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Short communication

Development and validation of an HPLC-UV method for iodixanol quantification in human plasma

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ABSTRACT

Iodixanol is a widely used iso-osmolar contrast medium agent. Similar to iohexol, it can also be a good exogenous marker for the measurement of glomerular filtration rate (GFR). This article describes the development and validation of an HPLC-UV method for quantification of iodixanol in human plasma. Internal standard, iohexol (20 µl, 1 mg/ml), and perchloric acid (30 µl, 20%, v/v) were added to plasma samples (300 µl), followed by neutralization with 10 µl potassium carbonate (5 M). Samples were centrifuged and 10 µl of the supernatant was injected onto a C_{18} EPS analytical column (3 µm particle size, 150 mm × 4.6 mm). The extraction method yielded >95% recovery for both iodixanol and iohexol. The mobile phase consisted of 0.1% (w/v) sodium formate buffer and acetonitrile. Iohexol and iodixanol peaks were eluted at ~5 and 9 min, respectively using a fast gradient method. The assay lower limit of detection was 2.0 µg/ml and lower limit of quantification was 10 µg/ml. The calibration curves, assessed in six replicates, were linear over an iodixanol concentration range of 10–750 µg/ml. Intra- and inter-day accuracy was >95% and precision expressed as % coefficient of variation was <10%. This method is simple, accurate, precise and robust and can potentially be used for iodixanol quantification in large-scale clinical studies.

1. Introduction

Chronic kidney disease is a worldwide epidemic and the number of patients suffering from this, and eventually reaching end stage renal disease (ESRD), is on the rise. An accurate method to measure kidney function, by the means of estimation of glomerular filtration rate (GFR), is therefore, required to diagnose chronic kidney diseases. GFR correlates well with the functional mass of the kidney [1]. It is measured as the renal clearance of a particular substance such as creatinine or inulin from plasma in unit time and typically expressed as ml/min [2].

In the clinical settings, calculated creatinine clearance (Clcr) values are widely used to estimate GFR [3]. Creatinine is produced endogenously during biosynthesis of aminoacids, is freely filtered through glomerulus and is excreted by the kidneys. Creatinine clearance can be calculated by collecting urine over a 24-h period or by measuring a single creatinine concentration in serum followed by calculation of Clcr using Cockcroft–Gault or comparable equations [3]. However, creatinine clearance often overestimates the GFR in different patient groups.

An ideal GFR marker is often considered as an agent that does not bind to plasma proteins, has no significant systemic metabolism and is freely excreted through glomerular filtration [4,5]. Traditionally, clearance of inulin, a simple sugar administered intravenously, is considered the gold standard for GFR measurement. The inulin clearance method has several disadvantages such as the unavailability of clinical grade dosage form for injection and the need for 24 h urine collection. Alternatively, the usefulness of contrast media agents for GFR estimation has been demonstrated by several investigators [6]. These agents include water-soluble iodinated pyridones [7], triiodinated benzoates (ionic monomers, e.g., iothalamate) [1,8] and nonionic monomers (e.g., iohexol) [6].

In the past decade, iodixanol (Visipaque®, Nycomed, Oslo, Norway) with a molecular weight of 1550.2 (iodine content 49.1%), has been introduced into the clinical practice (Fig. 1) [9]. Iodixanol is an iso-osmolar (low osmolality compared to other nonionic monomers and ionic compounds), nonionic dimeric and hydrophilic contrast medium agent; which produces less hemodynamic changes and in turn reduces the renal toxicity associated with the use of contrast media agents [9,10]. It is commonly used as a contrast agent during coronary angiography and contrast enhanced computer tomography (CECT) imaging of the head and body [11]. Iodixanol does not change GFR and is well tolerated in

