Scandinavian Journal of Laboratory Animal Science

2018, Volume 44, Number 4
ISSN 2002-0112

Original scientific article

Determination of iohexol in canine plasma – strong correlation between enzyme-linked immunosorbent assay, high-performance liquid chromatography, and neutron activation analysis

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Summary

Iohexol is a non-radioactive, iodinated, water-soluble radiographic contrast medium that is widely used in detection imaging for both clinical and scientific purposes. It has also been used as a marker for glomerular filtration rate (GFR) and intestinal permeability (IP) in both humans and animals, such as dogs, rats and cats. Currently, iohexol is determined mainly by high-performance liquid chromatography (HPLC) methods which limit its use in veterinary clinical practice. The aim of this study was to validate an enzyme-linked immunosorbent assay (ELISA) and its accuracy for the measurement of iohexol in canine plasma by comparison with HPLC and neutron activation analysis (NAA). Blank and iohexol-containing blood samples (n=100) from Beagle dogs were collected from the jugular vein in lithium heparin tubes before and after intravenous application of 3.0 g iohexol/dog via the cephalic vein.

The results of this study show that the correlation coefficients when comparing ELISA vs. HPLC (r=0.99), ELISA vs. NAA (r=0.99) and HPLC vs. NAA (r=0.98) are all excellent. In conclusion, the measurement of iohexol from canine plasma using ELISA is as reproducible and reliable as using HPLC or NAA. However, using ELISA for measuring iohexol may be more practical, economical and useful for clinical practice and research than using HPLC or NAA.

Introduction

Iohexol (5-[*N*-(2,3-dihydroxypropyl) acetamido]-2,4,6-triiodo-*N*, *N*'-bis(2,3-dihydroxypropyl) isophthalamide) is a non-radioactive, low-osmolar, iodinated, water-soluble radiographic contrast medium (Andersen et al. 2001). This molecule has been utilized as a practical and reliable marker of glomerular filtration rate (GFR) in humans, pigs, horses, donkeys, dogs, rats and cats (Gleadhill and Michell 1996; Schwartz and Furth 2007; Bexfield et al. 2008; Wang et al. 2012; Meucci et al. 2013; Hellqvist et al.

2015; Meucci et al. 2015; Passos et al. 2015; Schwertner and Weld, 2015). Iohexol has also played a fundamental role in assessing intestinal permeability (IP) (Andersen et al. 2001), having been successfully used as an IP marker in humans, dogs, horses and rats (Halme et al. 1997; Halme et al. 2000; Frías et al. 2009a; Klenner et al. 2009; Koskinen, Hewetson and Pöytakangas 2015).

Although the current gold standard contrast agent for assessment of GFR and IP is 51Cr-EDTA (Frías, Sankari and Westermarck 2004; Frías et al. 2009b), there is evidence that iohexol shares a similar IP and GFR pathway with this marker in dogs and humans, respectively (Klenner et al. 2009; Slack et al. 2014). However, due to its radioactivity, 51Cr-EDTA is used only by specialized institutions and not in routine clinical practice (Klenner et al. 2007).

The main detection techniques used for iohexol determination have been high performance liquid chromatography (HPLC) and neutron activation analysis (NAA), because the characteristics of these detection methods for assessment of the iohexol concentration are the best known and best validated to date (Albert et al. 2003; Soman, Zahir and Akhlaghi 2005; Klenner et al. 2007; Klenner et al. 2009; Pöytäkangas et al. 2010; Gerova et al. 2011; Meucci et al. 2013; Luis-Lima et al. 2014)

A further validation of a practical, reliable, accurate and less costly method for the determination of iohexol concentrations has been considered necessary, particularly for the clinical use of this marker (Frías, Sankari and Westermarck 2004; Klenner et al. 2009; Meucci et al. 2013; Frías et al. 2014; Jovanović et al. 2015;). Enzyme-linked immunosorbent assay (ELISA) has been widely used in veterinary clinical practice and research showing that it is a simple method to perform and deliver reliable results. However, there are to our knowledge no publications assessing the validity of an ELISA for measuring the concentration of iohexol in canine plasma. We tested the hypothesis that the results of an ELISA for determining iohexol in canine plasma are well correlated with the results of previously validated HPLC and NAA methods.

