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# Repeatability and reproducibility of quantitative contrast-enhanced ultrasonography for assessing duodenal perfusion in healthy dogs

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**ABSTRACT.** Contrast-enhanced ultrasonography (CEUS) with microbubbles as a contrast agent allows the visualization and quantification of tissue perfusion. The assessment of canine intestinal perfusion by quantitative CEUS may provide valuable information for diagnosing and monitoring chronic intestinal disorders. This study aimed to assess the repeatability (intraday variability) and reproducibility (interday variability) of quantitative duodenal CEUS in healthy dogs. Six healthy beagles underwent CEUS three times within one day (4-hr intervals) and on two different days (1-week interval). All dogs were sedated with a combination of butorphanol (0.2 mg/kg) and midazolam (0.1 mg/kg) prior to CEUS. The contrast agent (Sonazoid®) was administered using the intravenous bolus method (0.01 ml/kg) for imaging of the duodenum. Time-intensity curves (TIC) were created by drawing multiple regions of interest (ROIs) in the duodenal mucosa, and perfusion parameters, including the time-to-peak (TTP), peak intensity (PI), area under the curve (AUC), and wash-in and wash-out rates (WiR and WoR, respectively), were generated. Intraday and interday coefficients of variation (CVs) for TTP, PI, AUC, WiR and WoR were <25% (range, 2.27–23.41%), which indicated that CEUS was feasible for assessing duodenal perfusion in healthy sedated dogs. A further study of CEUS in dogs with chronic intestinal disorders is necessary to evaluate its clinical applicability.

**KEY WORDS:** canine, duodenum, quantitative CEUS, repeatability

Contrast-enhanced ultrasonography (CEUS) is a relatively recent imaging technique using microbubbles (MB) as a contrast agent (CA). Sonazoid®, a second-generation CA, which contains lipid-stabilized perfluorobutane (PFB) gas-filled microspheres, is highly stable *in vivo* [4]. Using CEUS, tissue perfusion can be evaluated in real-time based on the intensity of contrast enhancement, which is proportional to the MB concentration within microvessels [7, 27]. Moreover, adjunct quantitative analysis, which is performed by drawing a region of interest (ROI) on targeted tissue and obtaining a number of perfusion parameters, provides a more objective evaluation [10]. Quantitative CEUS has been utilized for the perfusion analysis of various abdominal organs in dogs, including the liver, spleen, kidneys pancreas and adrenal glands [19, 21, 24–26, 34, 35].

The assessment of canine intestinal perfusion may provide valuable information for the diagnosis and monitoring of dogs with chronic intestinal disorders. In human medicine, intestinal perfusion of Crohn's disease (CD) patients has been evaluated by CEUS. It revealed changes in the enhancement pattern [28] as well as perfusion parameters when compared with healthy controls [14]. CEUS has been utilized to estimate the disease activity and predict the treatment response in CD patients [8, 13, 30, 31]. It has also been reported to be correlated with the endoscopic severity [29] and shown to be comparable with Magnetic Resonance Enterography (MRE) [16]. In veterinary medicine, a few studies reported that qualitative and quantitative CEUS enable the characterization of intestinal perfusion in healthy dogs [18, 20, 25], but the application in clinical practice has yet to be established.

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Repeatability (intraday variability) and reproducibility (interday variability) assessment of quantitative CEUS in evaluating intestinal perfusion is important as a prerequisite before clinical translation. It is necessary to evaluate its feasibility and determine reliable perfusion parameters to detect pathological changes in the intestine. It is even more essential as this modality is aimed at serially repeated assessment (e.g. disease monitoring and treatment evaluation), so that changes related to disease activity or the treatment response can be differentiated from physiological changes and measurement errors. To our knowledge, information on this topic in healthy dogs is still limited. Therefore, this study was aimed to assess the repeatability and reproducibility of quantitative CEUS of the duodenum in healthy dogs.

