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Short communication

Development and validation of an HPLC-UV method for determination of iohexol in human plasma

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Abstract

An HPLC-UV analytical method for estimation of iohexol in human plasma was developed and validated. Protein precipitation and iohexol extraction from plasma (100 μl) was carried out by adding 800 μl perchloric acid (5%, v/v in water) containing iohexol related compound B as the internal standard followed by vortex mixing and centrifugation. The supernatant (90 μl) was then injected onto a μBondapak C_{18} column (150 mm × 3.9 mm, 10 μm) maintained at 30 °C. The mobile phase comprised of various proportions of acetonitrile and water with a total run time of 12 min and the wavelength of the UV detector was set at 254 nm. The extraction recovery of iohexol from plasma was >95% and the calibration curve was linear ($r^2 = 0.99$) over iohexol concentrations ranging from 10 to 750 μg/ml ($n = 8$). The method had an accuracy of >92% and intra- and inter-day CV of <3.7% and <3.6%, respectively. The method reported is simple, reliable, precise, accurate and has the capability of being used for determination of iohexol in clinical settings.

Keywords: Iohexol; GFR

1. Introduction

Glomerular filtration rate (GFR) is defined as the volume of plasma that can be completely cleared of a particular substance by the kidneys in a unit of time. The "Gold Standard" for determining GFR is to measure the clearance of an exogenous substance such as inulin, a polyfructose molecule with an average molecular weight of 5200 Da. Because of the lack of commercially available dosage forms, need for an infusion pump and multiple sample collection, GFR measurement using inulin becomes expensive and its reliability dependent on the method used for the analysis of the polyfructose molecule [1]. As an alternative to inulin, two major classes of agents have been introduced—the radio labeled chelating agents including ethylene diamine tetra-acetic acid (^{51}Cr-EDTA) or diethylene triamine penta-acetic acid (^{99m}Tc-DTPA) and radiological contrast media agents (including ^{125}I-iothalamate) [2]. Although simple and rapid, these methods require use of radiolabeled tracers, which complicates the procedure (special licensing, complicated handling, storage and disposal of waste) and excludes certain patients including pregnant women from investigations [3]. Use of non-radiolabeled contrast media agents including iohexol is therefore preferred for GFR estimation in human [4].

Iohexol (Fig. 1), with the trade name of Omnipaque™ (Nycomed) has a molecular weight of 821 Da. It is commonly used as a non-ionic X-ray contrast media agent [5] and for the measurement of GFR [6]. Iohexol does not bind to serum proteins and is 100% filtered through glomerulus, with no indications of tubular secretion or reabsorption [5,7]. Therefore, iohexol clearance should represent the GFR value and this makes iohexol an ideal marker for GFR determination. In fact, a good agreement was reported between the urinary clearance of inulin and the total body clearance of

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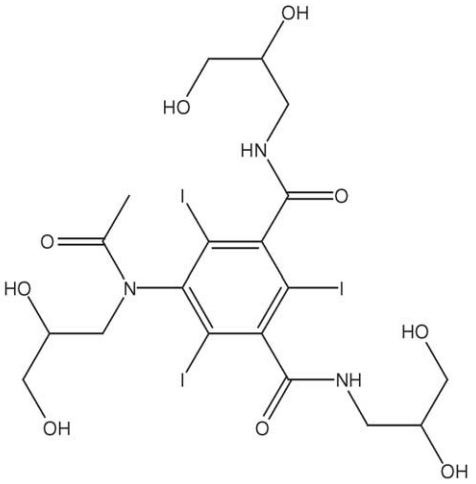

1,3-Benzenedicarboxamide, 5-[acetyl(2,3-dihydroxypropyl)amino]-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo

Fig. 1. Chemical structure of iohexol.

iohexol over a wide range of GFR values (6–160 ml/min per 1.73 m^2) [4].

Several HPLC-UV methods have been reported for iohexol estimation in human plasma [8], serum [9], urine and feces [8] in addition to CSF and brain tissue [10]. Capillary electrophoresis [11,12] and inductively coupled plasma-atomic emission spectroscopy [13] have also been employed for determination of iohexol in biological samples. However, because of insufficient details in the methodology or lack of rigorous validations in some of the published HPLC-UV methods, we could not reproduce the results of these papers. The aim of the current study is to develop a simple, rapid and reliable method for estimation of iohexol in human plasma using HPLC-UV technique and to validate the method according to guidelines provided by the Food and Drug Administration (FDA) of the United States [14].