Materials and methods

Plasma samples

The experimental protocols using dogs were approved by the local Ethics Committee for Animal Experiments of the University of Helsinki, Finland (license numbers STU 758 A and STU 776 A). All the dogs were cared for and used in experiments in accordance with the principles outlined in the Finnish and European legislation on the use of vertebrate animals for scientific purposes (European Community Council Directive 86/609/EEC, Council of Europe, 1986; Finnish Government, 1985; Finnish Government, 1996).

The plasma samples (n = 100) taken from 10 clinically healthy Beagle dogs were historical samples from an unrelated study in which iohexol had been used to determine GFR (Pöytäkangas et al. 2010). The dogs were maintained in indoor pens, spending about 4 h daily in outdoor runs. The environmental temperature indoors was maintained within a range of c.15-24°C. Prior to commencement of the study, each dog was subjected to a clinical examination and a complete plasma biochemical analysis and blood count to confirm the absence of disease.

The blood samples were collected from the jugular vein in lithium heparin tubes before and after intravenous application of 3.0 g iohexol/dog (Omnipaque 300 mg mL^{-1}; GE Healthcare, Helsinki, Finland) via the cephalic vein (Pöytäkangas et al. 2010). Subsequently, blood samples were kept on ice packs until being centrifuged (1,300 x g for 10 min). Plasma was drawn off and frozen at -20°C. Prior to analysis, plasma samples were thawed to room temperature and mixed thoroughly. All samples were analyzed in duplicate (Pöytäkangas et al. 2010). Iohexol concentration was analyzed by rapid high-performance liquid chromatography-ultraviolet (LC-UV) in the Faculty of Veterinary Medicine, Helsinki, Finland, prior shipping the samples to BioPhysics Assay Laboratory Inc (BioPAL, Worcester, MA, USA) for analysis using ELISA and NAA.

Immunoassay analysis

The ELISA (functional immunoassay technology (FIT)-GFR™ Iohexol kit, BioPAL, Worcester, MA, USA) was used according to the manufacturer's instructions. Iohexol standards were prepared using the ELISA kit diluent (0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 µg/mL), and then, to bring the samples within the active range of the standard curve, the plasma samples were diluted 1:300.

Fifty µL of standard or diluted sample were pipetted into wells of a 96-well coated plate, and then 50 µL of rabbit anti-iohexol were added to each well. The plate was incubated on an orbital shaker for 1 h, and immediately washed 3 times with a Tween 20 PBS wash buffer (Elx50 WasherBiotek Instruments, Inc., Winooski, VT, USA). Then, 100 µL of goat anti-rabbit IgG-HRP was pipetted into each well and again incubated for 30 min followed by a

second plate wash cycle. Substrate reagent (100 µL) was added to each well and incubated for 30 min at room temperature. Finally, stop reagent (100 µL) was added to each well.

The absorbance at 450 nm was recorded using software supplied with the plate reader (Multiskan® Spectrum, Thermo Electron Corporation, Waltham, MA, USA), and data from the standards were fitted to a 4-parameter logistic function. By interpolation, the concentration of iohexol present in each sample was determined.

Neutron Activation Analysis

Each plasma sample was centrifuged and 100 µL of plasma was transferred to a vial designed for neutron activation analysis (NAA), as described previously (Albert et al. 2003; Mandelbrot et al. 2007). Each vial contained a known amount of a metallic monitor to account for potential neutron-flux density variations during neutron activation (Reinhardt et al. 2001). All vials were activated by exposure to a field of neutrons. These vials were stored for 48 h to allow the short-lived activation products to decay. The concentration of the resultant radioactive nuclei in each vial was then measured by spectrographic analysis (Reinhardt et al. 2001). Iohexol standards were prepared in the same way.