## MATERIALS AND METHODS

Six beagle dogs (three males and three females, aged 1–4 years, weighing 8.8–12 kg) were enrolled in this study. All dogs were healthy on physical examination and did not present any clinical signs or hematologic (complete blood count and serum biochemistry) abnormalities related to gastrointestinal diseases. CEUS was conducted on 4 days over a 9-day period (i.e. 3 dogs underwent CEUS on days 1 and 8, while the remaining 3 dogs underwent CEUS on days 2 and 9). On a given day, the three dogs were each examined 3 times (i.e. at 9.00, 13.00 and 17.00). The scanning of 3 dogs was performed consecutively according to the same order for each examination (dogs 1, 2 and 3 for days 1 and 8; and 4, 5 and 6 for days 2 and 9). Dogs were fasted for 12 hr and sedated with a combination of butorphanol (0.2 mg/kg) and midazolam (0.1 mg/kg) before the procedure. Sedation was performed to improve animal cooperation throughout duodenal imaging. During the procedure, the heart rate (HR) and mean arterial pressure (MAP) of all dogs were monitored noninvasively using an oscillometric technique (BSM-5192, Nihon Kohden Co., Tokyo, Japan). All procedures were approved by the Hokkaido University Animal Care and Use Committee.

B-mode ultrasonography was performed prior to CEUS for general imaging of the duodenum, and neither focal nor diffuse abnormalities were found. For CEUS acquisition, dogs were positioned in left lateral recumbency, and the ultrasound probe was placed behind the last rib to achieve a longitudinal view of the duodenum. Contrast agent (Sonazoid®, Daiichi-Sankyo, Tokyo, Japan) administration and CEUS acquisition (Aplio XG; broadband linear probe, 5–11 MHz, PLT-704AT, Toshiba Medical Systems, Otawara, Japan) were performed based on the bolus method described in a previous study on the pancreas [21] with few modifications. In the current study, the mechanical index (MI), imaging depth and focal zone depth were adjusted to 0.20, 3 cm and 2 cm, respectively. All CEUS was performed by one sonographer (S.Y.L.) and one CA administrator (S.M.) throughout the study.

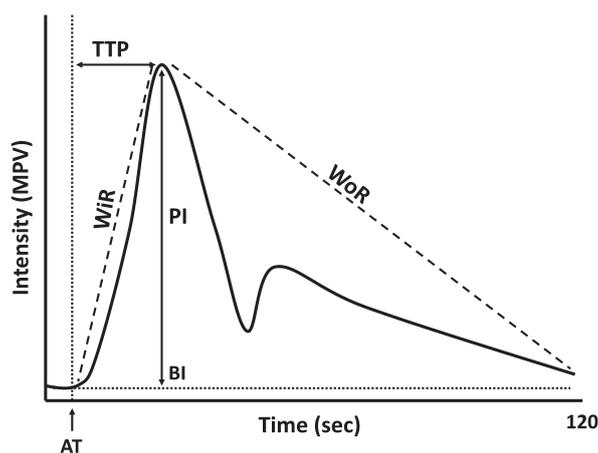
Quantitative analysis was done using image analysis software (ImageJ, US National Institutes of Health, Bethesda, MD, U.S.A.) by one observer (K.N.). One frame per sec for a total 120 sec was analyzed. Four ROIs were manually drawn as large as possible in the duodenal mucosa at approximately the same depth and without including big vessels or adjacent tissue. When a respiratory motion or duodenal movement was present, the ROIs were adjusted manually to maintain the same position within the duodenal mucosa and narrowed depth range. The analysis using the four ROIs was selected based on a preliminary study that indicated the best intraobserver agreement when compared with other methods (data not shown). If four ROIs could not be drawn due to motion artifacts, one, two or three ROIs were drawn instead. The software calculated the intensity within each ROI as a gray-scale level ranging from a mean pixel value (MPV) of 0–255. The intensity data were subsequently exported to commercial software (Microsoft Excel 2013, Microsoft, Redmond, WA, U.S.A.), followed by averaging the intensities obtained from the four ROIs. The averaged intensities were plotted against time to create a time-intensity curve (TIC).

A number of perfusion parameters were acquired from TIC, including the time-to-peak (TTP), which refers to the time from the arrival time (AT, time point when the intensity is above the baseline and followed by a further rise) to maximum enhancement; peak intensity (PI) refers to maximum enhancement with subtraction of the baseline intensity (BI, intensity at AT); area under the curve (AUC) refers to the area under the TIC curve above BI and is calculated from AT to 120 sec; wash-in and wash-out rates (WiR and WoR, respectively) refer to the slope of ascending and descending tracts of TIC, respectively. WiR and WoR were calculated by performing regression using the same Excel sheet to subsequent points that continued to increase from BI to PI and decrease from PI to the end of the recording, respectively (Fig. 1). Quantitative analysis for all scans was performed twice by the same observer to evaluate intraobserver variability.

