Original article

An automated spectrophotometric method for measuring canine ceruloplasmin in serum

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Abstract – An automated method for the determination of ceruloplasmin activity was developed and validated in canine serum. The method is based on the in vitro oxidase activity that this protein shows with substances such as p-phenylenediamine. In order to determine optimum assay conditions, the effects of the substrate concentration, buffer pH, reaction time and EDTA on the reaction were evaluated. The precision of the assay was good with within-run and between-run coefficients of variation lower than 10%. The method measured the ceruloplasmin values in a proportional and linear manner ($r = 0.99$) with a limit of detection of 0.0007 ± 0.0001 ΔAbs/min. A temperature of -20 °C kept the reagent stable for 30 days. The method is cheap and easy to adapt to any automated biochemical analyser, considerably decreasing the processing time required with the manual method. Additionally it allows to differentiate dogs with pyometra and trauma from clinically healthy dogs.

automated measurement / ceruloplasmin / dogs

1. INTRODUCTION

Ceruloplasmin (Cp) is an α2-glycoprotein which is considered as one of the major positive acute phase proteins in dogs [9]. It plays an important role in protecting host tissues from toxic oxygen metabolites released from phagocytic cells during inflammatory states [4]. Additionally it is involved in copper transport and antioxidant defence [11], the latter by inhibiting copper ion-stimulated formation of reactive oxidants and the scavengers H_2O_2 and superoxide [11].

Canine serum ceruloplasmin levels increase during infection, inflammation or trauma [2]. The increase is higher and evident earlier than in humans, peaking at about double normal values on the fourth day following surgery [2]. Measurement of this protein provides valuable information on the inflammatory status to clinicians in canine practice [12] and has recently been shown to be useful for monitoring the treatment of canine leishmaniasis [7].

In human medicine, methods for ceruloplasmin determination based on the oxidation of different compounds have become widely adopted for routine use in clinical chemistry laboratories. The most used substrates for these assays are p-phenylenediamine (PPD) or its N-dimetyl derivate and O-dianisidine dihydrochloride. But, despite

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the importance and clinical value that this protein could have in canine practice, only manual methods based on the PPD-oxidase assay have been reported to determine Cp concentrations in dogs [2, 12]. The main objective of the present report was to implement and validate an automated method for the determination of canine serum ceruloplasmin based on the use of the PPD substrate.

2. MATERIALS AND METHODS

2.1. Sample preparation

Two types of samples were collected and used for the assay:

– Samples obtained from clinically healthy dogs (healthy samples, $n = 15$). They were clinically healthy, presenting no abnormal findings at physical examination, and routine haematological and biochemical test results were unremarkable.

– Samples from dogs determined to have an inflammation (pathologic samples, $n = 25$) here defined as clinical cases of dogs with pyometra or traumas received at the Veterinary Clinical Hospital of the University of Murcia, Spain. The diagnosis was based on a clinical examination, standard haematology, clinical biochemistry profiles and different additional diagnostic tests such as radiography and echography.

Blood samples were obtained by cephalic venipuncture using serum tubes containing a coagulation activator and a gel separator (Tapval Aquisel, Barcelona, Spain). After 30 min, the tubes were centrifuged ($2\,000\,g$) for 15 min to obtain the serum. All serum samples were stored frozen ($-20\,°C$) until being tested (the maximum storage period was one month).

2.2. Apparatus

The analyses were performed in a multiple biochemical autoanalyser (Cobas Mira Plus, ABX Diagnostic, Montpellier, France).

2.3. Principle of the assay

The method employed in this work was based on that described by Sunderman and Nomoto [13], with the modifications of Lewis [6]. Ceruloplasmin catalyses the oxidation of PPD to yield a purple coloured product. The colour intensity is proportional to the concentration of ceruloplasmin in the sample [13].

2.4. Reagent preparation

Sodium acetate buffer (0.598 M) was prepared by dissolving 40.75 g of sodium acetate trihydrate (Panreac química, Barcelona, Spain) in distilled water and by adjusting the pH to 5.2 with glacial acetic acid and the volume to 500 mL (stability > 1 month at 4 °C) [6].

The solution of p-phenylenediamine HCl (0.0135 M) was prepared by dissolving 61.5 mg of PPD (Sigma Chemical Co., St. Louis, MO, USA) in 25 mL of sodium acetate buffer.

The reagent preparation listed above is only one out of several described in the following sections.

2.5. Assay procedures

In the reagent rack, the solution of PPD was the working reagent. Ten microlitres of the sample were mixed with 100 μL of the PPD solution. The increase in absorbance per min at 550 nm was measured and the results were expressed as values of an increase of absorbance per minute.