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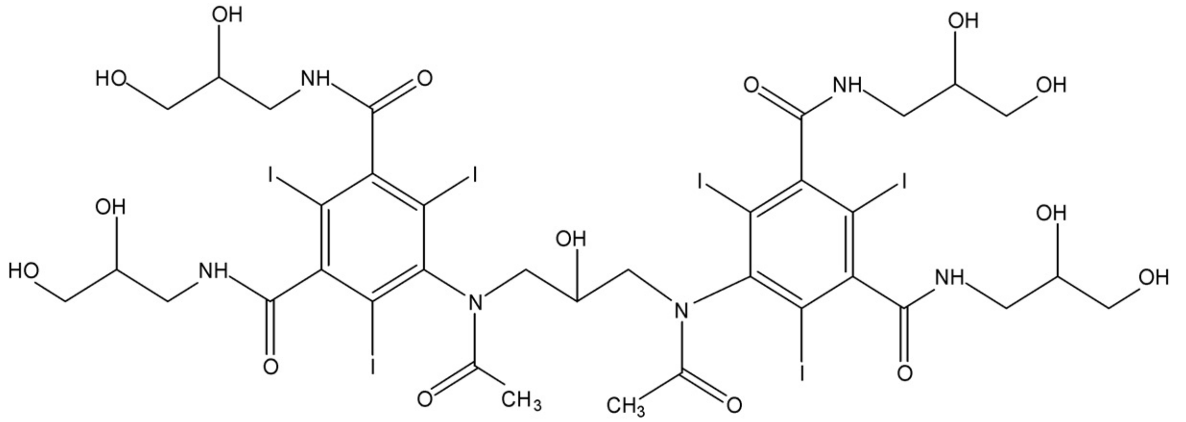

Fig. 1. Chemical structure of iodixanol. Iodixanol: 5,5′-[(2-hydroxy-1,3-propanediyl)bis(acetylimino)] bis [*N*,*N*′-bis(2,3-dihyd roxypropyl)-2,4,6-triiodo-1,3-benzenedicarboxamide].

patients with normal renal function or in patients with severe renal failure [12,13].

Upon intravenous administration of iodixanol, peak plasma concentration is achieved rapidly and it distributes exclusively in extracellular fluids with a distribution half-life of ~0.5 h. Iodixanol does not undergo systemic metabolism and >97% of the dose is excreted in urine by glomerular filtration [9,14]. Furthermore, the renal clearance of iodixanol shows a good agreement with the renal clearance of ^{125}I-iothalamate, which itself is a well-established GFR marker [15]. In conclusion, iodixanol may prove to be an ideal candidate for the measurement of GFR when compared to other iodinated contrast media agents.

Several methods have been published for quantification of iodixanol in plasma, urine and feces using HPLC-UV, X-ray fluorescence spectroscopy and neutron activation analysis [14,16–18]. The method described by Jacobsen [16] requires an automated online dialysis system that is not widely available. In addition, the other published methods lack detailed description of the chromatography or extraction procedures and present limited assay validation. In the present study, we have developed a simple, robust and rapid HPLC-UV assay, which utilizes a simple protein precipitation method and commercially available USP grade internal standard to quantify iodixanol in human plasma. The present assay, is a modified version of a method we have previously published to analyze the concentration of iohexol in human plasma [5]. Furthermore, the assay was validated according to guidelines provided by the Food and Drug Administration of the United States (USFDA) [19].

2. Experimental

2.1. Chemicals and reagents

Iodixanol (99% pure) and iohexol (99% pure, used as internal standard) were purchased from United States Pharmacopeia (USP, Rockville, MD). Perchloric acid (70%, v/v) was obtained from Acros Organics (Fair Lawn, NJ), HPLC grade acetonitrile and methanol from Pharmco Products (Brookfield, CT) and potassium carbonate from Fisher Scientific (Fair Lawn, NJ). Purified deionized water was prepared using Milli Q50 (Millipore, Bedford, MA) water purification system. For the preparation of in-house quality control and calibration samples, human plasma from normal donors was purchased from Rhode Island Blood Center (Providence, RI).

