood sampling is conducted in the field or at rehabilitation centres that often lack suitable equipment and are run by volunteers, adequate collection and storing procedure can be difficult to follow, and the risk of thawing samples in transit from the collection site to the laboratory is always of concern.

Augustinsson & Holmstedt (1965) pioneered a method for storing human blood without the need for complicated equipment or immediate refrigeration. It involved applying drops of whole blood onto filter paper and immediately drying the sample to prevent possible hydrolysis, and hence reactivation, of the bound enzyme fraction. Drying the samples also prevented bacterial degradation of the sample. Subsequently, ChE activity could be determined using the Augustinsson method (Augustinsson et al. 1978). Eriksson & Faijersson (1979) used this method to measure ChE activity in human blood dosed *in vitro* with ChE-inhibiting insecticides and found that blood samples stored on the filter paper at room temperature or deep frozen for at least 1 week had the same level of inhibition as at the start of the experiment. The amenability of this technique for the storage of avian blood would be an obvious advancement for assessing exposure of wild birds to ChE-inhibiting insecticides. To test the utility of using filter paper as a matrix to store avian blood, we conducted a preliminary laboratory study and measured ChE activity from dried blood spots on filter paper from captive ringed turtle-doves (*Streptopelia risoria*) dosed with the OP and carbamate insecticides diazinon, carbofuran and chlorpyrifos (Trudeau et al. 1995, Trudeau & Sans Cartier 2001). Birds were given between 0.5 and 0.05 LD_{50} to produce a wide range of ChE inhibition. Results revealed that the technique was applicable to avian species under laboratory conditions, provided the blood was rapidly dried after its application, and kept desiccated until analysis. In fact, the enzymes preserved on filter paper appeared to be more stable then in the homologous frozen aliquots up to six months after collection. The purpose of this present study was to collaborate with staff at wildlife rehabilitation centres located across Canada to assess the value of the validated laboratory collection technique for diagnosing exposure to cholinesterase-inhibiting OP and carbamate insecticides in wild birds under non-laboratory conditions.

Materials and methods

Plasma, whole blood and dried blood spot sampling and handling

In order to standardize the collection procedures as much as possible, every collaborator was provided with a complete blood collection kit and detailed procedures for the application, storage and shipment of plasma, whole blood and blood spotted on filter paper. Participants were asked to apply three blood spots per specimen on a Walthan #4 filter paper by filling three capillary tubes (44.7 µl) with whole blood, drawing across and allowing to soak into the filter paper in a uniform manner. The filter paper was then transferred to a container containing a desiccant (i.e. Drierite, Sigma-Aldrich, Oakville, Ontario, Canada; Cat. No. 44572) and

allowed to dry for 30 min, after which time it was placed in a prelabelled envelope containing a tablespoon of Drierite, and immediately sent via regular mail services to our laboratory (National Wildlife Research Centre (NWRC), Ottawa, Canada). Transit times (the number of days samples were in the mail) ranged from 4 to 21 days. Upon arrival at our laboratory the desiccant was replaced (if necessary) and the samples stored at 20°C pending ChE analysis. Immediately after the application of blood on filter paper, an aliquot of the whole blood and the plasma fraction were frozen and sent to our laboratory on dry ice where they were stored at −80°C until ChE analysis. Participants were also asked to provide the haematocrit value for each specimen and any pertinent information (i.e. species, age, blood collection date, etc.).

Test species

Blood samples from 29 different species (169 individuals) were sent to our laboratory (Table I). For some individuals, plasma, whole blood and dried blood spots were not all collected. A total of 120 plasma samples, 150 whole blood samples, and 166 dried blood spot samples were assessed for ChE activity.

Table I. Bird species, and the number of representative samples, from which blood was collected for the assessment of ChE activity.

Species	Banding abbreviation	Number
American crow	AMCR	3
American kestrel	AMKE	5
Bald eagle	BAEA	55
Barn owl	BNOW	4
Barred owl	BDOW	6
Broad-winged hawk	BWHA	9
Cooper's hawk	COHA	3
Eastern screech owl	EASO	2
Ferruginous hawk	FEHA	1
Golden eagle	GOEA	1
Great grey owl	GGOW	3
Great horned owl	GHOW	16
Great-blue heron	GBHE	3
Lanner hawk	LAHA	1
Long-eared owl	LEOW	3
Merlin	MERL	6
Northern goshawk	NOGO	5
Osprey	OSPR	2
Peregrine falcon	PEFA	5
Prairie falcon	PRFA	1
Red-shoulder hawk	RSHA	1
Red-tailed hawk	RTHA	13
Rough-legged hawk	RLHA	3
Saw-whet owl	SWOW	1
Short-eared owl	SEOW	9
Snowy owl	SNOW	1
Swainson's hawk	SWHA	3
Trumpeter swan	TRUS	1
Turkey vulture	TUVU	3