Rapid high-performance liquid chromatography-ultraviolet

Iohexol concentration in canine plasma samples was determined using rapid HPLC-UV previously developed and validated for the assessment of the GFR and IP (Pöytäkangas et al. 2010). In brief, trifluoracetic acid (TFA) was used for protein precipitation and iohexol extraction from canine plasma, followed by vortex mixing and centrifugation. 4-Aminobenzoic acid (para-aminobenzoic acid, PABA) was added as an internal standard. Samples were analyzed by an Agilent model 1200 series rapid resolution LC system (Agilent Technologies, Waldbronn, Germany). The mobile phase gradient was linear and consisted of methanol and water (pH 3.0, adjusted with TFA). Gradient stop time was 8 min and post-time 5 min. The flow rate was 1 mL/min. At a column oven temperature of 50°C the LC operating pressure was approximately 310 bar. Iohexol detection was carried out at a wavelength of 246 nm, and then using a Chemstation data system the results were calculated (Pöytäkangas et al. 2010).

Statistical methods

For each sample, the iohexol concentration measured by ELISA was compared to the concentration measured by NAA and HPLC using Bland and Altman analysis (Bland and Altman 1986). NAA, ELISA and HPLC were compared by calculating correlation coefficient, precision (standard deviation of bias), bias (the difference between a population mean of the measurements), and accuracy (absence of bias, percent error from the true value) (Albert et al. 2003; Walther and Moore 2005). Statistical analysis was performed by using GraphPad Prism 6.07 (GraphPad Software Inc., La Jolla, CA, USA).

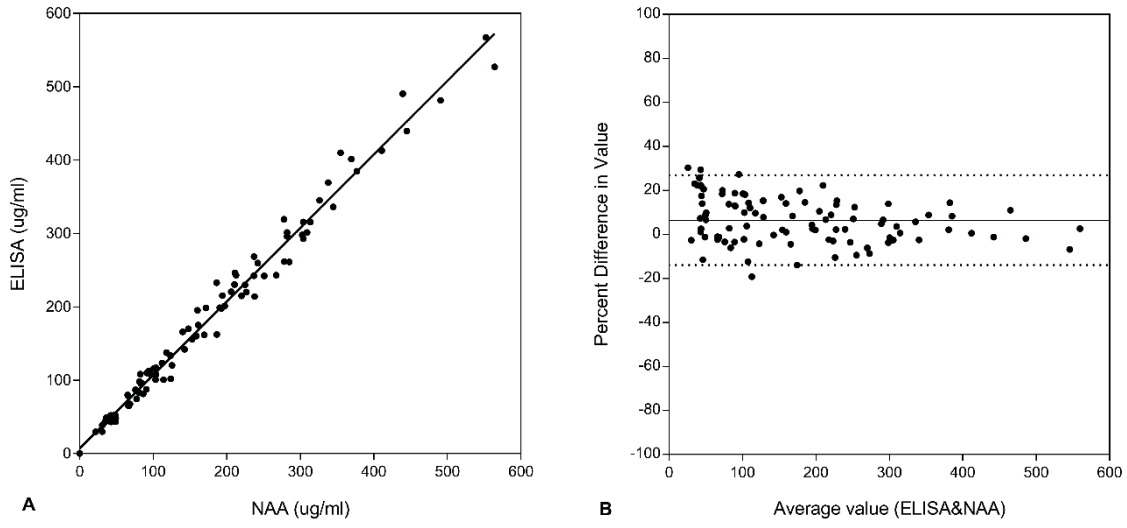

Figure 1. The iohexol concentration (µg/mL) in collected plasma samples (n = 100) measured by ELISA and NAA. A. ELISA values compared directly with NAA values. y = 1.002x + 7.164; r = 0.99 (p <0.0001). B. Difference against the mean iohexol value.