The concentration of a blank sample containing a PPD solution and distilled water was tested and the concentration obtained was subtracted from the final results.

2.6. Establishment of optimum assay conditions

In order to determine the optimum conditions of the assay, the maximum activity with lower background was calculated. In

order to measure the background, a blank sample containing the working reagents and distilled water was assayed.

2.6.1. Optimum concentrations of substrate

Solutions of PPD (0.0045–0.036 M) were prepared by adding 20.5, 41, 61.5, 82, 124 and 164 mg of PPD to 25 mL of acetate buffer. All solutions obtained were used to test two healthy samples, two pathological samples and distilled water.

2.6.2. Optimum pH of the buffer

Acetate buffer solutions ranging from pH 4.8 to 6.4 (4.8, 5.2, 5.6, 6, 6.4) were prepared and used for the testing of two healthy samples, two pathologic samples and distilled water.

2.6.3. Optimum reaction time

The time course of the reaction was examined by recording the change of absorbance every 25 s, when serum and PPD solution were mixed, until 300 s later. The assay was performed with a substrate concentration of 61.5 mg in 25 mL of buffer and a pH of 5.2. A serially diluted (1:2; 1:4; 1:8) serum from a dog with an inflammation was used for the analysis.

2.6.4. Effect of EDTA on the reaction

The effect of EDTA was assessed by adding EDTA disodium salt dihydrate (Panreac química, Barcelona, Spain) over the range 1.6–12.8 mg to 10 mL of the PPD solution. The solutions obtained were used for the PPD-assay of two healthy samples, two pathological samples and distilled water.

2.7. Validation of the assays

2.7.1. Imprecision

The within-run imprecision was calculated for 8 healthy and 8 pathological sam-

ples analysed 10 times in a single analytical run. Between-run imprecision was determined by analysing 3 healthy and 3 pathological samples on 6 different days within one month. All these samples were stored frozen at –20 °C in aliquots to avoid possible interferences due to repeated freezing and thawing.

2.7.2. Inaccuracy

Inaccuracy was evaluated indirectly by investigating linearity under dilution of two canine serum samples with a high concentration (0.0176 and 0.029 ΔAbs/min) of ceruloplasmin (pathological samples).

2.7.3. Limit of detection

The limit of detection of the assay was determined as the lowest concentration of ceruloplasmin which could be distinguished from a zero sample and was taken as the mean ± 2 standard deviations of 10 replicates of a blank sample, tested in one analytical run.

2.7.4. Stability of the reagent

To assess the stability of the reagents, a PPD solution (61.5 mg of PPD in 25 mL of buffer, pH:5.2) was prepared, divided into aliquots and stored at –20 °C, 4 °C and 20 °C. The solutions were used immediately after their preparation and 1, 4, 7, 15 and 30 days after for testing of the aliquots of 3 normal and 3 pathologic samples, that were stored at –20 °C. The results were compared each time with those obtained with freshly reagents prepared.
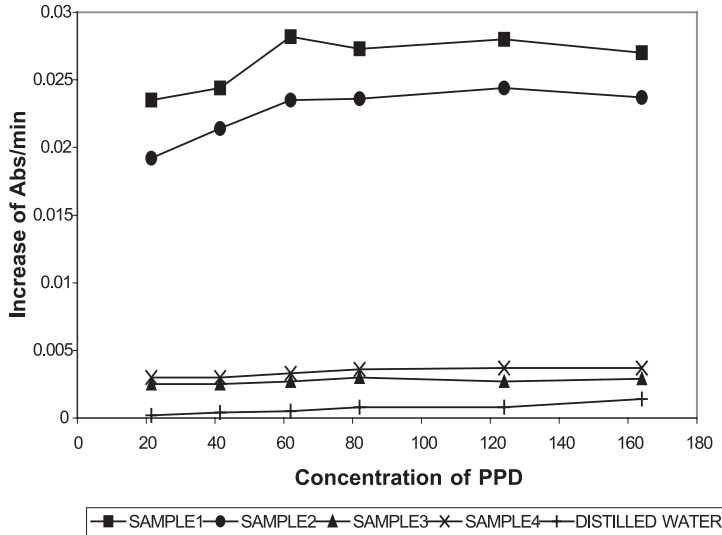
2.7.5. Application to biological samples

The assay was used to measure ceruloplasmin in samples obtained from three groups of dogs:
Group 1. Clinically healthy dogs ($n = 10$).
Group 2. Dogs with pyometra ($n = 10$).
Group 3. Dogs with diverse traumas ($n = 10$).