2. Experimental

2.1. Chemicals and reagents

Iohexol (99.99% pure) and the internal standard (IS) (iohexol related compound B: *N*,*N*′-bis(2,3 dihydroxy propyl)-5-nitro-l,3-benzenedicarboxamide) were obtained from US Pharmacopoeia (Rockville, MD). Perchloric acid (70%, v/v) was obtained from Acros Organics (NJ, USA) and HPLC grade acetonitrile and methanol were purchased from Pharmco Products (Brookefield, CT). Purified de-ionized water was prepared using Milli Q50 (Millipore, Bedford, MA) water purification system. For the preparation of in-house quality controls and calibration samples, human plasma containing citrate anticoagulant was purchased from Rhode Island Blood Center (Providence, RI).

2.2. Chromatographic conditions

The determination of iohexol was performed using an HPLC-UV analytical system, which consists of a Hitachi D-7000 series (San Jose, CA) interface with an autosampler fitted with a 200 μl sample loop, a quaternary pump, a column oven and a variable wavelength UV detector set at 254 nm. Peak areas were integrated using the Hitachi System Manager (HSM) software. Chromatographic separation of iohexol and IS was achieved using μBondapak C_{18} analytical column (150.0 mm × 3.9 mm, 10 μm particle size, Waters, Milford, MA). A 2 μm pore size pre-column filter (Supelco, Bellefonte, PA) was attached to the column and both the column and the pre-column filter were maintained at 30 °C. Mobile phase was filtered and degassed by passing them through 0.45 μm Nylon filters (Millipore, Bedford, MA) under vacuum.

Elution of iohexol and its IS from the chromatographic column was carried out using a fast gradient elution technique. The composition of mobile phase was rapidly changed (within 6 s) from an initial 4% acetonitrile to 14% acetonitrile in water (v/v) from fifth to ninth minute, after which the composition was changed back to 4% acetonitrile for the last 3 min comprising a total run time of 12 min. The flow rate was also changed during the assay run from 0.8 ml/min for initial 5 min to 1.2 ml/min for intermediate 4 min and back to 0.8 ml/min for the last 3 min. For analysis, the peak area of the major iohexol isomer was used because it constituted more than 80% of the combined peak areas and the ratio of both the isomer peaks remained constant at different iohexol concentrations under the current analytical condition. All calculations were performed using peak area ratios of the larger iohexol peak to the IS peak (peak area ratio) by the use of Microsoft Excel (MS OFFICE, 2000).

2.3. Calibration and quality control standards

Stock solutions of iohexol (stock solution I: 15 mg/ml and stock solution II: 10 mg/ml) and internal standard (1 mg/ml) were prepared in acetonitrile. There was negligible (<1.0%) degradation of iohexol stock solutions at freezer temperature (−20 °C) over the 12-month study period. A total of seven concentrations of iohexol including 10, 25, 50, 125, 250, 500 and 750 μg/ml in drug free plasma were used as calibrators. Three in-house quality control standards (QCs), containing iohexol at low (20 μg/ml), medium (175 μg/ml) and high (600 μg/ml) concentrations were also prepared in plasma and were used for assay validation. The stock solution I was used to prepare the highest calibrator (750 μg/ml) and quality control sample (600 μg/ml), while the remaining calibrators and quality control samples were prepared from stock solution II. Aliquots of the internal standard stock solution were diluted in 5% (v/v) perchloric acid to produce a working strength (10 μg/ml) internal standard solution. This was also used as precipitating reagent for plasma samples. Aliquots of the calibrators, quality control samples and reference standard solutions were stored at −20 °C until use. A total of eight standard curves were prepared and all calibrators or quality control samples were injected in triplicates.