2.2. Assay conditions

Quantitative analysis of iodixanol in human plasma was performed using an HPLC-UV analytical system. The instrument consisted of a Hitachi D-7000 series (San Jose, CA) interfaced with an auto-sampler fitted with a 200 μl sample loop, a quaternary pump, a column oven and a variable wavelength UV detector set at 254 nm. The Hitachi System Manager (HSM) software was used to integrate the chromatograms. All quantifications were based on the peak area ratio of iodixanol to internal standard and all calculations were performed using Microsoft Excel (MS office 2000). The analytical column used was a C_{18} EPS analytical column (3 μm particle size, 150 mm × 4.6 mm) manufactured by Alltech Associates (Deerfield, IL). A 2 μm pore size pre-column filter (Supelco, Bellefonte, PA) was attached to the column and both the pre-column and analytical column were maintained at 30 °C.

Iodixanol exists in three isomeric forms however, the mobile phase composition and gradient were adjusted such that all three isomers were eluted as a single peak at ~9.3 min. The optimum chromatographic conditions were achieved using a fast gradient system of sodium formate buffer and acetonitrile. Mobile phase was filtered and degassed by passing through a 0.45 μm nylon filter (Millipore, Bedford, MA) under vacuum followed by sonication for 15 min.

The mobile phase consisted of different percentage of Solution A (sodium formate buffer, 0.1% (w/v) pH 7.4) and acetonitrile. At the beginning of each run, Solution A was pumped for 1 min at a flow rate of 1 ml/min. At 1.1 min, the mobile phase composition was changed (over a period of 30 s) to 4% acetonitrile and 96% Solution A and the flow rate was increased to 1.2 ml/min. Between 6.6 and 11.0 min, the mobile phase composition was maintained at 30% acetonitrile and 70% Solution A at 1.2 ml/min flow rate. The column was then re-equilibrated from 11.1 to 15.0 min with 100% Solution A. To minimize carryover, the sample loop was washed between injections with 10% acetonitrile in deionized water. Before each batch of samples, the analytical column was equilibrated with Solution A for at least 1.5 h at a flow rate of 1 ml/min.

2.3. Protein precipitation method

Frozen calibrators, QC standards and working stock solution of internal standard were thawed at 37 °C using a thermostatic shaking water bath (Precision Scientific, Chicago, IL). Aliquots of calibrators and QC standards (300 μl) were added to 1.5 ml microcentrifuge tubes. To all samples, 20 μl internal standard (1 mg/ml in acetonitrile:water 10:90%, v/v) and 30 μl perchloric acid (20%, v/v) were added. Samples were vortex mixed for 3 min, sonicated for 5 min and centrifuged (10,000 × *g*) for 12 min at room temperature. After centrifugation, 250 μl of clear supernatant was pipetted into a fresh vial and 10 μl potassium carbonate (5 M) was added to neutralize excess acidity. The samples were then vortex mixed for 2 min and centrifuged for 12 min (10,000 × *g*) at room temperature followed by pipetting the clear supernatant (200 μl) into autosampler vial. The extracted iodixanol sample (10 μl) was then injected onto the analytical column. To check for interfering plasma peaks at the retention times of iohexol and iodixanol, a blank plasma sample was extracted and processed in the same way as the plasma samples containing these analytes.

2.4. Calibration curve

Stock (10 mg/ml) and substock (1 mg/ml) solutions of iodixanol and iohexol were prepared in 10% acetonitrile in water. A total of seven iodixanol concentrations (10, 50, 120, 300, 400, 500, 750 μg/ml) in drug-free plasma were used as calibrators and three in-house quality control standards (QCs), containing 20 μg/ml (low QC), 180 μg/ml (medium QC) and 600 μg/ml (high QC) iodixanol were used to estimate the accuracy and precision of the assay. Iodixanol stock solution (10 mg/ml) was used to prepare medium and high QC standards and all the calibrators except 10 μg/ml, and iodixanol diluted stock (1 mg/ml) was used for the lowest calibrator (10 μg/ml) and the low QC standard (20 μg/ml). A diluted stock solution of iohexol (1 mg/ml in acetonitrile:water 10:90%, v/v) was used as an internal standard. All the stock and diluted stock solutions, calibrators, QC standards and working stock solution of the internal standard were stored at −80 °C until use.