Figure 1. Optimum concentrations of substrate. Samples 1 and 2: Pathologic samples; Samples 3 and 4: Healthy samples.

2.8. Statistical analysis

The level of imprecision is stated quantitatively in terms of the coefficient of variation (CV(%)). Within-run and between-run CV were calculated as (standard deviation/average of replicated assays × 100. The investigation of linearity under dilution was accomplished by ordinary regression analysis. A wilcoxon signed rank test (significance accepted at $P < 0.05$) was used to compare the results obtained each day with the reagent stored at different temperatures and those obtained with a fresh reagent. A comparison of the results obtained in samples from clinically healthy dogs and samples from dogs with pyometra and diverse traumas was made using the Kruskall Wallis test; when significant results were obtained, the Mann Whitney non-parametric test of significance (with significance accepted at $P < 0.05$) was applied.

All statistical parameters were calculated using the SPSS statistical programme (SPSS Inc., Chicago, Ill., USA)

3. RESULTS

3.1. Establishment of optimum assay conditions

3.1.1. Optimum concentrations of substrate

The results obtained are presented in Figure 1. PPD solutions with concentrations of 61.5 mg of PPD in 25 mL of buffer for pathologic samples and with concentrations of 82 mg of PPD in 25 mL of buffer for healthy samples gave the highest values of an increase of absorbance/minute.

3.1.2. Optimum pH of the substrate

The maximum values of increase of absorbance/minute were obtained when the buffer pH was 5.2. The decrease or increase in the pH values caused a drop in the oxidase activity of ceruloplasmin (Fig. 2).

3.1.3. Reaction time

The different dilutions of the serum sample were linear and proportional throughout
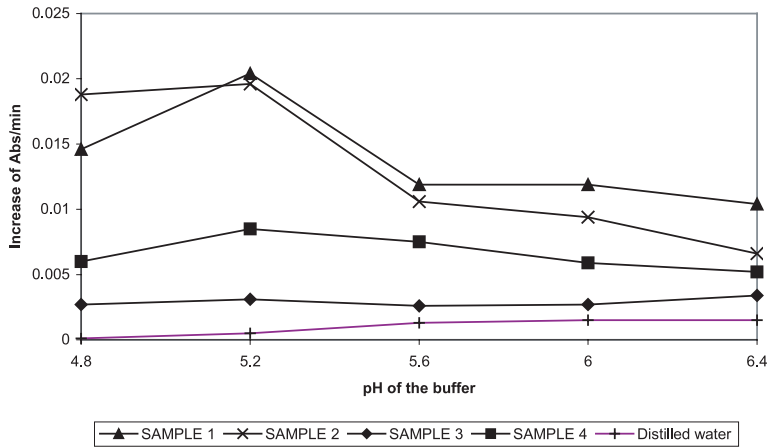
Figure 2. Optimum pH of the buffer. Samples 1 and 2: Pathologic samples; Samples 3 and 4: Healthy samples.
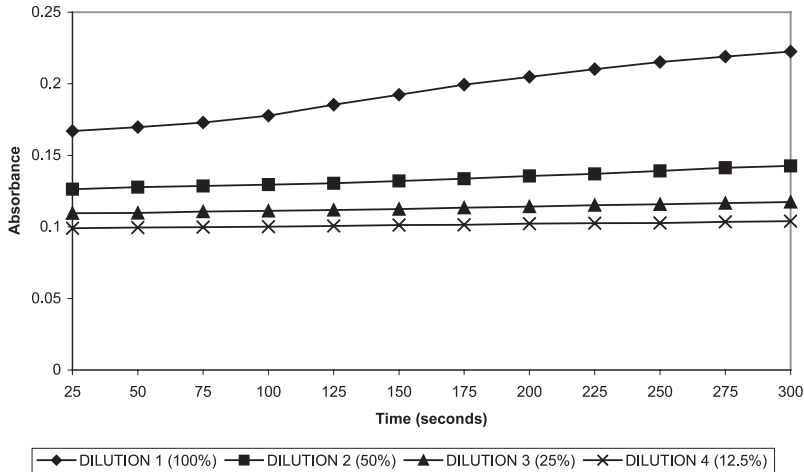
Figure 3. Reaction times.

the interval from 200 to 300 s after initiation of the enzymatic reaction (Fig. 3).

3.1.4. Effect of EDTA on the reaction

EDTA caused a drop in the increase of absorbance/minute of the samples and increased the reaction between PPD and distilled water (Fig. 4).

