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Bioanalysis

UHPLC–MS/MS method for iohexol determination in human EDTA and lithium-heparin plasma, human urine and in goat- and pig EDTA plasma

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Aim: Iohexol plasma clearance is used as an indicator of kidney function in clinical and preclinical settings. To investigate the pharmacokinetic profile of iohexol, a rapid, simple method for measurement of iohexol in different matrices and species was needed. **Materials & methods:** Iohexol was separated on an Accucore C18 column (Thermo Fisher Scientific, CA, USA). Detection was performed on a Thermo Scientific Quantiva tandem quadrupole mass spectrometer. The method was validated according to the requirements for bioanalytical methods issued by the US FDA and European Medicines Agency. **Conclusion:** We developed and validated a fast and efficient analytical method, suitable for analyzing iohexol in human EDTA plasma, human lithium-heparin plasma, human urine and goat- and pig EDTA plasma, using only one calibration line prepared in human EDTA plasma.

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Keywords: goat plasma • human plasma • human urine • iohexol • LC–MS/MS • pig plasma

Glomerular filtration rate (GFR) is measured to assess kidney function and monitor progression of kidney disease. In clinical practice, GFR is usually estimated based on the endogenous clearance of creatinine using equations such as the CKD-EPI formula [1]. Although practical, this method is imprecise and does not always reflect the true GFR, which precludes its use for scientific research to assess the renal hemodynamic profile of drugs [2,3]. Therefore, regulatory agencies like the European Medicines Agency (EMA) demand that during the drug development process, at least once, an investigation is performed in which actual GFR is measured [4]. A common method to measure GFR is to quantitate inulin clearance after an intravenous dose of inulin and measuring plasma- or urinary inulin clearance [3]. Because kidney function has a circadian rhythm, GFR was historically calculated as the mean of a 24-h inulin clearance. Next to being cumbersome and difficult to perform in the clinic, inulin for parenteral administration is no longer available and is thus being replaced by alternative markers such as radiolabeled compounds or contrast media. Radiolabeled compounds have their obvious drawbacks and the nonionic contrast agent iohexol is currently the most widely used alternative marker for GFR measurement in humans due to its favorable safety profile, high concordance with urinary inulin clearance and ease of use, for example, iohexol in urine and plasma is stable at room temperature, 4, -20 and -80°C [5–7]. In addition to the use of iohexol in clinical

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practice and clinical research, iohexol is also used to measure kidney function in animal models to evaluate the pathogenesis and treatment of kidney disease.

Various analytical methods have been described to measure iohexol in plasma or serum using a wide range of analytical methods, including x-ray fluorescence, HPLC–UV and LC–MS/MS, which have been reviewed elsewhere [8,9]. The published assays use different sample volumes (range: 10–1000 μl), anticoagulants (e.g., lithium-heparin- vs EDTA-coated tubes), sample preparation methods (acetonitrile vs trichloroacetic acid) and flow rates, resulting in a wide range of run times, retention times and lower LOD [7,10,11]. Most importantly, few studies use the stable isotopically labeled iohexol as internal standard (IS), recommended by the US FDA and EMA [12,13], which complicates the universal application of the described analysis across matrices because possible matrix effects may affect the results. In addition, it is unknown whether the iohexol measurement in human plasma can be used for quantification of iohexol in plasma of other species such as goats and pigs, which are commonly used large animal models of chronic kidney disease [14]. To the best of our knowledge, no single method is available for measurement of iohexol in different matrices and species that uses a stable isotope-labeled IS and can be performed with different anticoagulation agents. Therefore, the aim of this study was to develop and validate a rapid LC–MS/MS method for the quantification of iohexol in human EDTA plasma, in goat- and pig EDTA plasma, human lithium-heparin plasma and human urine using a stable isotope-labeled iohexol as IS.