Statistical analysis was done using statistical analysis programs (JMP pro 12.0.1, SAS Institute Inc., Cary, NC, U.S.A. and IBM SPSS Statistics V22.0, IBM Corp., Armonk, NY, U.S.A.). All perfusion parameters were evaluated for normal distribution using the Shapiro-Wilk test, and the results are expressed as the mean  $\pm$  standard deviation (SD). The following linear fixed effect model was used to analyze intraday, interday and intraobserver variability:

$$Y_{ijkl} = \mu + analysis_i + day_j + dog_k + (day \times dog)_{jk} + \epsilon_{ijkl}$$

where  $Y_{ijkl}$  is the  $l$ th value measured for dog  $k$  on day  $j$  in the  $i$ th analysis,  $\mu$  is the general mean,  $analysis_i$  is the differential effect of analysis  $i$ ,  $day_j$  is the differential effect of day  $j$ ,  $dog_k$  is the differential effect of dog  $k$ ,  $(day \times dog)_{jk}$  is the interaction term between day and dog, and  $\epsilon_{ijkl}$  is the model error. The SD of intraday variability was estimated as the residual SD of the model, the SD of interday variability as the SD of the differential effect of day, and the SD of intraobserver variability as the SD of the differential effect of analysis. The coefficients of variation (CVs) were calculated by dividing each SD by the mean and are written as a percentage (%). Based on previous studies of CEUS in humans and animals, CV <25% is considered clinically acceptable [20, 32]. The confidence intervals (CIs) were calculated by multiplying SD by 2.77 [2]. The CI represents the difference required between two analyses conducted at the same time or on a single individual for a true change to be detected with a probability of <0.05. In addition, the partial correlation coefficient between HR as well as MAP and perfusion parameters were analyzed.



**Fig. 1.** Schematic time-intensity curve describing wash-in and wash-out after bolus injection. The second peak indicated recirculation. The arrival time (AT) refers to the time point when the intensity rose above the baseline, followed by a continuous increase. The baseline intensity (BI) refers to the intensity at AT. Five parameters were analyzed: time-to-peak (TTP) refers to the time from AT to PI; peak intensity (PI) refers to the maximum enhancement subtracted by BI; area under the curve (AUC) refers to area under the TIC curve above BI, calculated from AT to 120 sec; wash-in and wash-out rates (WiR and WoR, respectively) refer to slope of ascending and descending tracts of TIC. WiR and WoR were calculated by performing regression to subsequent points that continued to increase from BI till PI and decrease from PI to the end of the recording.

## RESULTS

No dogs showed immediate or delayed adverse reaction (e.g., vomiting, syncope) after the bolus injection of Sonazoid<sup>®</sup>. Selected frames from a total of 36 CEUS examinations of the duodenum (six dogs; 3 times within one day and on 2 different days) were satisfactory for quantitative analysis. Four ROIs were drawn in selected frames of 34 CEUS examinations, while only one and two ROIs could be drawn in those of another two examinations due to motion artifacts.

Subjectively, the duodenum was enhanced within several sec after contrast injection (Fig. 2A). The contrast enhancement began from the perivisceral vessels, moved towards the duodenal lumen centripetally and involved all layers of the duodenal wall. Contrast enhancement was homogeneous along the imaged duodenal segment at PI (Fig. 2B). This was followed by contrast elimination during wash-out (Fig. 2C). The generated TIC showed biphasic decreases (Fig. 2D). From the arrival time, initial rapid wash-in and wash-out stopped approximately 20–30 sec after injection, followed by recirculation. The enhancement pattern of the duodenum and the generated TIC were consistent in all dogs.

