Rapid blood sampling method for measuring intestinal permeability by gas chromatography in dogs

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Introduction

The intestinal epithelium has properties that provide both a barrier against various pathogens and transport of nutrients with respect to luminal molecules. The barrier function of the gastrointestinal tract can be assessed by intestinal permeability evaluation using macromolecules that diffuse passively through the mucosa using different pathways, transcellular or paracellular [4].

The permeability tests use one or more probes given orally. The most widely used probes are the non metabolized sugars, mannitol and L-rhamnose that permeate via the transcellular pathway and lactulose and cellobiose that enter through the tight junctions (paracellular route). The excretion of the probe is then measured in urine samples. In dual-sugar tests, the permeability of the smaller and larger sugars (i.e. Lactulose and L-rhamnose) are compared and expressed as a ratio to assess their excretion. This method avoids any bias effect from many factors such as gastric emptying, intestinal transit time, renal clearance and completeness of urinary collection, as they would affect both sugars equally.

Radioactive isotope of chromium (Cr-EDTA) has also been employed successfully in dogs. Further to some interpretation interference [11], there are practical limitations in working with a radiochemical agent. Therefore, non-digestible sugars

SUMMARY

The aim of the study was to determine a new and simplified procedure usable in daily practice for the measurement of intestinal permeability by the sampling of blood. This is the first report of a permeability test in blood sampling by gas chromatograph in veterinary medicine.

Two trials were conducted in 12 beagles. Lactulose and mannitol were the two classical forms of sugars used in this test for the different ways in which they are absorbed within the intestines. They were orally administered and blood samples were collected at 0, 1, 2, 3, 4 and 5 hours. Plasma aliquots were analysed in a gas chromatograph the results of which were also compared with the analysis realised in urine samples collected at the same time in the 6 male dogs. A repeated time measures ANOVA and the non-parametric Wilcoxon signed rank test were used for statistics with a p inferior to 0.05 considered as significant.

Comparison of the two trials showed no significant statistical difference. From 3 to 5 hours, the Lactulose/Mannitol ratio remained stable. The evolution of the Lactulose/Mannitol ratio in the urine and blood from 1 to 5 hours was very well correlated (r²=0.9).

In conclusion, the evaluation of intestinal permeability through blood sampling using gas chromatography with one single blood sample taken between 3 and 5 hours appears to be an easy and repeatable test which could be applied in veterinary practice.

Keywords: Intestinal permeability, dog, lactulose, mannitol, gas chromatography, blood test.

RéSUMÉ

Le but de cette étude était de mettre en place une méthode nouvelle et simplifiée, utilisable en pratique courante, de mesure à partir du sang de la perméabilité intestinale. Deux essais ont été conduits sur 12 beagles. Le lactulose et le mannitol sont deux sucres classiquement utilisés dans ce test pour leur mode différent d’absorption intestinale. Ils ont été utilisés dans cette étude et administrés oralement. Des échantillons sanguins furent collectés à 0, 1, 2, 3, 4, 5 heures post administration. Des aliquots plasmatiques ont été analysés par chromatographie en phase gazeuse. Les résultats ont été comparés à ceux obtenus par échantillonnages urinaires collectés sur les 6 mâles aux mêmes temps.

Une analyse de variance en temps répétés et un test non paramétrique de Wilcoxon ont été utilisés pour les analyses statistiques avec p inférieur à 0.05 considéré comme significatif. La comparaison des 2 essais n’a pas montré de différence significative. De 3 heures à 5 heures, le ratio est resté stable. L’évolution du ratio lactulose /mannitol dans l’urine et le sang de 1 à 5 heures était fortement corrélé (r²=0.9).

Pour conclure, l’évaluation de la perméabilité intestinale sur prise de sang par chromatographie en phase gazeuse avec un unique échantillon prélevé entre 3 et 5 heures semble être un test facile, répétable et applicable en pratique vétérinaire.

Mots-clés : Perméabilité intestinale, chien, lactulose, mannitol, chromatographie en phase gazeuse, test sanguin.

Keywords: Intestinal permeability, dog, lactulose, mannitol, gas chromatography, blood test.
are more often used. In many respects, they fulfil several requirements of an ideal probe: stable, non-toxic, biologically inert and confined to an extracellular location. The calculation of lactulose/rhamnose ratio or lactulose/mannitol ratio are the principal methods used in evaluating the intestinal permeability with sugars.

Measurement of intestinal permeability has been used as an investigative and diagnostic tool for intestinal diseases in both man and dog [4, 12, 15, 20].