Material & methods

Chemicals & reagents

Iohexol (5-[acetyl(2,3-dihydroxypropyl)amino]-1-N,3-N-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide) and the IS $^{2}H_5$-iohexol were obtained from Toronto Research Chemicals, Inc. (Toronto, Canada). Formic acid (98–100% pure) and trichloroacetic acid (for analysis) were obtained from Merck (Darmstadt, Germany). Methanol absolute was from Biosolve (Valkenswaard, The Netherlands). Water was obtained as ultra-pure water generated by a Milli-Q Advantage A10 system (Millipore B.V., Amsterdam, The Netherlands). Blank human EDTA plasma was obtained from Bio-Connect (Huissen, The Netherlands).

Donor samples for method development & validation

Blank human lithium-heparin plasma and human urine samples were obtained from healthy volunteers according to local hospital standard operating procedures. Blank goat- and pig plasma was obtained from healthy animals that were kept at a certified animal facility (Central Laboratory Animal Research Facility, Utrecht University, The Netherlands). Animal experiments were approved by the Animal Experiments Committee (Utrecht, The Netherlands) and performed in accordance with national guidelines for the care and handling of animals.

LC–MS/MS

The Thermo Scientific Vanquish UHPLC system (Thermo Fisher Scientific) consisted of a binary pump H, split sampler HT (temperature controlled at 10°C) and column compartment. The UHPLC was coupled to a Thermo Scientific Quantiva tandem quadrupole mass spectrometer.

Chromatographic separation was performed on a reversed phase Thermo Scientific Accucore C18 column (2.6 μm, 50 × 2.1 mm), maintained at 40°C. The mobile phase was a gradient which consisted of 1% formic acid in water and 1% formic acid in methanol at a flow of 1 ml/min.

The mass spectrometer operated in positive mode with electrospray as ionization technique.

The spray voltage was 4000 V, the vaporizer temperature was 350°C and the ion transfer tube temperature was set at 140°C. The sheath gas pressure and auxiliary gas pressure were 50 and 20 arbitrary units, respectively. Selected reaction monitoring mode was used for the detection of iohexol and the IS. MS data were collected and processed with Thermo Scientific Xcalibur software version 4.1.

Preparation of standard solutions, calibration standards, quality controls & samples

For the plasma analysis a calibration curve, 0.05, 0.15, 1.00, 10.0, 100, 300, 600 and 750 mg/l iohexol in human EDTA plasma was prepared from an iohexol stock solution of 30 mg/ml in water. For the independent quality controls (QCs), blank EDTA plasma was spiked with a separate iohexol stock solution, resulting in concentrations of 0.05 (LLOQ), 0.150 (low), 300 (medium) and 600 (high) mg/l iohexol. The IS solution was prepared by dissolving 10 mg of $^{2}H_5$-iohexol in 50 ml trichloroacetic acid (10%).

To 100 µl plasma samples, calibration curve samples and QCs, 50 µl of IS solution was added in safe-lock tubes. The samples were vortexed for 1 min prior to centrifuging at 9500 g during 5 min. After precipitation, 10 µl of supernatant was diluted 100-times with water in an autosampler vial. After vortexing for 1 min, 40 µl was injected into the LC–MS/MS.

Method validation

To evaluate whether the assay was reliable and acceptable, the assay was validated according to the FDA and EMA guidelines on bioanalytical method validation [12,13]. The validation protocol included an evaluation of the following parameters: selectivity, linearity, accuracy and precision, dilution integrity, matrix effect, recovery and stability in matrix. In addition, matrix comparisons between human EDTA plasma and goat EDTA plasma, pig EDTA plasma, human lithium-heparin plasma and human urine were evaluated.

Selectivity

Selectivity of the analytical method was investigated by measuring the responses of six blank EDTA plasma samples. The responses of co-eluting components of these samples should be <20% of the signal of the analyte at the LLOQ. Also, the signal at the IS mass transition should not exceed 5% of the peak height of the IS.

