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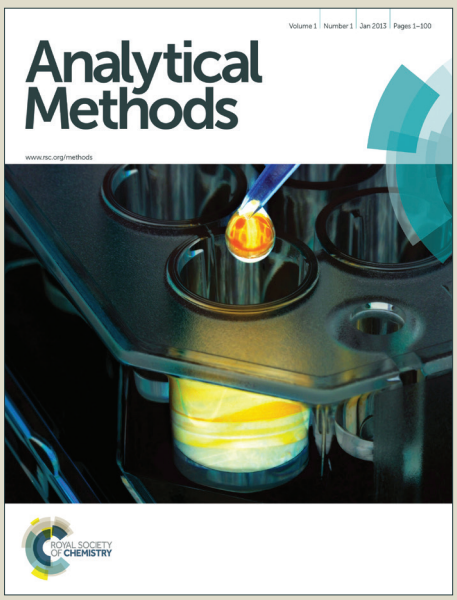

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Highly sensitive method for quantification of iohexol

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Abstract

Background: Iohexol (1-N,3-N-bis(2,3-dihydroxypropyl)-5-[N-(2,3-dihydroxypropyl) acetamid-2,4,6-triiodobenzen-1,3-dicarboxamid) is used for accurate determination of the glomerular filtration rate (GFR) in chronic kidney disease (CKD) patients. However, high iohexol amounts might lead to adverse effects in organism. In order to minimize the iohexol dosage required for the GFR determination in humans, the development of a sensitive quantification method is essential. Therefore, the objective of our preclinical study was to establish and validate a simple and robust liquid-chromatography-electrospray-mass-spectrometry (LC-ESI-MS) using the multiple reaction monitoring mode for iohexol quantification.

Methods: In order to test whether a significantly decreased amount of iohexol is sufficient for reliable quantification, a LC-ESI-MS approach was assessed. We analyzed the kinetic of iohexol in rats after application of different amounts of iohexol (15 mg-150 µg/rat). Blood sampling was conducted at four time points, at 15, 30, 60, and 90 min after iohexol injection. The analyte (iohexol) and the internal standard (iothalamic acid) were separated from serum proteins using centrifugal filtration device with a cut-off of 3 kDa. The chromatographic separation was achieved on an analytical Zorbax SB C18 column. The detection and quantification were performed on a high capacity trap mass spectrometer using positive ion ESI in the multiple reaction monitoring (MRM) mode. Furthermore, using real-time polymerase chain reaction (RTPCR) the effect of iohexol on early regulated gene expression in thyroid and renal cortex was tested to determine a threshold of physiological active iohexol concentrations.

Results: A linear correlation of the iohexol amount and mass-signal (MS) intensity was found in the range of 50 pg-40 ng ($r^2 = 0.998$). The lowest limit of quantification (LLOQ) was 50 pg. The intra- and inter-day accuracies were between 91.2 % and 98.7 %. The intra- and inter-day

precisions were between 2.7 % and 9.2 %. The recovery rate of iohexol was determined in the range of 100.8 % ± 10.9 %. The gene expressions test revealed that iohexol dosages exceeding 0.5 mg/kg induce a group of genes in thyroidal tissue that comprises transcription factors and genes of cellular stress response. Namely, Ptgs2, Smad7, Jun, Srf, Dusp1, Timp1 and others are up-regulated within 15 min after iohexol i.v. application.

Conclusion: This mass-spectrometric based method has been proved to be sensitive, selective and suitable for the quantification of iohexol in serum. Due to high sensitivity of this novel method the iohexol application dose as well as the sampling time in the clinical routine could be reduced in the future in order to further minimize side effects in humans.

INTRODUCTION

Chronic kidney disease (CKD) is characterised by a progressive loss of renal function[1]. CKD is one of the major worldwide public-health problems affecting about 11 % of the population[2]. Kidney damage is quantified by the glomerular filtration rate (GFR). GFR is determined by different renal clearance techniques. Endogenous creatinine or cystatin C clearances are commonly used for the estimation of GFR (eGFR) [3]. However, for an accurate measurement of the GFR (mGFR) the use of an exogenous filtration marker is essential[4]. Different exogenous agents are used in the clinical routine for the GFR determination including inulin[5], iohexol[6], ^{51}Cr-EDTA[7], ^{125}I-iothalamate or ^{99m}Tc-diethylenetriaminepentaacetic acid (DTPA)[8]. Since these agents may induce adverse effects in different tissues, the mGFR determination is only indicated for patients at a high renal risk.