In human medicine, the intestinal permeability test was tried in numerous situations. In 1995, BJARNASON et al. [4] realized a review of studies based on this technique and reported around 200 references. Since 1995 the test has been widely used in a large number of health and disease studies. In dermatology, the test was evaluated and is today used in atopic patients or patients with both adverse food reactions and dermatological symptoms. Nevertheless, the results remain conflicting. Even if a majority of authors have highlighted an increase in the L/M ratio [2, 3, 14, 17, 19, 26] associated with the presence of the symptoms, some authors reported a lack of response or a limited variation [13].

In veterinary medicine, this method has been used in normal dogs (i.e. dogs used for sporting activities [6]), in dogs with gastro-intestinal diseases [1, 20, 21, 23] and in dogs with traumatic injury [24]. Except studies on gluten-sensitive enteropathy [10, 12, 25], only one study has reported the potential interest of this test in dogs with food allergies associated with intestinal symptoms [20]. To our knowledge, not one study has been conducted in atopic dogs or dogs with dermatitis due to adverse food reactions. In human dermatology, this test is considered as a rapid, precise and non-invasive tool to diagnose food reactions [17] but it has never been used in veterinary medicine. So, its evaluation could be of great interest in veterinary dermatology and may give clinicians a new tool for diagnosis and/or the follow up of dogs with hypersensitivity diseases.

To evaluate intestinal permeability, trials are mainly done on urine samples. Dogs must remain in a cage between 5 and 24 hours after oral administration of sugars to collect the total urine volume either through a metabolic cage or urinary catheterisation or cystocentesis. Unfortunately, a large proportion of companion dogs do not spontaneously urinate in a cage and catheterisation or cystocentesis are needed but could be disapproved of by owners. In a practical and clinical goal, only a very simple protocol based on limited numbers of blood samples would be appropriate in order to use this test. In the literature, two studies [9, 22] have reported the use of blood samples for intestinal permeability test: one with sugars and high performance liquid chromatography (HPLC) and the second with Cr-EDTA. SORENSEN et al. [22] showed a good correlation between the ratio obtained with a 5-h urine sample (classical sample method) and a 2-h blood sample and proposed to use this one, unique blood sample to evaluate the ratio with this HPLC method.

The aim of this study was to determine a new procedure using gas chromatography for the measurement of intestinal permeability in blood samples. Such a technique would be acceptable by owners and applicable in daily clinical practice for the evaluation of dogs with atopic dermatitis or adverse food reaction dermatitis.

Materials and Methods

Animals

The experiment was conducted in 12 beagles (6 males and 6 females). The dogs were housed in closed indoor runs for the duration of the study. They were fed a dry expanded diet during the study. All dogs received routine vaccinations and were treated for endoparasites.

Males were 1.1 years old (8 to 14 months) and weighted 12.3 kg (8.5 to 15.8 kg). Females were 4.9 years old (3 to 6.5 years) and weighted 15.2 kg (9.3 to 16.4 kg).

Dogs were placed into metabolic cage during trials. Not one of the dogs urinated during these experiments.

Experimental Design

The day of the test, dogs remained unfed 12 hours before administration of the sugar solution and water was taken away 3 hours before and until the end of the test. A baseline of blood samples was collected before the start of each experiment to verify the absence of the administered sugars.

A solution containing 150 mg/mL of lactulose (Biogaran, Colombes, FRANCE) and 75 mg/mL of mannitol (Sigma Aldrich, Steinheim, GERMANY) was orally administered to each dog (2 mL of solution/kg) [16]. A blood sample was collected in 5 mL heparinised tubes at 60 (T1), 120 (T2), 180 (T3), 240 (T4) and 300 (T5) minutes after oral administration of the sugars from the jugular vein.

Two trials were realized for each dog at an interval of 8 weeks.

During the first trial, to compare this new method with a classical method of urine sampling, specimens (10ml) were also collected in the 6 male dogs by uretral catheterisation at the same times (60, 120, 180, 240, 300 minutes). Excluding the 10ml of urine extracted every hour, bladder drainage was absent. So the ratio at 5 hours is equivalent at the ratio at the fifth hour with a difference of 40 ml of urine collected.

Preparation of Blood Samples

Samples were processed using the method of FARHADI et al. [7].

Plasma was removed from the tube by centrifugation (5000 t/min, 15 min, 27°C), then labelled and stored at −20°C further processing at a later date. A plasma aliquot (0.3 mL) was combined with 12 μL of an internal standard solution of myo-inositol (Sigma Aldrich, Steinheim, GERMANY) (20 mg/mL) and then deproteinized by precipitation with 39 mL of metaphosphoric acid (Grosseron Laboratoires Merck, Nantes, FRANCE) (16% w/v) in a bain-marie (70°C, 1h). The supernatant was extracted after centrifugation...
(15300 t/min, 90 min, 27°C), quantified, frozen (-20°C) and finally lyophilized.