2.4. Sample preparation

Frozen calibrators and quality control standards were thawed at 37 °C using a thermostatic shaking water bath (Precision scientific, Chicago, IL). Aliquots of the plasma samples (100 μl) were added to 1.5 ml micro centrifuge tubes (Simport, Quebec, Canada). To all samples, 800 μl of 5% perchloric acid containing internal standard (10 μg/ml) was added. This was vortex mixed for 3 min and sonicated for 5 min in an ultrasonic water bath (Cole-palmer, Vernon Hills, IL). The tubes were then centrifuged for 10 min in a microcentrifuge unit. The resulting supernatant was decanted and 90 μl of this supernatant was injected onto the analytical column, previously equilibrated with acetonitrile and water at 4:96% (v/v) and maintained at 30 °C.

2.5. Assay validation

All validation steps were conducted following FDA guidelines [14]. To evaluate specificity, blank samples of plasma containing no iohexol or IS were analyzed to check for the presence of interfering peaks at the elution time of iohexol and IS. Furthermore, to investigate possible chromatographic interference by drugs administered to transplant recipients including immunosuppressive agents and other drugs, plasma samples from 24 kidney transplant recipients prior to the administration of iohexol were analyzed. The sensitivity of the analytical technique was expressed as the lower limit of quantification (LLOQ) which is the minimum plasma concentration of iohexol that can be quantitatively determined with peak height to base line ratio of atleast 10:1 and the limit of detection (LOD) as peak height to base line ratio of 3:1. The analytical recovery of iohexol was assessed by comparing the peak area ratio of QCs with the peak area ratio (analyte peak area/IS peak area) of the reference standards prepared in methanol. The closeness of mean test results obtained by the method to the actual concentration of the analyte and the degree of agreement among the individual results for multiple analytical runs of the same sample was taken as the accuracy and precision, respectively. To evaluate stability, aliquots of QCs were subjected to three cycles of freeze and thaw (freezing for 24 h at −20 °C and thawing unassisted at room temperature). For short-term stability test, the aliquots of the QCs were thawed at room temperature and kept at this temperature for 8 h (the duration of analysis for a typical batch) before analysis.

2.6. Patient samples

Patient samples were obtained from 24 kidney transplant recipients at Rhode Island Hospital, Providence, Rhode Island. The protocol was approved by the Institutional Review Board at the hospital and written informed consent was obtained from each patient. On the study day, a cannula was inserted into the antecubital vein of each arm, and a baseline blood sample was drawn. Bolus IV injection of 10 ml of Omnipaque (647 mg/ml of iohexol corresponding to 300 mg iodine/ml; Nycomed, Oslo, Norway) was administered, followed by 10 ml of saline solution to wash the line. Blood samples for iohexol analysis were drawn from the contralateral vein of the other arm in 10 ml blood collection EDTA tubes (BD Vacutainer, NJ) before iohexol dose and at approximately 0.25, 0.5, 1, 1.5, 2, 3, 4, 5 and 7 h post dose. The blood samples were centrifuged for 10 min at 1500 × *g* and room temperature and the separated plasma was stored at −70 °C until analysis. The values of iohexol clearance were calculated by the use of non-compartmental pharmacokinetics analysis implemented in WinNonlin Software (version 4.1, Pharsight Corporation, Mountain View, CA) and normalized to a body surface area of 1.73 m^2.

3. Results

A typical blank plasma chromatogram is shown in Fig. 2(A). Fig. 2(B) illustrates chromatogram of iohexol and IS in extracted plasma sample from a representative patient sample. Iohexol was eluted as two isomers at 5.4 and 5.8 min, whereas the IS eluted at 8.4 min and no interfering peaks were observed at the time of iohexol peaks or the IS. The specificity of the method was tested by analyzing plasma samples before the administration of iohexol dose from 24 kidney transplant recipients on triple immunosuppressive therapy including cyclosporine or tacrolimus, mycophenolate mofetil and prednisone. In addition the patients were taking many other medications including statin lipid lowering agents, insulin, aspirin, anti hypertensive agents and antibiotics. No interfering peaks