Iohexol is among widely used markers for the determination of mGFR. It has been described to be suitable for accurate GFR measurement in adults and children[6, 9-11]. It is a chemically stable, non-ionic, low-osmolar, X-ray contrast medium with the monoisotopic molecular mass of 820.9 Da. Intravenously applied, iohexol is eliminated from plasma exclusively by glomerular filtration[11]. It has been shown that there is no tubular secretion, metabolism or reabsorption of iohexol by the kidney [12]. Furthermore, iohexol exhibits low protein binding of less than 2 %[13]. Iohexol was initially used in angiographic and urographic procedures[14].

The concentration of iohexol is presently quantified in the clinical routine by applying high performance liquid chromatography (HPLC) on deproteinized serum samples[15]. The sensitivity of this method is characterized by a limit of quantification of 10 µg/ml corresponding to 1 µg iohexol owing to detection using the UV absorbance[16, 17]. The usage of iohexol clearance determined by HPLC analysis was extensively validated and established in the clinical routine[18]. However, the application of UV absorbance as a detection method might imply disadvantages. Besides the limitation due to the insufficient sensitivity the detection

using UV absorbance might lead to false positive results due to co-detection of co-eluting substances from the chromatographic column. Since the sample preparation usually only includes a simple protein removal step using high concentrated acid the sample contains a high number of UV-absorbing serum components.

Furthermore, HPLC-based approach requires relatively large quantities of iohexol for reliable GFR measurement. However, iohexol administered in these amounts may cause adverse effects in patients[19, 20]. It has been demonstrated that the treatment of iodinated contrast media interferes with iodide uptake in the human thyroid gland [21]. Furthermore, there is still a risk of contrast medium induced nephropathy especially in patients at a high renal risk[22-24]. Moreover, recent studies have shown that radiographic contrast agents might have an effect on hemostatic processes and may be important for thromboembolic complications[25]. Furthermore, the high concentrations used in the HPLC-approach increase the exposure and result in longer sampling. The decreased application of reduced iohexol doses lead the linear iohexol elimination phase at an earlier time point and thus reduces the sampling time period.

Accordingly, there is a strong need to reduce the application dose of iohexol without loss of accuracy of the mGFR determination. As a consequence, the quantification of iohexol in serum has to be performed by a more sensitive method. Mass spectrometry provides significantly higher sensitivity as well as selectivity compared to the conventional HPLC method. Furthermore, due to the possibility to acquire fragmentation spectra the identity of the underlying substance can be further verified. Therefore, we established and validated a LC-ESI-MS based method, which is able to detect and quantify serum iohexol at a low ng/ml range.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

The reference standard of iohexol (Histodenz) and primer sets with 6-FAM and TAMRA labeled probes were obtained from Sigma-Aldrich (Hamburg, Germany). Iothalamic acid (Conray30^{TM}) was purchased from GE Healthcare (Munich, Germany). LC-MS-grade water was purchased from LAB-SCAN (Gliwice, Poland). LC-MS-grade acetonitrile was obtained from Honeywell (Seelze, Germany). HPLC-grade formic acid was purchased from Merck (Darmstadt, Germany). ImProm-II TM Kit was purchased from Promega (Mannheim, Germany). qPCR MasterMix Plus was obtained from Eurogentec (Köln, Germany).

QUANTIFICATION OF IOHEXOL BY MASS SPECTROMETRIC-BASED APPROACH

All analyses were performed using an Agilent 1200 series (Böblingen, Germany) liquid chromatographic system, interfaced to HTC mass spectrometer (Bruker-Daltonic, Bremen, Germany), equipped with an electrospray ionisation source (ESI). The analytes were separated on a Zorbax SB C18 column (150 mm×0.5 mm, 5µm, Agilent Technologies, Santa Clara, USA). The column temperature was maintained at 50°C. The mobile phase consisted of 0.1 % formic acid in water (v/v) (eluent A) and 0.1 % formic acid in acetonitrile (v/v) (eluent B). The flow rate was 50 µl/min. The total run time was 16 min. The following gradient was used for the separation: 0 - 5 min 0 - 100 % B, 5-7 min 100 % B, 7-7.5 min 100 - 0 % B ,7.5 - 15 min 0 % B. The ESI source was set in positive ion mode. The ion spray voltage was set to 4,000 V. The dry temperature was set at 300°C and the dry gas at 8 l/min. The nebuliser gas was set at 20 psi. The maximal accumulation time was set to 200 ms. The mass spectrometer was operated in "multiple reaction monitoring" (*MRM*) mode. The following ion transmissions were used to detect iohexol: m/z 821.8 -> 803.8; and iothalamic acid: m/z 614.7 -> 583.6. The following extracted ion chromatograms were used for quantification of iohexol: m/z 656.7 ± 0.5 and iothalamic acid m/z 455.7 ± 0.5 for iothalamic acid. All data were acquired and processed using Compass 1.3 Software (Bruker-Daltonik, Bremen, Germany). Calculations including calibration curve regressions, sample

concentrations values and statistics were performed with GraphPad Prism 5.0 Software (GraphPad Software, San Diego, USA).