The dried residue was submitted to a reaction of oximation, for 1 hour at 70°C, in a solution of pyridine (Sigma Aldrich, Steinheim, GERMANY). 25 mg per mL of hydroxylamine (Sigma Aldrich, Steinheim, GERMANY), at the ratio of 400 µL of pyridine solution per mL of supernatant obtained before lyophilization.

An aliquot (60 mL) of the supernatant was transferred to a small conical tube and the sugar oximes were silylated with 60 µL of N-trimethylsilylimidazole (Interchim Laboratoire Pierce, Paris, FRANCE) for 30 min at 70°C.

Two or 3 aliquots of each blood sample were prepared and sealed in an autosampler vial for assay in a chromatograph.

PREPARATION OF URINE SAMPLES

Urine was stored at –20°C for further processing at a later date. A urine aliquot (0.3 mL) was combined with 12 µL of an internal standard solution of myo-inositol (20 mg/mL), frozen (-20°C) and finally lyophilized. The dried residue was submitted to a reaction of oximation and silylation, under the same conditions as that of the blood sample.

Two or 3 aliquots of each urine sample were also prepared and sealed in an autosampler vial.

ASSAY OF SUGARS

Gas chromatography has been evaluated as a simple, rapid, sensitive and reproducible technique for intestinal permeability testing [5, 7, 8].

Analyses were performed in a gas chromatograph (Hewlet Packard 6890, Palo Alto, USA), equipped with a flame ionization detector and a DB-1 capillary column (15m x 0.53 mm ID, 1.5 µm film thickness; J & W Scientific, Folsom, USA). For analysis, detector temperature was maintained at 280°C and the injector at 250°C, splitless. The carrier gas was helium with a constant flow of 40 ml/min. The injection volume was 1 µL.

The initial oven temperature of 220°C was held for 5 minutes and then increased by 4°C/min to 260°C which was maintained for 7 minutes. The total running time was 22 minutes.

Between each sample run, a solvent run with Hexane was performed to clean the column and to avoid ghost peaks.

The identification of peaks for the different sugars was assessed previously to the in vivo test. Each sugar was added separately to plasma aliquots and prepared for the analysis by the method described in this article. Their corresponding times of retention were noted (results not shown).

The amount of each sugar was measured by its area under the curve, quantification was evaluated by the comparison with the internal standard sugar.

STATISTICAL ANALYSIS

Statistical analysis was performed with statistical software (Statview 4.1, SAS Abacus Concept, Inc., Berkeley, California, USA). All continuous data were expressed as mean ± SEM. A repeated time measures ANOVA and the non-parametric Wilcoxon signed rank test were used to examine the permeability results within the different groups. Significance was declared at p≤0.05.

Results

CHROMATOGRAPHIC SEPARATION IN PLASMA

The peaks corresponding to the 2 sugars used (lactulose, mannitol) were consistently well resolved (Figure 1) and standard curves showed a linear relationship between concentration and area under the response-peaks (results not shown) with an excellent coefficient of correlation (respectively 0.98 and 0.99). The retention time was 2.9 minutes for the mannitol and 13.5 for the lactulose.

EVOLUTION OF PLASMA SUGARS CONCENTRATIONS

Mannitol was faster absorbed in plasma than lactulose. It obtained its highest concentration at T1 than that of lactulose at T3.

The concentration of mannitol decreased throughout the trial (Figure 2) whereas the concentration of lactulose slightly increased until T3 and then decreased (Figure 3).

EVOLUTION OF THE PLASMA RATIO L/M

The comparison of both trials with the same dogs at an interval of 8 weeks showed no significant difference (p = 0.11).

When considering the time of sampling, a statistically significant and marked increase of the ratio L/M was observed between T1 and T2 (p<0.05) and T2 and T3 (p<0.05). From T3 to T5, the ratio remained stable (respectively T3-T4 p = 0.13, T4-T5 p = 0.31) (Figure 4).

Between the two assays the coefficients of the variation were respectively for T1, T2, T3, T4 and T5: 6.9%, 23.4%, 9.9%, 5.9% and 9.4%.

For each time, an inter-individual variation of the L/M value was present (Figure 5). The excretion ratios of L/M were 0.098±0.012 at T1, 0.156±0.010 at T2, 0.235±0.016 at T3, 0.267±0.023 at T4 and 0.300±0.025 at T5 (Figure 4).

The evolution of the L/M ratio in urine and blood, in males, from T1 to T5, correlated well (r²=0.9, p<0.05) (Figure 6). As in the SORENSEN et al. study [22], an excellent correlation of the ratio results (r²=0.8, p<0.05) was observed between the 2-h blood sample and the 5-h urine sample with no significant difference (p = 0.11) (respectively 0.37±0.031 versus 0.107±0.092).
FIGURE 1: Chromatogram of a plasma sample. The peaks of mannitol, myo-inositol and lactulose are observed with their retention time, respectively at 2.9, 4.5 and 13.5 minutes.