2.5. Assay validation

All validation steps were conducted according to the FDA guidelines [19]. A total of six calibration curves were prepared on six different days to evaluate inter-day accuracy. In addition, in-house QC standards were run in replicates of six to evaluate intra-day precision. To verify the assay specificity and to check for the absence of interfering peaks at the elution time of iodixanol and IS, blank samples of drug-free plasma were analyzed. Sensitivity of the analytical technique was expressed as the lower limit of quantification (LLOQ) which is the minimum plasma concentration of iodixanol that can be quantified by measuring peak height to baseline ratio of at least 10:1 and the limit of detection (LOD) as peak height to baseline ratio of 3:1. The analytical recovery of iodixanol was assessed by comparing the peak area ratio of QCs extracted from plasma with the peak area ratio of reference standards prepared in the same way as the stock solution. Stability of stock solutions of iodixanol and internal standard was checked by keeping both the stock solutions at room temperature (∼25 °C) for 6 h and comparing the accuracy against freshly prepared stock solution. The freeze thaw stability was characterized at two different temperatures −20 °C and −80 °C, by subjecting low and high QCs to three consecutive freeze–thaw cycles (freezing temperature −20 °C or −80 °C for 24 h and thawing unassisted at room temperature, $n = 3$). The short-term stability of samples was checked by keeping the QC standards at room temperature for 4 h and analyzing fresh QC standards in parallel. The low, medium and high QC standards were checked for autosampler stability by storing extracted samples for 12 h at room temperature and were compared with freshly extracted QC standards. The sample dilution step involved in patient sample analysis was validated using plasma sample with high concentration (2400 μg/ml, $n = 3$).

2.6. Patient samples

To evaluate the iodixanol clearance, in an on-going clinical study, patients were recruited at Rhode Island Hospital, Providence, USA. The study protocol was reviewed and approved by the Institutional Review Board of the hospital and written informed consent was obtained from all the patients. On the first day of study, the patients were administered a clinically relevant dose of iodixanol (Visipaque^{TM} 320 mg/ml, GE Healthcare Inc., Princeton, NJ). The average dose of iodixanol was 1200 mg/kg administered by intravenous infusion method. On the second study day, typically 2 weeks later, a small dose (5 ml of Visipaque^{TM} 320 mg/ml) of iodixanol was administered. On both days, blood samples were collected using BD Vacutainer (Becton Dickinson, Franklin Lakes, NJ) containing EDTA from contralateral arm at 2, 3 and 4 h after the completion of IV dose and the exact time was recorded. The blood was centrifuged at 1500 × *g* and the plasma was stored at −80 °C until analysis. On the day of concentration determination, all patient plasma samples from the first day of the study (clinical dose) were diluted four times.

The iodixanol clearance was estimated using one compartmental intravenous infusion model implemented in WinNonlin software (Version 5.0.1, Pharsight Corporation, Mountain View, CA) and the GFR values were calculated using Brochner–Mortensen equation: $\mathrm{CL}_p = (0.990778 \times \mathrm{CL}_1) - (0.001218 \times \mathrm{CL}_1^2)$ [20]. Individual subjects' body surface area (BSA) were calculated by the use of DuBois and DuBois formula that is $\mathrm{BSA}\,(\mathrm{m}^2) = 0.20247 \times \mathrm{height}\,(\mathrm{m})^{0.725} \times \mathrm{weight}\,(\mathrm{kg})^{0.425}$ and all the GFR values were normalized for a BSA of 1.73 m^2 [15].

3. Results

Iohexol and iodixanol were eluted at ∼5 and 9 min, respectively (Fig. 2). A protein precipitation method was utilized to extract iodixanol from plasma samples. No interfering peak was observed around 5 or 9 min at which iohexol or iodixanol peaks elute. The LOD of the method was 2.0 μg/ml and its LLOQ was 10 μg/ml. To evaluate the reproducibility of the extraction method, three QC standards were extracted and analyzed in six replicates. The average recovery was 95.6 ± 3.5% to 98.8 ± 2.0% for low to high iodixanol QC standards (Table 1) and for the internal standard it was >95%. All calibration curves were linear over the iodixanol concentration range of 10–750 μg/ml with an average coefficient of determination (r^2) of 0.999.