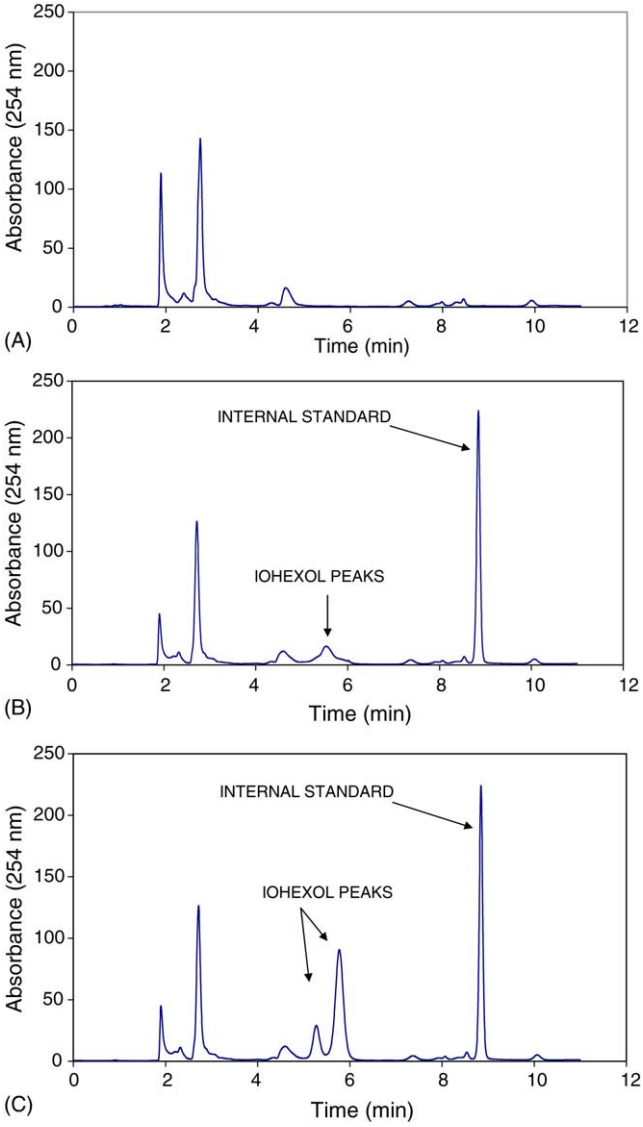

Fig. 2. (A) Chromatogram of iohexol free (blank) plasma. (B) Chromatogram of an extracted plasma sample at LLOQ (10 μg/ml) iohexol concentration and internal standard (5 μg/ml). (C) Chromatogram of an extracted plasma sample from a representative kidney transplant recipient containing iohexol (125 μg/ml) along with internal standard (50 μg/ml).

were observed at the elution times of iohexol isomers or the internal standard. Iohexol LOD and LLOQ were found to be 6 and 10 μg/ml, respectively. The assay was linear over iohexol concentration range of 10–750 μg/ml with an average correlation coefficient (r^2) of 0.999 ($n = 8$). The accuracy of the estimated iohexol concentration was more than 90% at

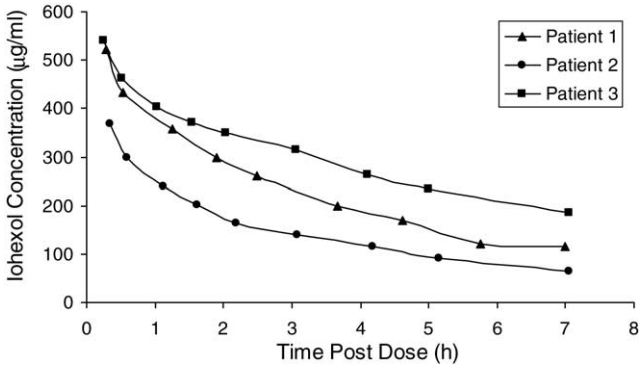

Fig. 3. Iohexol concentration vs. time profile from three representative kidney transplant recipients. The values of iohexol clearance were estimated to be 45.2, 69.5 and 26.1 ml/min per 1.73 m^2 for patients 1–3, respectively.

three QC concentrations (Table 1). The precision expressed as inter-day coefficient of variation (CV%) ranged from 1.6% to 3.2% (Table 1) and the intra-day CV% ranged from 0.5% to 3.7%.

The liquid-liquid extraction method for extraction of iohexol from plasma samples had an average recovery ranging from 95.0 ± 1.8% to 97.0 ± 1.1% for low to high QC standards. The recovery was reproducible over six replications performed over 6 different days. The IS had an average recovery ranging from 96.0 ± 1.7% to 98.0 ± 1.5%.