Results

No evidence of gastrointestinal or renal disease was found in any of the dogs used in the study. All dogs were in good body condition, no abnormalities were identified on clinical examination, plasma biochemical analysis and blood count. Iohexol tolerance was excellent in the dogs and there were no adverse effects.

The range of iohexol concentrations measured with the ELISA was 0–600 µg/mL. The correlation coefficients when comparing ELISA vs. HPLC (r=0.99), ELISA vs. NAA (r=0.99), and HPLC vs. NAA (r=0.98) were all strong (Figs 1A, 2A & 3A). For the direct comparison between ELISA and NAA, the bias was 6.52 µg/mL, the precision was 10.42 µg/mL, and the accuracy was such that 97% of the measured values were within 2 standard deviations (SD) of the mean (Fig. 1B). For the comparison between ELISA and HPLC, the bias was -5.123 µg/mL, the precision was 17.5 µg/mL, and the accuracy was such that 94% of the measured values were within 2 SD of the mean (Fig. 2B). Finally, in the comparison between HPLC and NAA, the bias was -1.36 µg/mL, the precision was 15.55 µg/mL, and the accuracy was such that 94% of the measured values were within 2 SD of the mean (Fig. 3B).

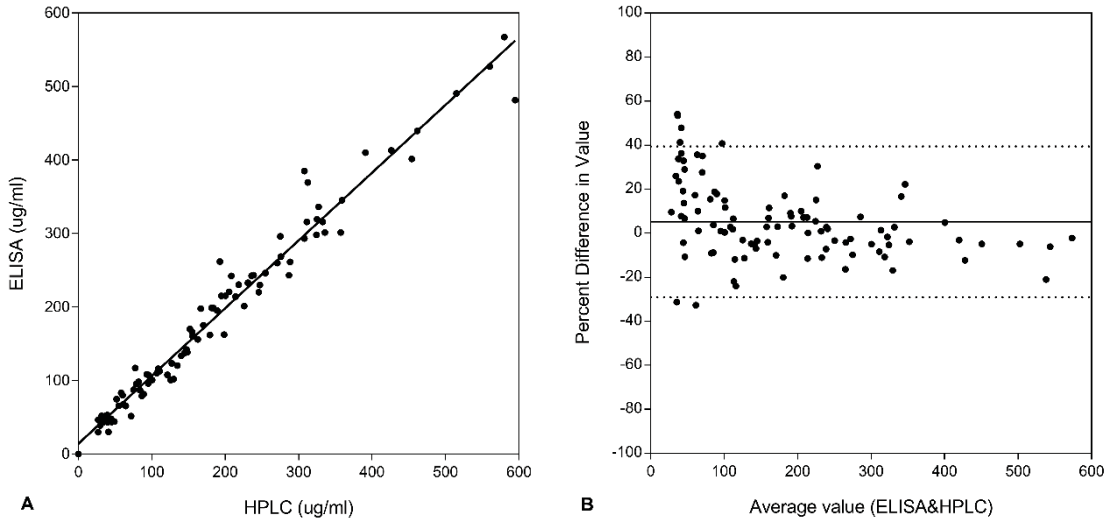

Figure 2. The iohexol concentration (µg/mL) in collected plasma samples (n = 100) measured by ELISA and HPLC. A. ELISA values are compared directly with HPLC values y = 0.9218x + 14.04; r = 0.99 (p <0.0001). B. Difference against the mean iohexol value.