FIGURE 2: Evolution of the plasmatic ratio mannitol/myo-inositol (internal standard) during 5 hours after administration of sugars (Mean ± SEM).

FIGURE 3: Evolution of the plasmatic ratio lactulose/myo-inositol (internal standard) during 5 hours after administration of sugars (Mean ± SEM).

FIGURE 4: Evolution of the lactulose/mannitol ratio in plasma during 5 hours after oral administration of sugar solution (Mean ± SEM).

FIGURE 5: Inter-individual variation of the lactulose/mannitol ratio for each time of sampling in the 12 beagles.

FIGURE 6: Correlation curve of lactulose/mannitol ratio in function of sampling time between urine and blood, and during 5 hours after oral administration of sugar solution.
Discussion

To the authors’ knowledge, this is the first report of a permeability test in blood sampling by gas chromatograph in veterinary medicine. This study suggests that measurement of mannitol and lactulose and the calculation of the ratio may be achieved in plasma with comparable results to that of a classical method in urine.

Pre-treatment of the plasma for the analysis of sugars creates compounds that can bind to the column of the chromatograph and produce different parasite peaks. However, they don’t interfere with sugar detection. The peaks of mannitol, myo-inositol and lactulose are clearly identifiable and are proportional to their concentration in plasma. Moreover, the absence of variation between both trials showed that this pretreatment has no or little effect on the sugar dosage.

Despite the collection of 10 mL of urine every hour, the authors observed an excellent correlation between the 2-h blood sample and the 5-h urine sample as in the SORENSEN et al. study [22]. A bias in the results due to the successive samples was unlikely. The reason could be that, at the same time, the relative fraction collected from the bladder decreased: 1/3 of the diuresis at 60 min, 1/5 at 120 min, 1/7 at 180 min and 1/9 at 240 min (if we consider, in the dog, a mean diuresis of 60 ml/kg/day), whereas, with time, the value of the ratio increased. Despite this correlation (5-hour urine sample and 2-hour blood sample), the values obtained later than 2 hours (3 to 5 hours) seemed more appropriate for sampling due to the stable and constant values. An evaluation based on a blood sample performed at 3-5 hours could give a good estimation of the intestinal permeability. This procedure would be simpler and more convenient than a urine collection during 5 to 24 hours for the daily practice and more acceptable for pet owners.

Comparison of this study with others using permeability test in dog by blood sample [9, 22] is difficult due to the disparity of methods, of sugars used (rhamnose versus mannitol), the difference of age and breed. However, mannitol/lactulose ratio in the present study and rhamnose/lactulose ratio in SORENSEN et al. study [22] showed a similar kinetic result throughout the experiment. Furthermore, despite differences in analytical methods, lactulose/rhamnose [22] and lactulose/mannitol ratios from T3 to T5 had similar measurements which included results between 0.23 and 0.3.

Although of a same breed and similar age group, inter-individual variations were mild. This inter-individual variation of the L/M ratio, associated with those known based on age and breed [18, 27], suggested that the ratio could be employed for individual diagnosis in atopic dogs, only if the increase of ratio is important.

In the case of food allergies and hypersensitivity in humans, VENTURA et al. [26] demonstrated that the L/M ratio progressively increased in function to the severity of clinical symptoms, in comparison with normal subjects, from the subgroup of patients suffering from pruritus to patients suffering from angioedema and/or anaphylaxis. Moreover, a study in children with atopic dermatitis [19] showed a positive association between the lactulose to mannitol ratio and the severity of the eczema. Thus, from results of human research, it seemed that the more severe the cutaneous or intestinal symptoms were, the more the ratio increased. The use of such testing in dogs with canine atopic dermatitis or adverse food reaction dermatitis would correspond to evaluate dogs with no or slight intestinal disorders and inconsistent severity of skin symptoms. Thus, one may hypothesise a limited and subtle variation of the ratio. But, the probable slight increase of the ratio in such populations associated with a large inter-individual variation could make this test inadaptable for a diagnosis tool. In return, it could be efficiently used for the follow up of one atopic dog throughout treatment where the patient is its own control.

Conclusion

The evaluation of intestinal permeability by blood sample and analysis with gas chromatography is practicable in dogs. A unique blood sample taking between 3 and 5 hours could be an easy, good and repeatable test applicable in clinical practice and acceptable by owners. Thanks to this new method, tests in dogs with hypersensitivity dermatitis (atopy, food adverse reaction) is the next, clinical step. It will allow to assess and determine the interest of this ratio in the diagnosis and/or the follow up of these populations.

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