Carry over

To investigate if there is any unacceptable carry over, a double blank EDTA plasma sample was injected after the higher LOQ calibrator. According to the EMA guidelines, for carry over to be acceptable, the signal of the analyte needs to be <20% of the signal at LLOQ level and <5% of the signal of the IS.

Linearity

On three different days, a calibration curve of eight calibration samples ranging from 0.05 to 750 mg/l was analyzed. In every validation run, for at least 75% of the calibration concentrations, the back-calculated concentrations of the calibrator may not exceed 15% of the nominal concentrations (20% for the lowest calibration samples [LLOQ]).

Accuracy & precision

To determine accuracy and precision, QC samples with varying iohexol concentrations (LLOQ, low, medium and high) were analyzed in quintuplicate on three different days. Iohexol concentrations were calculated using the calibration curve. Accuracy was calculated as concordance percentage of the determined value to the nominal value and should be between 85 and 115% for the QCs (80–120% at the LLOQ). Precision was determined as the uncorrected CV for both intra and interday variability and was acceptable when the CV was <15% for the QCs (<20% at the LLOQ).

Dilution integrity

Dilution integrity was evaluated by measuring QC samples with a concentration exceeding the ULOQ of the calibration curve and after 1:10 dilution with blank human EDTA plasma. The diluted QC samples were analyzed in quintuplicate and calculated using the calibration curve. The accuracy is allowed to range from 85 to 115% and precision should not exceed 15%.

Matrix effect & recovery

Matrix effect and recovery were investigated in six different batches of blank human EDTA plasma spiked on two concentrations (QC levels low and high). For the evaluation of the matrix effect, the matrix factor (MF) was calculated. According to the EMA guidelines the ratio of the peak heights of iohexol and the IS in the presence and absence of human EDTA plasma were calculated [5]. By dividing the MF of iohexol by the MF of the IS, the IS-normalized MF was determined. The CV of the IS-normalized MF should be <15%.

In all batches, the recovery was measured by comparing the peak heights of extracted human EDTA plasma QC samples with those of spiked extracts of blank human EDTA plasma. The recovery should be reproducible resulting in CVs <15%.

Table 1. Gradient elution program for iohexol.

Time (min)	1% FA in water (%)	1% FA in methanol (%)
0.00	100	0
0.10	100	0
0.40	70	30
0.50	5	95
0.79	5	95
0.80	95	5

FA: Formic acid.

Stability

The stability of iohexol in matrix (human EDTA plasma) has been evaluated under different storage conditions over a 13 day period: at room temperature, in the refrigerator (4°C) and as processed sample in the autosampler. Three freeze–thaw cycles at -20°C were tested. Stability experiments were performed by analyzing QC samples low and high in fivefold. The concentrations of the stability samples were calculated on a freshly prepared calibration curve. Iohexol was classified to be stable if the accuracies were between 85 and 115% compared with the nominal concentrations and the CVs should not exceed 15%.

Matrix comparison: goat plasma, pig plasma, human lithium-heparin plasma & human urine

To evaluate whether the developed method was also applicable to other matrices, matrix comparison were performed. QC samples (LLOQ, low, medium and high) in goat EDTA plasma, pig EDTA plasma, human lithium-heparin plasma and human urine were analyzed in quintuplicate, and iohexol concentrations were calculated using the calibration curve in human EDTA plasma. As concentrations of iohexol in human urine are expected to be at least 25-times higher than in human plasma [15], the iohexol concentrations in QCs in human urine were 30-fold higher than in QCs in plasma. Human urine QC samples were diluted with water (1:30, v:v) before further sample preparation as described in the 'Sample preparation' section. The QCs were considered acceptable if the mean iohexol concentration per matrix did not deviate $>15\%$ of the nominal concentration except for the LLOQ (deviation $\leq 20\%$). Furthermore, CVs need to be $<15\%$ ($<20\%$ for LLOQ).

Results & discussion

UHPLC conditions

At a flow rate of 1.0 ml/min the gradient shown in Table 1 was run in a total runtime of 0.8 min. This gradient resulted in a retention time of 0.5 min for both iohexol and the IS.