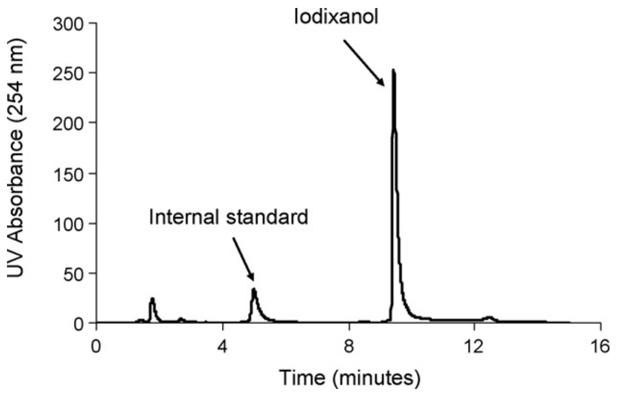

Fig. 2. Chromatogram depicts the separation of iodixanol (500 μg/ml) and internal standard, iohexol, extracted from human plasma. Iodixanol and the iohexol were eluted approximately at 5 and 9 min using C_{18} EPS analytical column (3 μm particle size, 150 mm × 4.6 mm) and acetonitrile and buffer (0.1% sodium formate) as mobile phase.

Table 1
Recovery, and intra- and inter-day accuracy and precision results for the assay

Sample	Nominal iodixanol concentration (μg/ml)	Recovery[a]	Intra-day accuracy (%)	Inter-day accuracy (%)	Intra-day precision (%CV)[b]	Inter-day precision (%CV)[b]
Low QC	20	98.8 ± 2.0	96.7 ± 6.5	98.0 ± 8.9	6.7	9.1
Medium QC	180	95.6 ± 3.5	101.5 ± 6.3	99.0 ± 4.0	6.2	4.1
High QC	600	98.2 ± 1.5	102.1 ± 8.2	102.4 ± 1.6	8.1	1.6

Plus-minus values are mean ± S.D.

[a] Recovery is expressed as accuracy (%).

[b] Intra- and inter-day precision is expressed as coefficient of variation (%CV).

Table 2
Stability results for iodixanol expressed as percentage accuracy

Sample	Nominal iodixanol concentration (μg/ml)	Freeze–thaw stability ($n = 6$)		Short-term stability ($n = 3$)	Autosampler stability ($n = 3$)
		Temperature −20 °C	Temperature −80 °C		
Low QC	20	99.4 ± 3.3	96.9 ± 3.1	99.4 ± 2.3	99.6 ± 0.8
High QC	600	100.5 ± 1.4	103.2 ± 3.3	98.4 ± 0.5	99.8 ± 0.5

Plus-minus values are mean ± S.D.

Stock solutions of iohexol or iodixanol were stable at working conditions and the accuracy was comparable to the freshly prepared stock solution. In addition, the short-term stability of plasma samples was >98%. The stability of iodixanol in plasma samples was examined after three freeze–thaw cycles at two different temperatures. The plasma samples were significantly stable at both −20 °C and −80 °C with average accuracy >95% for low or high QC standards (Table 2). Samples were checked for short-term stability of iodixanol over a period of 4 h. No appreciable loss of iodixanol was detected using the experimental conditions described in Section 2 (Table 2). Furthermore, autosampler stability experiment showed that iodixanol was fairly stable for 12 h in the autosampler (Table 2). The effect of sample dilution was studied by spiking blank plasma with high concentration of iodixanol (2400 μg/ml) followed by diluting four times to simulate the effect of dilution of clinical samples. The average ± S.D. accuracy for high concentration samples diluted to 600 μg/ml was 102.5 ± 5.35% ($n = 6$).