Extraction of blood ChE from the filter paper matrix

Dry blood spots were cut from the filter paper and placed in a disposable 12 × 75-mm glass test tube and extracted with 1.47 ml of a 1% aqueous solution of Triton X-100. After 30 min of vigorous shaking with a mechanical shaker kept at 4°C, the filter paper was removed, and 300 µl of the eluate used to assess ChE activity. Triton-X has been reported to have various effects on ChE activity in some animals (Carakostas & Landis 1991), so whole blood haemolysates were prepared with Triton-X and with water, and the ChE activity in both compared. Plasma samples were diluted in order to represent the equivalent amount of plasma enzymes that were found in the blood haemolysates (25 µl of plasma were added to 2.97 ml of water) and 300 µl were used for the ChE activity determination.

Determination of ChE activity

The activity of ChE from dried blood samples, haemolysates and plasma was analysed with a Hewlett Packard diode array spectrophotometer (HP8452A, S/N 261A00276) at 30°C using Augustinsson's method as described in Eriksson & Faijersson (1979), but without the addition of a selective inhibitor for butyrylcholinesterase. The activity measured was, therefore, the combined activity of acetylcholinesterase and butyrylcholinesterase. The change in absorbance was monitored at 324 nm for 1 or 2 min. Augustinsson's method is based on the hydrolysis of propionylthiocholine (PrThI) and the reaction of thiocholine with 4,4′-dithiopyrine (4-PDS). The reaction product, 4-thiopyridone is measured at 324 nm. This spectrophotometric method was preferred to the standardized Ellman kinetic assay because the reaction product in the Ellman assay is measured at 405 nm, which corresponds to the absorption region of haemoglobin. For the assessment of ChE in plasma samples, 100 µl was diluted to 2 ml with phosphate buffer (0.5 M, pH 8.0), and 100 µl of this suspension analysed as described above. ChE activity measured in plasma was converted to µmol min^{-1} l^{-1} of blood to allow direct comparison with the blood values following the equation:

$$C = P(100 - H)/100,$$

where C is the corrected plasma activity (i.e. per 1 litre of blood), P is the measured plasma activity (i.e. per 1 litre of plasma), and H is the haematocrit level as a percentage.

In order to evaluate the day-to-day variation, Precinorm E (Boeringer Mannheim 125113), a lyophilized control serum to which enzymes have been added, was used. All ChE determinations were performed in duplicate and repeated if results differed by more than 5% at normal levels and 15% at low levels. The hydrolysis of the substrate in the absence of the sample (reagent blank) was determined daily and subtracted from the total hydrolysis value measured in the presence of the enzyme.

Time-series study

During the course of this study, three bald eagles with known insecticide poisoning were taken to one of the rehabilitation centres. The time delay between exposure and admission is not known, but crop contents were surgically excised from two of the birds; the third had an empty crop but regurgitated a pellet spontaneously. The crop contents and the pellet were all found to contain fonofos, a common cause of poisoning

at the time. Blood was collected on the day of admission, and then sequentially collected for up to 1 month to determine if the technique was sensitive enough to be used as a biomonitor of individual changes (e.g. progression of inhibition or rebound to normal values due to metabolic detoxification) in ChE inhibition. For comparison, ChE inhibition was measured from dried blood spots and whole blood haemolysates.

Statistical analysis

Normality of the data was tested using a Lillifors test. If a data set was revealed to be non-normal, it was $\log_{10}$ transformed and the normality retested. The relationship between ChE activity measured in whole blood haemolysates prepared in Triton-X 100 and water was determined using a paired t-test. Values of ChE activity measured in whole blood haemolysates prepared in Triton-X 100 were subtracted from values measured in water, and an ANOVA was conducted to determine if this difference (i.e. potential inhibition) was related to species. Correlations between ChE activity measured in (1) whole blood haemolysates prepared in Triton-X 100 and water, (2) whole blood haemolysates prepared in Triton-X 100 and dried blood spots, and (3) plasma and dried blood spots were conducted using Pearson correlations.