Mass spectrometric conditions

Best sensitivity for iohexol and $^{2}H_5$-iohexol was achieved measuring the transition of $[M+H]^+$ precursor ions to their most stable product ion. For iohexol and the IS the transitions 822.0 → 803.9 m/z and 827.0 → 808.9 m/z, using a collision energy of 22 V, were selected.

Method validation

Selectivity

The peak heights of co-eluting peaks in six different single-donor lots of blank human EDTA plasma samples were $<1.7\%$ of the peak height of iohexol at LLOQ level and $<0.01\%$ of the peak height of the IS. In Figure 1, a chromatogram of blank human EDTA plasma is shown. As there were no interfering peaks at the retention times of iohexol and $^{2}H_5$-iohexol, the method was accepted to be selective.

Carry over

Injection of a double blank EDTA plasma sample after the injection of the higher LOQ calibrator resulted in a signal of 1.8% of the signal of iohexol at LLOQ level and 0.002% of the signal of the IS $^{2}H_5$-iohexol. These results were within the acceptance criteria of the EMA guidelines and therefore carry over was acceptable.

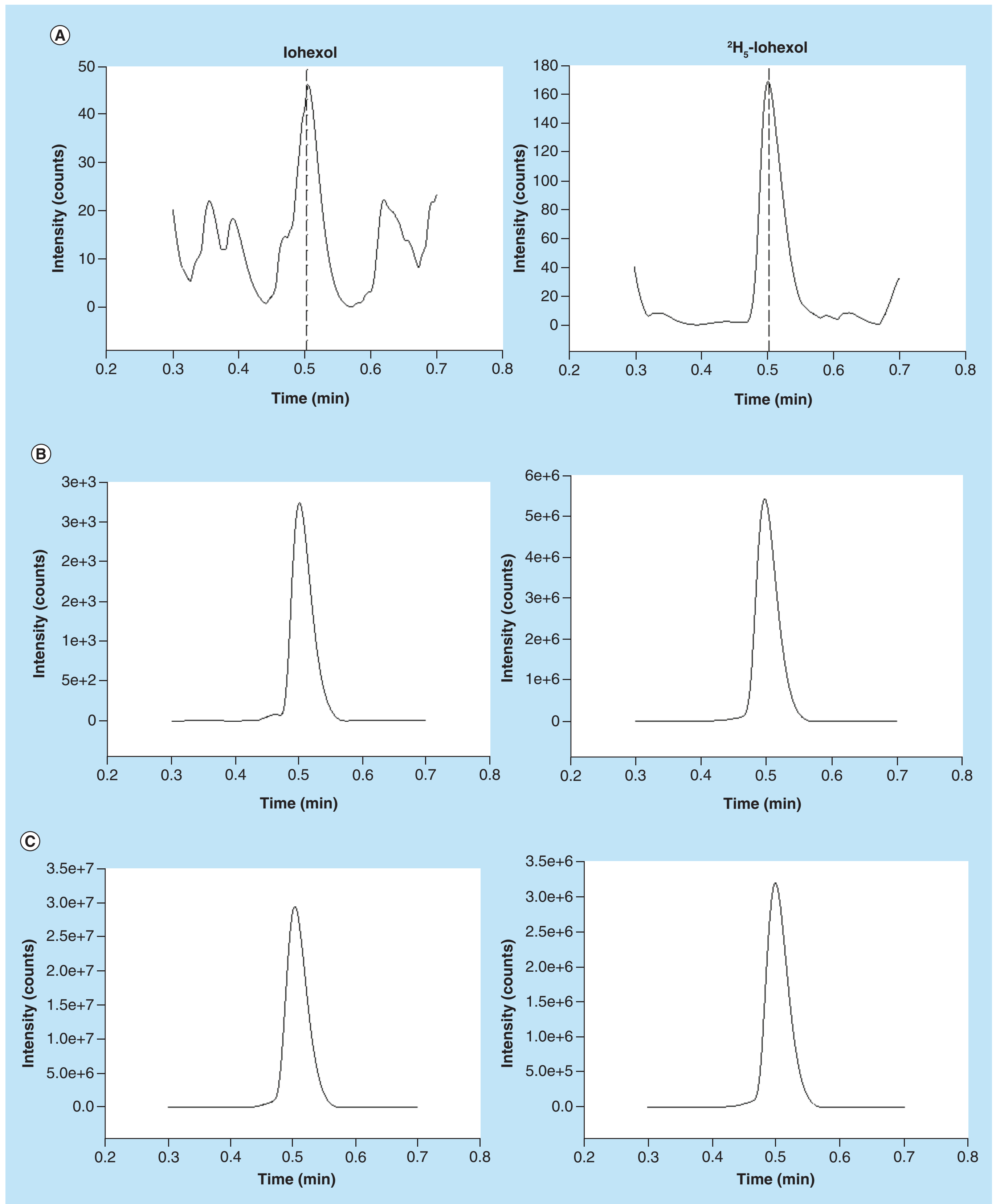

Figure 1. Selected reaction monitoring chromatograms of iohexol and the internal standard.

Table 2. Results of accuracy and precision measurements for iohexol quality control samples.

QC level	Nominal concentration (mg/l)	Accuracy (%)	Precision (%CV)	
			Within run (n = 5)	Between run (n = 15)
LLOQ	0.05	95.2	3.5	1.7
Low	0.15	94.4	1.7	1.2
Medium	300	95.5	6.9	0.0
High	600	96.4	0.9	3.0
Dilution	100†	106.1	3.0	2.8

†Postdilution (1:10).
Matrix used: EDTA plasma.
QC: Quality control.

Linearity

The calibration curve consisted of eight calibrators with iohexol concentrations ranging from 0.05 to 750 mg/l in human EDTA plasma and was analyzed on three different days. The peak height ratios of iohexol and the IS were fitted versus the concentration, using linear regression with 1/x as the weighing factor. The calculated biases were between -4.4 and 4.2% and the correlation coefficient (R^2) was 0.9996, proving the linearity of the calibration curve.