3.2. Validation of assays

3.2.1. Imprecision

Mean ceruloplasmin values obtained for samples assayed in this study were in a range of 0.0008–0.0318. The between-run CV obtained were higher than the within-run, but all CV were lower than 10% (Tab. I).
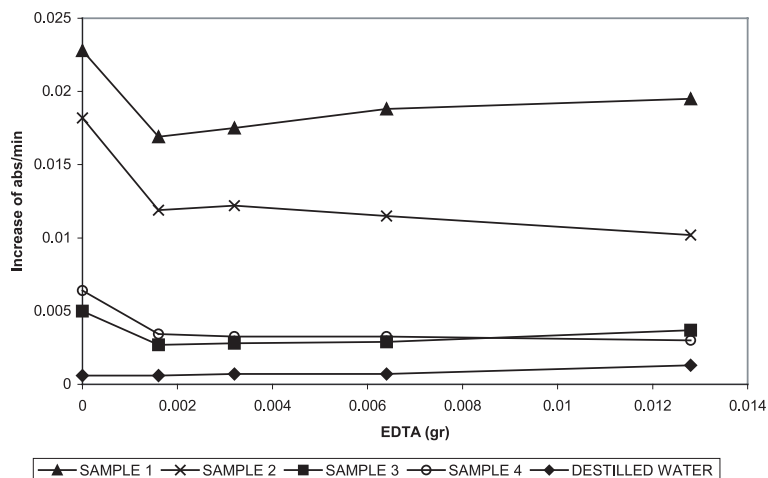
Figure 4. Effect of EDTA on the reaction. Samples 1 and 2: Pathologic samples; Samples 3 and 4: Healthy samples.

Table I. Precision of the PPD assay.

Samples	Within-run precision (n = 8)		Between-run precision (n = 3)	
	MEAN (ΔAbs/min) ± SD	CV (%)	MEAN (ΔAbs/min) ± SD	CV (%)
Normal	0.0016 ± 0.00013	8.1	0.0027 ± 0.00026	9.6
Pathologic	0.0299 ± 0.0013	5.2	0.0283 ± 0.0023	8.1

CV = coefficient of variation; SD = standard deviation.

3.2.2. Inaccuracy

The dilution of two serum samples with high levels of ceruloplasmin resulted in linear regression equations with a correlation coefficient of 0.99 (Tab. II).

3.2.3. Limit of detection

The detection limit calculated was 0.0007 expressed in values of an increase of absorbance/minute.

3.2.4. Stability of the reagents

Table III shows the values obtained with reagents freshly prepared and stored at different temperatures. No significant differences were seen between the results obtained with a PPD solution freshly prepared and stored at –20 °C. However significant increases in the results were seen after 7 days when the solution was stored at 20 and 4 °C. The PPD solution stored at 20 °C exceeded the absorbance limit of the test and could not be used on day 30.

3.2.5. Application to biological samples

Table IV shows that ceruloplasmin values in dogs with pyometra or traumas were significantly higher than in healthy dogs.

4. DISCUSSION

The quantification of the concentration of acute phase proteins has been established

Table II. Linearity under dilution.

	Sample 1	Sample 2
y-Intercept (95% CI)	0.00068 (–0.00040; 0.00177)	–0.00010 (–0.00044; 0.00023)
Slope (95% CI)	0.975 (0.854; 1.095)	1.006 (0.978; 1.035)
Coefficient of correlation	0.99	0.99

CI: Confidence interval.

Table III. Ceruloplasmin values obtained with fresh prepared and stored reagents at 20, 4 and –20 °C.

	Storage conditions	Median (ΔAbs/min)	Range (ΔAbs/min)
Day 0	Fresh reagent	0.0201	0.0047–0.0274
Day 1	Fresh reagent	0.0199	0.0037–0.0270
	20 °C	0.0216	0.0054–0.283
	4 °C	0.0208	0.0040–0.0283
	–20 °C	0.0189	0.0039–0.0277
Day 4	Fresh reagent	0.0225	0.0040–0.0283
	20 °C	0.0367	0.0058–0.0447
	4 °C	0.0246	0.0066–0.0315
	–20 °C	0.0224	0.0038–0.0297
Day 7	Fresh reagent	0.0222	0.0034–0.0289
	20 °C	0.0511*	0.0043–0.0691
	4 °C	0.0418*	0.0090–0.0474
	–20 °C	0.0238	0.0047–0.0285
Day 15	Fresh reagent	0.0287	0.0040–0.0342
	20 °C	0.0713*	0.0058–0.1072
	4 °C	0.0820*	0.0134–0.1023
	–20 °C	0.0272	0.0035–0.0306
Day 30	Fresh reagent	0.0305	0.0043–0.0362
	20 °C	----	-------
	4 °C	0.0893*	0.0198–0.1358
	–20 °C	0.0282	0.0017–0.0329