Results

ChE activity measured in whole blood haemolysates with and without Triton-X 100, on filter paper, and in plasma was not normally distributed (Lillifors $p < 0.01$). Log_{10}-transformed ChE activities measured in whole blood haemolysates with and without Triton-X 100 were normally distributed (Lillifors $p > 0.05$); $\log_{10}$-transformed ChE activities measured on filter paper were essentially normally distributed (Lillifors $p = 0.04$) but $\log_{10}$-transformed ChE activities measured in plasma were not normally distributed (Lillifors $p < 0.01$). Inspection of this normal probability plot, however, illustrated that departure from normality was slight and due to skewness on the left tail, so it was decided that all $\log_{10}$-transformed variables would subsequently be analysed using Pearson correlations.

ChE activity measured in whole blood haemolysates prepared in Triton-X 100 and dried blood spots were significantly correlated ($r = 0.74$, $p < 0.001$) (Figure 1A), as was ChE activity measured in plasma and dried blood spots ($r = 0.68$, $p < 0.001$) (Figure 1B). ChE activity measured in whole blood haemolysates prepared in Triton-X 100 and water were strongly correlated ($r = 0.95$, $p < 0.001$) (Figure 2A). However, activity determined in Triton-X 100 (mean = 882.7, standard deviation = 400.1) was significantly lower ($p < 0.001$) than that measured in the presence of water alone (mean = 937.0, standard deviation = 414.7) by roughly 6%. The degree of inhibition varied by species ($p < 0.001$), and only six species did not exhibit Triton-X 100 induced inhibition (Figure 2B). The difference between ChE activity analysed with Triton-X 100 and with water was always above zero (e.g. no inhibition) in samples collected from Lanner hawks ($n = 1$), peregrine falcons ($n = 6$), red-shoulder hawks ($n = 1$), great blue herons ($n = 3$) and long-eared owls ($n = 8$). Over the course of the monthly bleeding of the three intoxicated bald eagles, ChE activity returned to 'normal', unexposed levels, and activity measured from dried blood spots closely matched that from whole blood haemolysates (Figure 3).

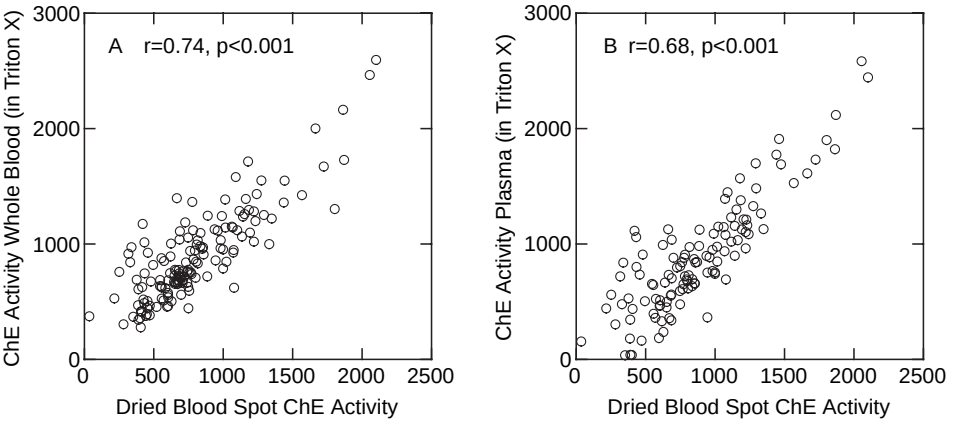

Figure 1. Correlations between (A) ChE activity (μmol min^{-1} l^{-1}) measured in whole bird blood haemolysates prepared in Triton-X 100 and dried blood spots ($n = 150$); and (B) ChE activity (μmol min^{-1} l^{-1}) measured in bird plasma and dried blood spots ($n = 120$).

Discussion

The results suggest a strong correlation between ChE activity in whole blood, plasma and blood eluted from the filter paper, provided that specific collection, shipment and storage guidelines are strictly followed. ChE activity measured from filter paper was better predicted from activity measured in haemolysates (55% of the variance explained) then from plasma (46% of the variance explained). This present study has demonstrated that monitoring pesticide exposure can be conducted using very elementary blood sampling, preserving and shipping procedures, and being rather inexpensive and simple, this technique is amenable to field situations when centrifuging and cooling devices are not available. Moreover, this technique is sensitive enough to be used as a diagnostic tool to identify exposure to ChE-inhibiting pesticides. Over the course of 1 month, repeat bleeding of exposed Bald eagles showed that dried blood spots and whole blood haemolysates showed similar trends when it came to tracking the recovery of ChE titres from an initial inhibition level of more than 50% of normal to levels considered 'normal' for the species. The use of Triton-X 100 did appear to inhibit ChE activity in the majority of species tested, but this reduction was only 6% when compared with preparations conducted in water alone. Though this inhibition is slight, the benefit of Triton-X 100 in this assay should be addressed further.