Accuracy & precision

Based on the measurements of the QC levels LLOQ, low, medium and high, accuracies and precisions were calculated. The results are presented in Table 2. The mean accuracies of quintuplicate QCs were between 95 and 97% and thus fulfill the EMA/FDA requirements of 85–115%. Precisions were <7% and meet the acceptance criterion of 15% (at LLOQ 20%).

Dilution integrity

After a tenfold dilution with blank human EDTA plasma accuracy and precision were 106.1 and <4%, respectively, as shown in Table 2. Therefore, dilution of samples with concentrations up to tenfold of the ULOQ was accepted.

Matrix effect & recovery

The matrix effect, expressed as the MFs of the six different lots of blank human EDTA plasma spiked at low and high concentration, was 1.055 and 0.970, respectively, for iohexol. For the IS $^2H_5$-iohexol, the matrix effect for low and high concentration was 1.146 and 1.048, respectively. These factors resulted in IS normalized MFs (CV) of 0.921 (1.3%) for QC low and 0.925 (1.9%) for QC high. The CVs were <2.0% indicating that the differences between the six lots of blank human plasma were not significant.

The recoveries for iohexol were 109.9% (CV 3.2%) at QC low and 104.7% (CV 0.9%) at QC high, whereas the recoveries for $^2H_5$-iohexol were 101.0% (CV 0.8%) and 101.5% (CV 2.2%), respectively. The results show that the developed and validated sample preparation yielded good and reproducible recoveries.

Stability

The results of all stability tests of iohexol in matrix under different conditions are summarized in Table 3.

As the accuracies were between 91 and 99%, iohexol was stable in human EDTA plasma for thirteen days at room temperature, in the refrigerator, after sample preparation in the autosampler and for three freeze–thaw cycles. This is in line with previous findings on the stability of iohexol [5–7].