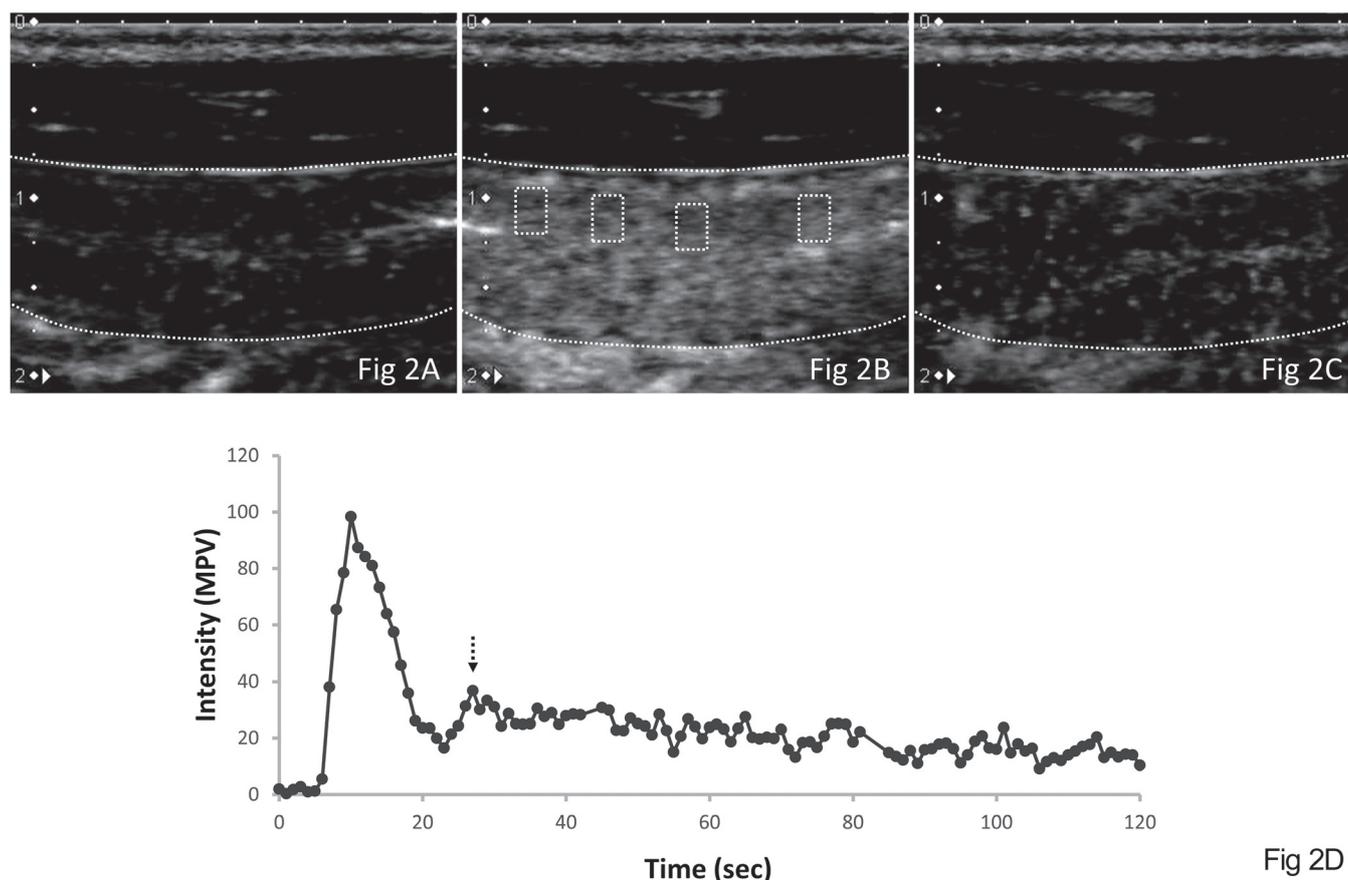
The mean  $\pm$  SDs, and intraday, interday and intraobserver SDs, CVs and CIs for all measured perfusion parameters derived from TIC of duodenal CEUS as well as hemodynamic parameters (HR and MAP) are summarized (Table 1). Intraday and interday CVs for TTP, PI, AUC, WiR and WoR were less than 25% (range, 2.27–23.41%). Intraobserver CVs for all perfusion parameters ranged between 2.27 and 8.30%. A significant partial correlation was indicated between HR and two of five perfusion parameters, TTP ( $P=0.012$ ) and WiR ( $P=0.007$ ). A negative partial correlation was indicated between HR and TTP ( $r=-0.444$ ), while a positive partial correlation was shown between HR and WiR ( $r=0.471$ ). No significant partial correlation was observed between MAP and any perfusion parameter.