*$P < 0.05$.

as a valuable tool providing diagnostic information in animals with inflammation, infection or trauma [3]. With ceruloplasmin, some studies have shown that its measurement can be useful for the detection of an inflammatory state in dogs [12] and additionally it can be an aid for the diagnosis and the evaluation of the response to treatment in canine leishmaniasis [7, 8]. However, one of the current limitations of the routine use of ceruloplasmin determination in canine practice is that only manual and time consuming methods have been available. For this reason a new automated method for the

Table IV. Ceruloplasmin values obtained in healthy dogs, dogs with pyometra and traumas. Group 1: healthy dogs, Group 2: dogs with pyometra, Group 3: dogs with traumas.

Group	Median	Range
Group 1 ($n = 10$)	0.0040	0.0032–0.0046
Group 2 ($n = 10$)	0.0158*	0.0108–0.0214
Group 3 ($n = 10$)	0.0157*	0.0105–0.0210

* $P < 0.05$.

determination of ceruloplasmin activity was developed and is fully described in this work. The method is based on the in vitro oxidase activity that ceruloplasmin shows with substances such as p-phenylenediamine [5].

4.1. Optimum assay conditions

The concentrations of substrate that gave the highest values of absorbance/minute were 61.5 and 81.5 mg of PPD in 25 mL of acetate buffer, but a concentration of 61.5 was selected because it gave the highest values of ΔAbs/min for pathologic samples, permitting a better discrimination between healthy and pathological samples with a lower background. According to these results, initial concentrations of PPD lower than those used by other authors [2, 12, 13] should be used to measure Cp in the serum of dogs.

A pH value for the buffer of 5.2 was selected as the optimum for the PPD oxidase assay since it provided the maximum absorbance values with minimum background. This pH value was lower than those specified in previous reports [2, 12].

The reading times (200–300 s) that were established in this study to ensure the linearity of the reaction produced a decrease in incubation times compared with previous methods described for ceruloplasmin measurement [1, 2, 6, 10, 12, 13]. This implies an increase in sample throughput rate and a faster ceruloplasmin analysis.

Other authors [1, 10] added a small quantity of EDTA to the acetate buffer in order to suppress nonenzymatic oxidation of PPD, which is mainly due to trace contamination of cupric ions. However, on the contrary to previous authors, our results show that EDTA modifies Cp values in canine serum, increasing the background. The addition of EDTA to the buffer would not be recommendable for Cp measurements in canine samples.

The results obtained in the present report are expressed in values of an increase of absorbance per minute. This unit was used, instead of others such as mg/L [6], UI/L [10, 12] or oxidase units [1, 2] since it allows to clearly distinguish the changes that the different parameters studied (concentration of substrate, pH, times of lecture and addition of EDTA) produce during ceruloplasmin oxidase activity. Additionally the results could be reported in concentration units by using a standard with a known concentration of canine ceruloplasmin in order to calibrate the assay. Unfortunately, to our knowledge, suitable reference preparations of canine ceruloplasmin are not commercially available. However a standard of human ceruloplasmin could be used to transform the ΔAbs/min values obtained with canine samples to g/L, since parallel dilution studies made at the authors' laboratory indicate that diluted clinical samples gave acceptable parallel dilution curves to the human standard curve (data not shown).

4.2. Validation

Our validation showed a good assay precision with within-run and between-run CV lower than 10% in both normal and pathologic samples respectively. These CV were lower than those obtained by Solter et al. [12] with a manual method for canine samples. The automation of the assay could contribute to the decrease observed in the CV. The method measured the Cp values in a proportional and linear manner ($r = 0.99$) (at least, below 0.026 ΔAbs/min) with a limit of detection of 0.0007 ± 0.0001 ΔAbs/ min.

Storage at –20 °C kept the PPD stable for 30 days. In the present study, this was longer than the stability obtained when stored at 20 and 4 °C and longer than that reported by other authors [10].

Significant increases in Ceruloplasmin concentration were observed in dogs with pyometra or trauma when compared to control animals, supporting the results of previous studies revealing increases in diseased dogs [7, 8, 12].

Overall, the method for ceruloplasmin measurement described in the present study shows adequate analytical performance characteristics (imprecision and accuracy). It is cheap and easy to adapt to any automated biochemical analyser, considerably decreasing the processing time required with the manual method. Additionally it allows to differenciate dogs with pyometra and trauma from healthy subjects. It is expected that the description of this method and the promising results obtained with this acute phase protein in clinical trials will contribute to a wider use of ceruloplasmin determination in canine routine practice.

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