Matrix comparison

For matrix comparison, the QCs in goat- and pig EDTA plasma and in human lithium-heparin plasma and human urine were calculated using the calibration curve prepared in human EDTA plasma. The calculated values for accuracy and precision are reported in Table 4. As the accuracies of all matrices were between 89 and 108% for QC levels low, medium and high and between 95 and 119% for the LLOQ and precisions were <3.3%, matrix comparison was accepted for all tested matrices. Therefore, we showed that iohexol concentrations in goat EDTA plasma, pig EDTA plasma and human lithium-heparin plasma can be calculated using a calibration curve prepared

Table 3. Stability of iohexol in human EDTA plasma.

Condition	Period	QC level	Nominal concentration (mg/l)	Accuracy (%)	Precision (%CV; n = 5)
Room temperature	13 days	Low	0.15	94.5	5.4
		High	600	92.8	0.7
Refrigerator (5°C)	13 days	Low	0.15	91.4	1.9
		High	600	92.5	0.4
Autosampler (10°C)	13 days	Low	0.15	98.5	1.6
		High	600	98.7	1.1
Freeze–thaw (at -20°C)	3 cycles	Low	0.15	96.6	2.9
		High	600	96.2	6.5

QC: Quality control.

Table 4. Results of matrix comparison measurements for iohexol quality control samples in goat plasma, pig plasma, human lithium-heparin plasma and human urine.

Matrix	QC level	Nominal concentration (mg/l)	Accuracy (%)	Precision (%CV; n = 5)
Goat EDTA plasma	LLOQ	0.05	119.0	1.8
	Low	0.15	107.5	1.0
	Medium	300	98.6	1.8
	High	600	99.5	0.6
Pig EDTA plasma	LLOQ	0.05	111.2	1.7
	Low	0.15	103.2	1.2
	Medium	300	101.4	1.5
	High	600	101.6	1.1
Human lithium-heparin plasma	LLOQ	0.05	114.5	2.6
	Low	0.15	112.0	2.3
	Medium	300	100.2	1.0
	High	600	103.4	0.5
Human urine[†]	LLOQ	0.05	95.1	3.3
	Low	0.15	89.0	1.4
	Medium	300	95.2	1.1
	High	600	94.6	0.4

[†]Postdilution (1:30).
QC: Quality control.

in human EDTA plasma. Furthermore, after a 30-fold dilution with water, human urine samples could be calculated on the calibration curve in undiluted human EDTA plasma.

Pharmacokinetic study samples in man, pig & goat

The concentration of iohexol was successfully measured in EDTA plasma after intravenous bolus dose of 3.225 g iohexol in a healthy human subject and 1.5 g iohexol in a healthy goat and pig (Figure 2). All concentrations were above the LLOQ of 0.05 mg/l. As expected, the maximum concentrations are observed at the first time point of sampling. 1 h post dosing, the slopes of the concentration time curves of the goat and pig are in the same order of magnitude, indicating similar half-lives. In contrast, in the human subject, clearance seems to be much slower as the slope is more shallow.

Conclusion

We have successfully developed and validated a LC–MS/MS method for rapid and efficient analysis of iohexol in human EDTA and lithium-heparin plasma, human urine and in goat- and pig EDTA plasma.

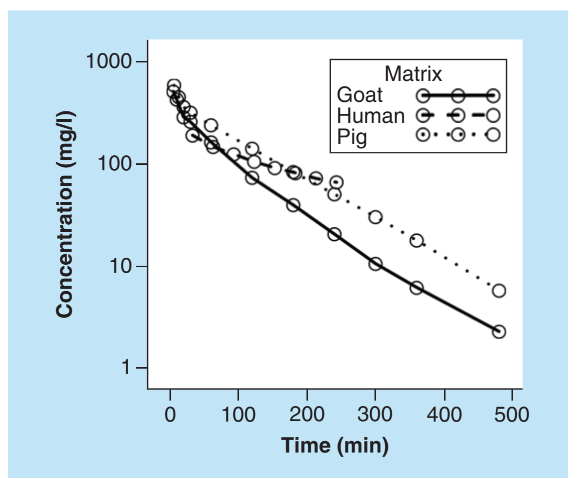

Figure 2. Individual iohexol concentration time profiles in human, goat and pig EDTA plasma.