Fig. 3 depicts the iodixanol concentration vs. time profile from three representative patients who were administered clinically relevant large (Fig. 3A) and small dose (Fig. 3B) of iodixanol on two separate occasions. The iodixanol clearance values estimated from the data and expressed as ml/min/1.73 m^2 were 72.5 and 72.3 for the patient 1, 83.4 and 72.7 for the patient 2 and 92.5 and 86.9 for the patient three, for large and small doses of iodixanol, respectively.

4. Discussion

Nephrotoxicity is a well-documented side effect of contrast media agents. Several studies have shown that nonionic contrast media agents, including iodixanol, can be relatively safe and well tolerated in patients at high risk of developing nephropathy [21]. Iodixanol is a safe and effective nonionic contrast medium which offers several advantages in clinical practice and could be used as a reliable GFR marker in patients with severe renal failure [7,15].

In the present study, we report a simple, rapid and economical HPLC-UV analytical method that potentially can be used for GFR determination in large-scale clinical trials. The method utilizes a commercially available C_{18} EPS column that is robust and reproducible.

Commercially available iodixanol exists in three isomeric forms (*endo–endo*, *exo–endo* and *exo–exo*). However, this method does not separate these isomers as the peak separation may compromise the LLOQ. All the samples were analyzed at 254 nm, as λ_{max} of iodixanol and its internal standard is close to 254 nm. The current method has an LLOQ of 10 μg/ml and LOD of 2.0 μg/ml, which is lower than the method reported by Svaland et al. [14], however, it is higher than the method reported by Jacobsen [16]. In clinical practice, iodixanol is given as a large dose (320 mg I/ml) and our method covers the dynamic range of 10–750 μg/ml for iodixanol.

Jacobsen [16] has reported an HPLC method coupled with online dialysis system. Current method, utilizes a very simple protein pre-

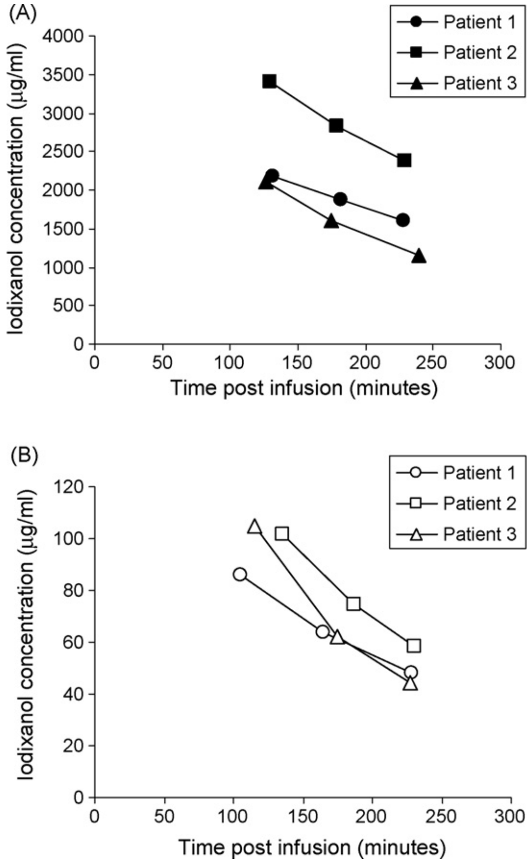

Fig. 3. (A) Iodixanol concentration–time profiles obtained in three representative patients (patients 1–3) after a large iodixanol dose (filled marks) and (B) iodixanol concentration–time profiles obtained in patients 1–3 after a small iodixanol dose (unfilled marks).

cipitation method using 20% (v/v) perchloric acid. All the extracted samples were neutralized using potassium carbonate (5 M) followed by centrifugation, which increased the stability of extracted samples. The neutralization of extracted samples with potassium carbonate increased the stability of the sample.

5. Conclusion

The present study reports a simple, rapid, and robust, validated [14] assay technique that can be used for quantitative determination of iodixanol in human plasma matrix.

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