It must be pointed out that our technique varies from other standardized human methods, specifically in relation to handling tissue blanks, duration of the assay and the ambient temperature when conducting the assay, and drying procedures. In many of the species analysed there was considerable non-enzymatic activity measured in the blood tissue. Substrate blanks values were removed from the activity measured but the tissue blank was not, as it is unnecessary with plasma. This did not preclude the comparison of the activity measured in blood haemolysate versus the dried blood spots because the assay conditions were strictly the same but, in future analysis, we suggest investigating whether subtracting the tissue blank is actually required. In order to decrease the tissue blank values, Wilson et al. (1996) recommends a pre-incubation time of 5–10 min of all assay components except the substrate. However, this is not

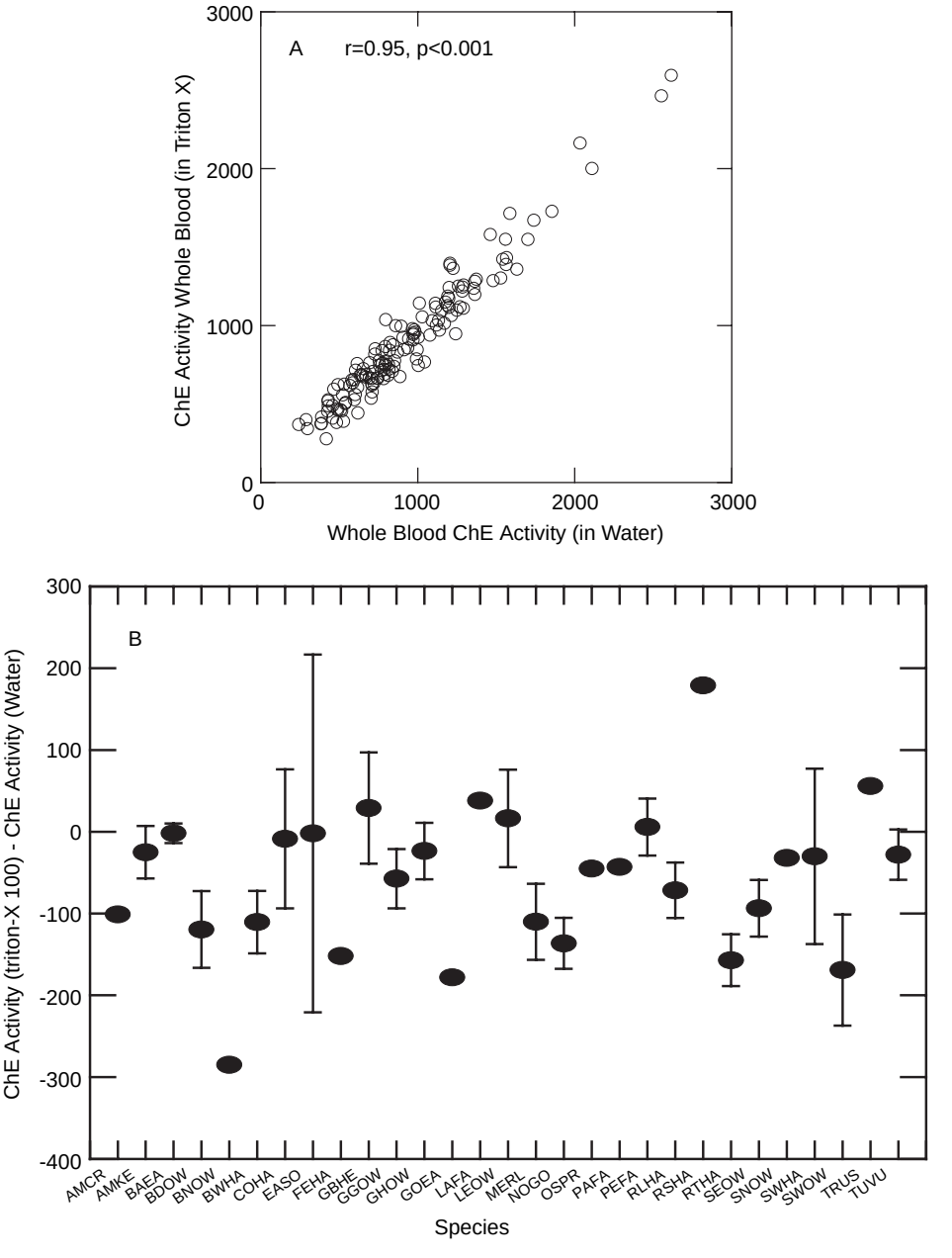

Figure 2. (A) Correlation between ChE activity (μmol min^{-1} l^{-1}) measured in whole bird blood haemolysates prepared in Triton-X 100 and water ($n = 150$). (B) Species, noted by banding abbreviation (Table I), -related differences between ChE activity (μmol min^{-1} l^{-1}) measured with water and Triton-X 100.

possible when dealing with carbamate-inhibited samples as increased time can result in enzyme reactivation.