## DISCUSSION

This study evaluated the repeatability (intraday variability) and reproducibility (interday variability) of perfusion parameters derived from duodenal CEUS of healthy sedated dogs. The results showed that the repeatability and reproducibility of duodenal CEUS in healthy sedated dogs were clinically acceptable, with the coefficient of variation (CV) for all perfusion parameters (including TTP, PI, AUC, WiR and WoR) being less than 25%.

Intraday and interday CVs for all perfusion parameters ranged from 2.27 to 23.41% (Table 1). In a study using a mouse tumor model, CVs ranging from 3.74 to 29.34% were considered acceptable for CEUS-derived perfusion parameters obtained from three repeated injections [11]. In another previous study of quantitative CEUS for abdominal organs of healthy cats including the small intestine, a CV of less than 25% for repeated examinations within a short time interval was considered acceptable [20]. In human medicine, a study of renal perfusion using CEUS also considered a change of more than 25% between two measurements to be significant [32]. Referring to these studies, the cut-off value of the CV required for a perfusion parameter derived from CEUS to be considered acceptable is 25%.

Low intraobserver CVs (range, 2.27–8.30%) for all perfusion parameters were documented in our study. Intraobserver variability indicates the size of variation when a CEUS image is analyzed more than once consecutively by the same observer. Intra-reader variability was examined in a study evaluating the reproducibility of hepatic hemodynamics with CEUS in healthy volunteers and



**Fig. 2.** Sequence images of the duodenum (dashed outline) following Sonazoid® administration in one representative dog (A–C) and the generated time-intensity curve (D). (A) The image of the duodenum during the arrival time of contrast agent [6 sec in this dog]. (B) Homogeneous enhancement along the imaged duodenal segment at peak intensity [10 sec in this dog]. Multiple ROIs (dashed box) were drawn at the same depth within the duodenal mucosa. (C) Contrast wash-out at the end of recording [120 sec]. (D) TIC showed a biphasic decrease. Rapid initial wash-in and wash-out (within 23 sec shown here), followed by recirculation (dashed arrow).

**Table 1.** Intraday, interday and intraobserver variability of perfusion parameters derived from the duodenal CEUS and hemodynamic parameters

Parameters	Mean ± SD	Intraday			Interday			Intraobserver		
		SD	CV (%)	CI	SD	CV (%)	CI	SD	CV (%)	CI
<b>Perfusion</b>										
Time-to-peak (sec)	4.40 ± 0.90	0.10	2.27	0.28	0.87	19.68	2.40	0.10	2.27	0.28
Peak intensity (MPV)	106.50 ± 11.50	13.71	12.87	37.97	9.12	8.57	25.27	4.61	4.33	12.78
Area under curve (MPV/sec)	3,474.12 ± 816.53	221.00	6.36	612.17	519.98	14.97	1,440.30	173.93	5.01	481.78
Wash-in rate (MPV/sec)	26.80 ± 6.50	1.59	5.92	4.40	6.27	23.41	17.37	2.22	8.30	6.16
Wash-out rate (MPV/sec)	-0.85 ± 0.09	0.05	6.44	0.15	0.08	9.11	0.21	0.05	6.44	0.15
<b>Hemodynamic</b>										
Heart rate (beat/min)	78.42 ± 15.94	25.83	32.94	71.56	4.95	18.31	39.78	NE	NE	NE
Mean arterial pressure (mmHg)	90.40 ± 12.25	4.95	5.48	13.71	8.59	9.50	23.78	NE	NE	NE

MPV, mean pixel value. SD, standard deviation. CV, coefficient of variation. CI, 95% confidence interval for mean. NE, not examined.

patients with liver cancer, and CVs within a range of 5–15% were reported. In this study, a single reader was assigned to analyze all scans twice. The author considered the documented CVs to be almost perfect [12].

Variability of perfusion parameters derived from CEUS could be related to internal and external factors. Internal factors are correlated with the animal physiology, such as cardiac output, blood pressure and heart rate. In the current study, a significant partial correlation was observed between HR and two perfusion parameters, TTP and WiR. A low HR resulted in a longer TTP and lower WiR. Low intraday and interday CVs of MAP were indicated, but those of HR were high, possibly influenced by the physiological status, level of excitement and sedative effect (Table 1). This could be the cause of the relatively higher interday CV for TTP and WiR in comparison with those for the other three parameters (PI, AUC and WoR).