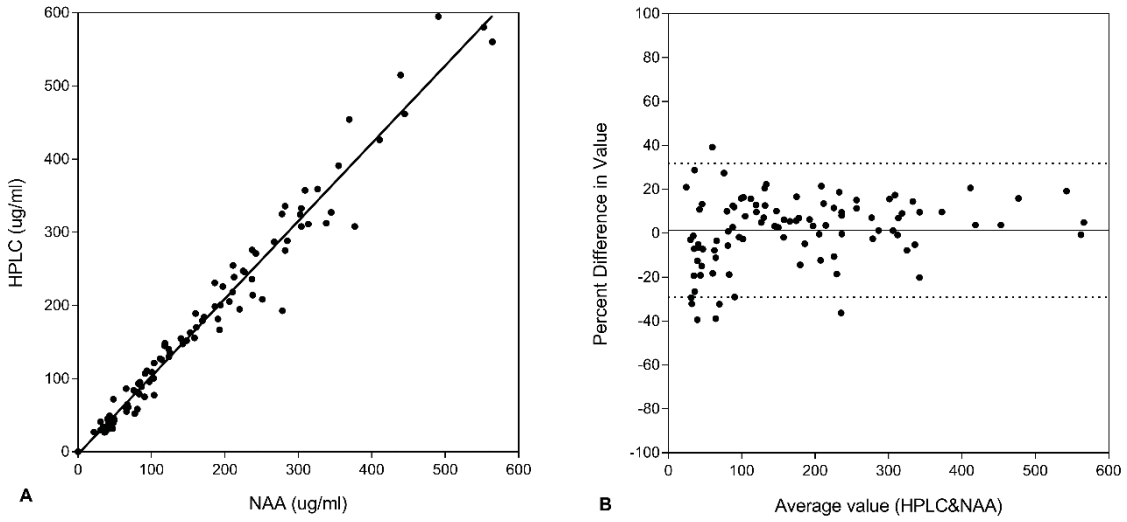

Figure 3. The iohexol concentration (µg/mL) in collected plasma samples (n = 100) measured by HPLC and NAA. A. HPLC values are compared directly with NAA values y = 1.062x – 3.273; r = 0.98 (p <0.0001) B. Difference against the mean iohexol value.

Discussion

In view of the excellent correlation between ELISA, HPLC and NAA, these methods may be considered comparable detection techniques for the determination of iohexol concentration in canine plasma. However, HPLC and NAA have many disadvantages. Although HPLC is a fast, reliable, and the most widely used technique for analysis of iohexol (McMaster 2007; Pöytäkangas et al. 2010; Meucci et al. 2013) its cost and technical complexity may make it impractical to use for clinical research and as a routine detection test for assessing renal function in patients (Rocco et al. 1996; McMaster 2007; Slack et al. 2014). NAA has advantages as a reproducible technique for detecting iohexol (Albert et al. 2003; Mandelbrot et al. 2007). However, the requirement of a nuclear reactor to work with radioactive materials and specific licenses make it an unsuitable method for routine clinical diagnostics or research projects (Albert et al. 2003; Loveland, Morrissey and Seaborg 2005; De Groot 2008). The main benefits of the ELISA method are its high sensitivity, high sample throughput, short time of analysis and relatively low cost when compared to HPLC and NAA (Hendrikje et al. 1997; Khan et al. 1998).

ELISA would have a greater application in clinical and experimental research not only for being a non-radioactive method, but also for being quick and easy to perform even for non-specialists, less technically demanding and allowing the use of a small sample size (Hendrikje et al. 1997; Khan et al. 1998; Alamdari et al. 2005). Moreover, because iohexol is a clinically accessible reagent, and as the ELISA reagents are widely available and provided in kit format, this approach may enable identical results and better standardization to be achieved among core laboratories and independent investigators.

The validation of new diagnostic techniques for analysis of iohexol concentration in canine plasma facilitates the assessment of GFR and IP in dogs, revealing that ELISA is as reproducible and reliable as HPLC or NAA. When comparing the advantages and disadvantages of the different methods, ELISA may be more economical and accessible for clinical practice and research than HPLC or NAA. In addition, a simple and reliable method for the determination of iohexol as a non-radioactive marker of GFR and IP may help not only veterinary clinicians to diagnose renal and intestinal dysfunction but also researchers to better investigate the role of the kidney and intestinal integrity in different canine disease models.

Acknowledgements

We are grateful to Juhana M. Honkavaara and Marja Raekallio for providing the plasma samples.

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