In our method, readings were made at 15-s intervals for 1–2 min. Marden et al. (1994) recommends running the assay for at least 3 min. We feel that if in the future

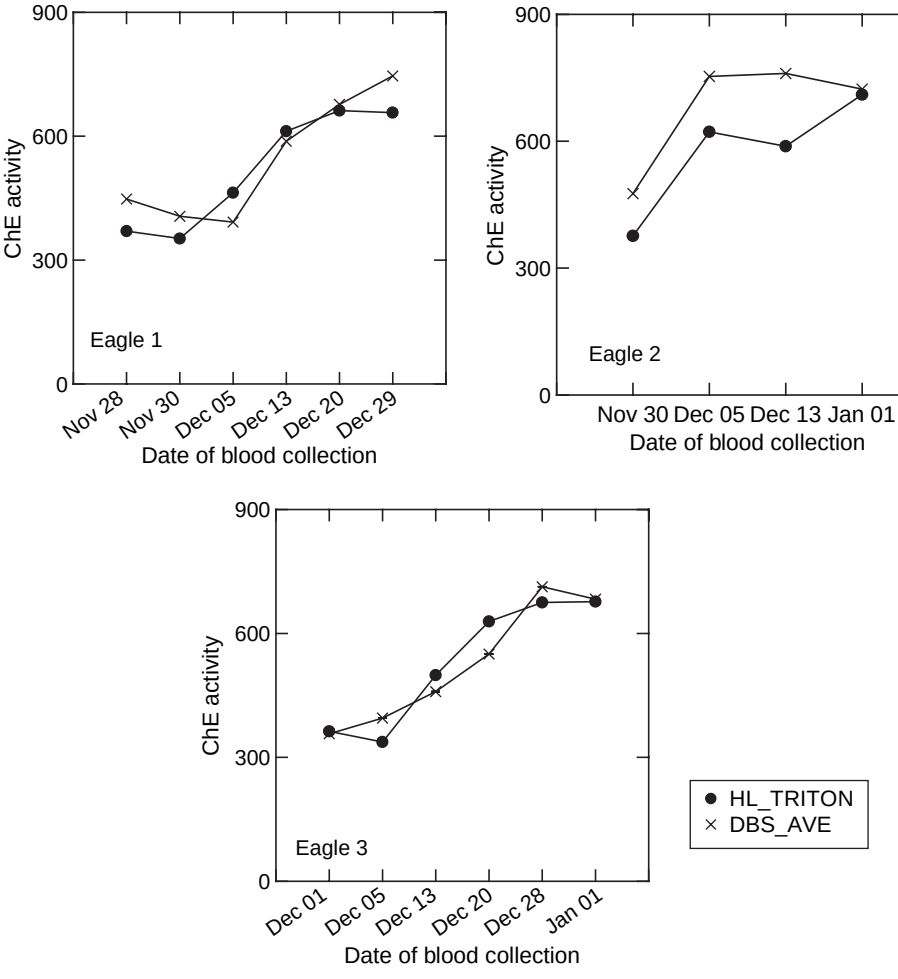

Figure 3. ChE activity (μmol min^{-1} l^{-1}) from three individual bald eagles exposed to ChE-inhibiting pesticide measured from whole blood (•) and from dried blood spots (X). Each graph depicts the results from an individual bird.

analyses the tissue blank is subtracted, then there is no need to increase reading time as it would not change the diagnosis and it would only diminish the throughput of samples. This decision would have to be reconsidered if a more automated method was used (e.g. microplate reader). Regarding temperature, maximum biochemical activity is usually obtained with temperatures approaching the bird's body temperature. In our laboratory an assay temperature of 30°C was chosen instead of 37 or 42°C as was the case in Augustinsson's method because these high temperatures may cause problems with carbamate-inhibited samples and are more difficult to maintain over the course of the assay. Some recommend working at 22–25°C, but we preferred to work at a slightly higher temperature because of the difficulty in maintaining a standardized temperature from day to day. Moreover, 30°C is closer to avian physiological temperature and thus results of ChE activity more likely biologically relevant.