Future perspective

Quantification of iohexol plasma- or urine clearance after intravenous administration is rapidly becoming the gold standard for measurement of GFR in man and in large animal models of chronic kidney disease. The higher sensitivity of the presented method allows for measurement of lower concentrations and can be readily implemented in preclinical and in clinical practice. In research settings, comparison of iohexol clearance between species and matrices can be affected by the current use of different detection methods. Use of the simple method presented in this paper overcomes the potential problem of matrix effects between sample types and species and allows for quantitative comparison of the iohexol clearance between species and matrices. This is of particular importance in development of drugs that affect kidney function and in safety studies that require precise measurement of kidney function, which can now be tested in a preclinical setting, prior to first-in-man studies.

Author contributions

J Stevens and MA Wessels were responsible for acquisition of data, data analysis, drafting and revision of the manuscript. DJ Touw was responsible for study conception and design. J Roggeveld, RA Koster and DJ Touw were responsible for the method development. MA Wessels, J Roggeveld and RA Koster were responsible for the formal analysis and validation of the method. CCJ Dekkers and MK van Gelder were responsible for the project administration and clinical- and preclinical investigations. RT Gansevoort, HJL Heerspink and DJ Touw were responsible for supervision. All authors were involved in revision of the manuscript.

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Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Glomerular filtration rate is used as an indicator of kidney function and can be determined by quantification of iohexol plasma- or urine clearance after intravenous administration in clinical and preclinical settings.
- Thus far, no single method is available for measurement of iohexol in different matrices and species that uses a stable isotope-labeled internal standard and can be performed with different anticoagulation agents.
- To investigate the pharmacokinetic profile of iohexol, a rapid, single method for measurement of iohexol in different matrices and species was needed.

Materials & methods

- Chromatographic separation was performed on a reversed phase Thermo Fisher Scientific Accucore C18 column (2.6 μm, 50 × 2.1 mm; Thermo Fisher Scientific, CA, USA) at 40°C.
- The Thermo Fisher Scientific Quantiva tandem quadrupole mass spectrometer operated in positive mode with electrospray ionization at 4000 V, 350°C and the ion transfer tube temperature was set at 140°C.
- The assay was validated according to the US FDA and European Medicines Agency (EMA) guidelines on bioanalytical method validation.

Results & discussion

- For the first time, a rapid (1.0 min) and reliable analytical method to measure iohexol in different matrices and species was established and fully validated according to EMA/FDA requirements.
- The assay in human EDTA plasma was selective and linear in the range of 0.05–750 mg/l.
- The LLOQ was 0.05 mg/l and a tenfold dilution of a quality control sample with an over-the-curve concentration was acceptable.
- The assay recovery, intra and interday precision, accuracy and stability fulfilled the requirements for bioanalytical methods issued by the FDA and EMA.
- For matrix comparison, the overall range of mean accuracies of quintuplicate low-, medium- and high quality controls for goat- and pig EDTA plasma, human lithium-heparin plasma and human urine was between 89 and 108% versus 95 and 119% for the LLOQs.
- The overall precision was <3.3%, resulting in an accepted matrix comparison for all tested matrices.

Conclusion

- A robust and fast UHPLC–MS/MS method for analyzing iohexol in different matrices and species was established.
- Validated in pig- and goat EDTA plasma, human lithium-heparin plasma and urine.
- Higher sensitivity allows for quantification of lower concentrations.
- Appropriate approach for pharmacokinetic studies in man, pig and goat.

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