The concentration of iohexol in freeze–thaw and short-term stability evaluation were not significantly different from the fresh calibrators. The accuracy for the QC samples ranged from 94% to 101% and 97% to 101% after the freeze–thaw stability and short-term stability testing, respectively.

Fig. 3 depicts iohexol concentration versus time profile from three representative kidney transplant recipients. The values of iohexol clearance were estimated to be 45.2, 69.5 and 26.1 ml/min per 1.73 m^2 for patients 1–3, respectively. In total, 206 plasma samples were analyzed using the analytical method described. The accuracy of the QC samples on a day to day basis during the analysis of the patient samples were less than 10%. The mean ± S.D. of all concentrations measured between 0.25 and 7 h post intravenous administration of iohexol was 272.5 ± 133.1 μg/ml (concentration range: 33.9–674.0 μg/ml). The maximum concentration (C_{max}) measured at 15 min after dose was 483.1 ± 108.9 μg/ml ranging from 261.0 to 674.0 μg/ml. The minimum concentration (C_{min}) measured at 7 h post dose was 123.5 ± 61.1 μg/ml (range: 33.9–225.4 μg/ml).

Table 1
Precision and accuracy data for iohexol

Samples	Actual concentration (μg/ml)	Observed concentration (mean ± S.D., μg/ml)	Accuracy (%)	Inter-day precision (CV%)
QC 1	20	18.5 ± 0.6	92.6	3.2
QC 2	175	172.9 ± 3.9	98.4	1.6
QC 3	600	602.7 ± 9.4	100.9	1.6

The data represent the mean of the assay accuracy and precision values obtained for eight sets of standard curve and QC samples. QC: quality control samples.

4. Discussion

The use of iohexol clearance test is a widely accepted method for estimation of GFR [4]. Considering no commercial methods are currently available for the estimation of iohexol in biological fluids, a rapid, precise and specific method for determination of iohexol in human plasma is required to ensure the success of the GFR investigation. In the current study, we report a simple, rapid and reliable HPLC-UV analytical method for the estimation of iohexol using a commercially available internal standard.

In the initial stage of this work, the wavelength for maximum UV absorption of iohexol (λ_{max}) was found to be 244 nm. However, due to high baseline noise at this wavelength further analysis was performed at 254 nm. This wavelength provided a maximum intensity with minimum interference. The method comprises of a simple protein precipitation step using perchloric acid followed by the injection of the supernatant onto the analytical column. The extraction process was efficient as the recoveries of both iohexol and the IS were more than 95%. Using a fast gradient elution technique, both iohexol and IS were eluted from the column with minimum interfering peaks. Commercially available iohexol consists of two geometric isomers, complexes of endo and exo forms. Although both peaks were detected using the current chromatographic method, the peak area of the major isomer was used for calibration curve and other calculations.

In the current study the LLOQ was found to be 10 μg/ml with a peak height to baseline noise ratio of 10:1. This LLOQ is better than 25 μg/ml reported by Shihabi et al. [9] but comparatively higher than 2.8 μg/ml that was reported by Edelson et al. [8]. The available clinical data suggests that serum iohexol concentration lies around 40 μg/ml at 4 h after iohexol administration (Omnipaque 300; 5 ml, 647 mg of iohexol/ml corresponding to 300 mg of iodine/ml) [15,16]. In addition in our own clinical study, the lowest observed concentration of iohexol at 7 h after the dose was approximately 33 μg/ml that is well within the dynamic concentration range of our HPLC method.

In addition, the robustness of the method makes it easy for an operator to learn the technique quickly and to generate reproducible results. The method indeed is very economical with an approximate cost per sample of less than two US dollars for the supply and material. In fact, a single analytical column under the assay condition has lasted for the entire period of method validation and clinical study.

In conclusion a simple, robust, precise and reproducible method is developed for determination of iohexol in human plasma using a commercially available internal standard. The method was then validated according to the FDA guidelines set forth for the bioanalytical method validation for human studies [14] and was successfully used in a clinical study to determine GFR in kidney transplant recipients. This method has an adequate degree of robustness and simplicity to be used in determination of GFR in clinical studies.

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