METHOD VALIDATION

The validation of the method was performed using calibration standards and quality controls (QC) The quantification method was validated for selectivity, linearity intra- and inter-day precision, accuracy, and recovery.

The linearity of the assay was assessed using calibration standards at 6 different amounts within the range 50 pg-40 ng for iohexol as well as the internal standard iothalamic acid. Six point calibration curves at amounts of 50 pg, 156 pg, 625 pg, 2.5 ng, 10 ng, 40 ng were prepared in extracted blank human serum. The standard curve was calculated using peak area ratio of the analyte vs. internal standard. It was subjected to reciprocal y weighted linear regression.

The selectivity of the method was assessed by comparing blank serum samples with spiked quality controls. Any chromatographic peaks were detected at the respective retention time for respective MRM transmission.

The intra-day (n=3) and inter-day (n=3 within 3 separate days) precisions and accuracies were determined using spiked quality controls. Low quality control (LQC) spiked with 200 pg iohexol, middle quality control (MQC) spiked with 2 ng iohexol and high quality control (HQC) spiked with 20 ng iohexol represented the entire range of the calibration curve. Precision was calculated from the coefficient of variation (% CV) of replicates. Accuracy was calculated by comparison of the measured concentration of spiked analyte with expected concentrations (% bias). Coefficient of variation in the range of mean observed concentration did not exceed 15 % at all concentrations. Accuracy was within 15 %. At LLOQ accuracy was within 20 % and precision was less than 20 %.

The recovery of iohexol and the internal standard was evaluated by comparison of peak area of extracted quality controls vs. peak area of samples spiked after extraction procedure representing 100 % recovery.

PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES

Standard stock solutions of iohexol and iothalamic acid (10 mg/ml) were prepared in LC-MS grade water in polypropylene tubes. The quality control samples (QC) were prepared by spiking blank serum of healthy subjects (n = 3) with iohexol at a low concentration of 200 ng/ml (LQC) corresponds to 200 pg in the measured sample, middle concentration 2,000 ng/ml (MQC) corresponds to 2 ng in the measured sample and high concentration 20,000 ng/ml (HQC) corresponds to 20 ng in the measured sample. Standard spiking solution of iothalamic acid was prepared at concentration 5 µg/ml in polypropylene tubes by diluting the standard stock solution with blank serum.

For quantification of concentration-mass signal intensity correlation, a serial dilution in the range of 40 pg-40 ng for iohexol and 50 pg-40 ng for iothalamic acid with extracted blank serum was prepared.

PREPARATION OF RAT SERUM SAMPLES

All procedures conformed with national legislation and EU directives for the use of animals for scientific purposes and were approved by the competent regional authority.

Male HsdRCCHan:WIS rats (8 weeks old) were obtained from Harlan (Limburg, Netherlands). Different quantities of iohexol (15 mg, 1.5 mg, 150 µg) of iohexol were administered to rat by tail vein application (*vena caudalis mediana*) in 0.1 % formic acid. Animals were sacrificed under deep anesthesia at different time points after application (15,

30, 60, 90 min) to obtain blood samples (n=3 per time point and dosage). The blood samples were centrifuged at 2,000 g for 15 minutes in order to separate serum from blood cells at room temperature. The serum samples were stored at -80 °C prior use. For preparation frozen samples were thawed at room temperature and vortexed. The internal standard (iothalamic acid) was added to each sample (5 ng in the measured sample). The sample was centrifuged using a 3 kDa filter unit and centrifuged at 10,000 rpm for 1.5 hours at 4 °C. The filtrate was collected and frozen at -20 °C until measurement.