It also appeared that one of the most important factors affecting the stability of the enzyme on filter paper is humidity. The National Committee for Clinical Laboratory

Standards (NCCLS) has published a document entitled *Blood Collection on Filter Paper for Newborn Screening Programs* (2003) where it is recommended that human samples be air-dried at room temperature for at least 3 h. However, under field conditions this is not always a feasible situation (i.e. collection conducted under various temperature and humidity levels). We recommend that samples be dried in a closed container with desiccant material. We would recommend the use of small packages of desiccant (Multiform Desiccant Products) along with a humidity indicator, or indicating Drierite, for the shipping of the dried blood spot to avoid direct contact of the Drierite with the filter paper. Collection methods may need to be adapted for work in extremely humid conditions in order to prevent early exhaustion of the desiccant material.

In order to be able to make a proper biochemical 'diagnosis' of carbamate or OP pesticide exposure, baseline values of ChE activity need to be known. In this study, tissues were provided by rehabilitation centres and it is, therefore, not known whether the values were indeed indicative of 'normal' levels because any number of these individuals might have been exposed to cholinesterase inhibitors. In our laboratory, we have a database with plasma and brain ChE values but we do not have any values measured with whole blood samples using PrThI as the substrate. We have tried over the years to keep the assay conditions consistent in order to be able to compare the results obtained. The assay parameters are not ideal for every species studied, but we have tried to maintain comparability of measurements. Because of the nature of the ChE enzyme and its large variability (due in part to animal age, circadian and circannual rhythms, sex, reproductive status and nutritional status, environmental temperature, pathophysiological states and disease: for a discussion of the subject (see Rattner & Fairbrother 1991) it would require considerable effort to optimize assay conditions for all the tissues that were received. Moreover, a study by Hunt et al. (1995) stressed the importance of optimizing not only the ChE assay but also the ChE reactivation techniques for each new species/pesticide combination. We know that a greater sensitivity could be obtained with a better characterization of the enzymes and optimization of the assay and reactivation conditions, but this would only have been feasible if sample supply was unlimited, and the pesticides involved were known. However, before starting to optimize conditions for each species, we have to consider the purpose of each study and the degree of precision that is needed to achieve the goals. In the context of wildlife exposure, one has to evaluate whether the optimization of the assay parameters would help in making a better, more sensitive diagnosis. In the case of whole blood, an inhibition rate of 30–50% below the normal value for the species is likely indicative of carbamate or OP pesticide exposure. A larger database of baseline ChE activity values would appear to be, in the long term, a more useful tool in the evaluating pesticides exposure than species-specific optimization of technique.

In the light of the results from this study, it appears that the use of filter paper as a matrix for storing dried blood is a feasible technique for assessing ChE activity in wild birds. With humans, the collection of blood spots on filter paper has been extensively used for a number of years with newborn screening programmes, and filter papers is now an acceptable matrix for a large number of analytes. The previously mentioned NCCLS publication contains a detailed description of techniques for blood collection on filter paper, specifications for specimen matrix and shipment, blood-spot-handling precautions for DNA analysis, etc. One of the suggestions in this document is that a punch be used to cut out a spot of a constant and specific diameter from the dried blood. In our method, blood was quantitatively transferred on the filter paper and the

whole spot cut out and used for the assay. Using the NCCLS technique means that the blood sample collection is facilitated because there is no need to quantify the amount of blood on the filter paper. The use of a spot of a known diameter (cut out with a punch) could be useful with the birds because some collectors have reported difficulty in collecting the blood in a field situation and/or with small birds. With the use of a standardized filter paper, handlers would simply have to puncture the bird vein and apply a spot of blood on the filter paper, without the burden on having to collect precise amounts.

The results suggest that a correlation exists between ChE activity in whole blood (measured with the standard techniques) and blood eluted from dried spots stored on filter papers, provided that specific collection, shipment and storage guidelines are strictly followed. This present study has demonstrated that monitoring pesticide exposure can be conducted using very elementary blood sampling, preserving and shipping procedures, and being rather inexpensive and simple, this technique is amenable in field situations when centrifuging and cooling devices are not available.

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