External factors influencing the variability of perfusion parameters might include the scanning and analysis processes. Continuous scanning of the duodenum was challenging due to its peristaltic movement. Therefore, during off-line analysis, ROIs were carefully placed in the mucosa to avoid noise and artifacts. The result of analysis by manual placement of ROI could be influenced by the human error, but it was better than automatic analysis at avoiding problems related to intestinal or respiratory motion. The software for automatic analysis utilized in our preliminary study was not able to filter out the influence of such motions. Furthermore, it was difficult to perform repeated scanning exactly in the same segment of the duodenum and place the ROI at the same depth. We have minimized the spatial variation by standardizing the transducer approach to obtain a longitudinal view of the duodenum.

The enhancement pattern of the duodenum and generated TIC in the current study are consistent with those of previous studies of intestinal CEUS in dogs and cats [9, 18, 19, 21], which depicted the mural vascularization of the duodenal wall [15, 22]. Recirculation, which was detected after the first wash-out in our TIC, was the same as that described in healthy cats [9]. However, it was not described in the TIC of other previous studies on dogs [18, 19, 21], probably due to differences in the type of microbubble contrast agent, dosage and image setting between the current and previous studies. Recirculation is likely to cause only a small increase in the intensity, often smooth and gradual, as most of the MBs are destroyed due to ultrasound beam exposure during the initial circulation [33].

Perfusion parameters derived from TIC provide a more objective way to evaluate hemodynamic changes in the duodenum. TTP, WiR and WoR were correlated with the blood flow velocity, while PI and AUC were correlated with the blood volume within the corresponding ROI [10]. These parameters may change with chronic inflammation that causes a vascular rearrangement in the intestine due to physiological and pathological angiogenesis [1, 5]. Increases in the angiogenesis and microvessel density were investigated in the intestine of patients with inflammatory bowel disease (IBD), resulting in increased regional blood flow. The blood flow was reported to increase only in the mucosa and submucosa, and remained unchanged in the muscular layer [6, 17]. Even though differences between canine chronic enteropathy (CE) and human IBD have been reported [3], similar pathological changes in the intestinal perfusion could be seen.

The values of TTP, PI, AUC, WiR and WoR in the current study may serve as a reference for future examination of duodenal perfusion in clinical practice. In addition, the 95% confidence intervals (CIs) provided in the current study encompass the actual range of the mean for each perfusion parameter in healthy dogs (Table 1). In other words, true alterations related to pathological changes can only be considered, if the values decrease below or increase above this interval. A further study in dogs with chronic intestinal disorders should be performed to confirm whether the evaluated parameters have adequate sensitivity and specificity.

TTP in the current study was shorter compared with a previous report [21], because we selected the arrival time of the contrast agent as the starting point instead of contrast injection. The method of measuring TTP in our study was determined to minimize the influence of systemic blood flow and/or the contrast injection speed, and yielded better repeatability and reproducibility. The PI and AUC were higher than those recorded in the same report due to different scan settings. Therefore, the reference value provided in the current study should be used only for examination with the same protocol.

This study had several limitations. First, this study only evaluated the repeatability and reproducibility of duodenal CEUS in sedated dogs. Therefore, the repeatability and reproducibility of this method in unsedated dogs are unknown. A lower repeatability of hepatic vein CEUS was documented in conscious dogs when compared with sedated dogs [23]. Second, even though perfusion parameters derived from duodenal CEUS demonstrated acceptable intraday and interday CVs in the current study, these results cannot guarantee that this technique is applicable for other sonographers and/or other US machines. Interobserver variability was also not assessed in this study. Third, the dogs enrolled in the current study did not present any symptoms or laboratory findings related to gastrointestinal disorders, but the absence of inflammatory lesions could not be confirmed because the histopathological evaluation of the duodenum was not performed.

From the results, it can be concluded that quantitative CEUS was feasible in assessing duodenal perfusion in healthy sedated dogs. TTP, PI, AUC, WiR and WoR demonstrated adequate intraday, interday and intraobserver CVs. A further study in dogs with chronic intestinal disorders is necessary to evaluate the clinical applicability of duodenal CEUS.

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