RELATIVE mRNA EXPRESSION ANALYSES BY REAL-TIME POLYMERASE CHAIN REACTION (RTPCR)

Kidney cortex and thyroid were taken directly after scarification of rats and cut to representative pieces of app. 30 mg. Tissues were immediately frozen on dry ice and stored at – 80°C. Frozen tissue samples were ground in liquid nitrogen and nucleic acids were isolated by TRIzol and chloroform extraction followed by precipitation in aqueous ethanol. DNA was eliminated by deoxyribonuclease I digest. cDNA was synthesized using the ImProm-II TM Kit. Relative gene expression was determined by real-time quantitative polymerase chain reaction using ABI Prism Sequence Detection System (Applied Biosystems ABI Prism 7700 Sequence Detector, Germany). cDNA samples were amplified with a PCR mix containing Taq polymerase and primer sets with 6-FAM and TAMRA labeled probes in 384-well microtiter plates. 1-10 ng of cDNA samples were run in triplicates in reaction volumes of 20 µl (384-well MTP) under standard thermocycler conditions. TaqMan probe sets were generated from NCBI Genbank mRNA sequence files (http://www.ncbi.nlm.nih.gov/genbank) using Primer3Plus Software. Relative gene expression was calculated using the delta Ct term dCt (User Bulletin No. 2, Applied Biosystems, Germany) related to endogenous controls ribosomal protein L32 and beta-actin. Induction factor of relative mRNA expression represents

the quotient of mRNA levels of respective genes after exposure to iohexol at the given time points and dosages divided by mRNA expression levels of these genes at non-induced baseline levels.

GFR CALCULATION

GFR was calculated as the plasma clearance of iohexol according to the formula:

$$Cl = \frac{D * B}{Io}$$

where D represents the application dose (µg), B the slope of the curve in the linear elimination phase (1/min) and I_0 the Y intercept, when X=0 of the extrapolated curve (95 % confidence Intervals) in the linear elimination phase (µg/ml).

RESULTS

The chemical structure of iohexol is given in **Figure 1A** and the internal standard iothalamic acid in **Figure 2A**. Since molecular structures of iothalamic acid and iohexol are highly comparable, iothalamic acid was used an internal standard in this study. The MRM transmission parameters were optimised for both iohexol and internal standard. The quantification was performed using MRM mode. The MRM transmissions for iohexol were m/z 821.8 -> 803.8 (**Figure 1 B-D**) and for internal standard m/z 614.7 -> 583.6 (**Figure 2 B-D**).

The schematic preparation protocol for all serum samples is given in **Figure 3A**.

The method was validated by analysing an appropriately prepared calibration, and quality control standards in three consecutive batches to demonstrate acceptable intra- and inter-batch accuracy and precision over the desired range of concentration. Furthermore the method was validated in terms of selectivity and recovery.

The selectivity was determined by quantification of quality controls at low, middle and high iohexol concentrations compared to blank serum samples. The retention times for iohexol and iothalamic acid were 4.7 min and 3.3 min respectively (**Figure 3B**). No interfering peaks from endogenous compounds were observed at the retention times of iohexol and internal standard in the respective MRM transitions (**data not shown**).

The calibration curve was determined in the range of 50 pg-40 ng for iohexol. The calibration curve was calculated by plotting the ratio of peak area of extracted ion chromatogram vs. amount of corresponding compound. The linear calibration curve of iohexol was calculated as y=0.004197x + 0.01197 (**Figure 3C**). The calibration curve showed a coefficient of determination (R^2) of 0.998. The linear regression of the calibration curve was weighted by reciprocal y.

The lowest limit of detection (LLOD) was estimated as the amount of iohexol resulting in a mass-signal intensity three times higher than noise ($S/N \geq 3$). The LLOD of iohexol was calculated as 2 pg. The lowest limit of quantification (LLOQ) was calculated at the lowest amount of the calibration curve with a precision not exceeding 20 % and accuracy within 15 % of the actual value. The LLOQ for iohexol was calculated as 50 pg.

The inter- and intra-day precisions and accuracies with three replicates of LQC (200 pg), MQC (2 ng) and HQC (20 ng) of iohexol were analyzed. The inter-day precision and accuracy were assessed over three separate days. The inter- and intra-day precision expressed as percent coefficient of variation (% CV) ranged from 2.7 % to 12.1 %. The inter- and intra-day accuracy defined as percent of nominal values was between 91.2 % and 98.7 %. The analysis of the accuracy and precision is given in **Table 1**.

The mean relative recovery rate of iohexol was calculated as 100.8 ± 10.9 %.

The aim of this study was development of a reliable and sensitive method for iohexol quantification in rat serum in order to estimate the minimal iohexol application dose for the GFR determination in the clinical routine. Therefore, we analysed serum concentration-time profiles after application of different amounts of iohexol. The quantification was carried out by using a LC-ESI-MS method. The results indicate that the mass spectrometry-based method validation over the analytical range of 50 pg-40 ng is appropriate to quantify levels of iohexol present in the circulation following an injection of 15 mg, 1.5 mg, or 150 µg. The serum-concentration versus time curves are shown in **Figure 4**. The serum-concentration versus time curves obtained after application of 15 mg iohexol did not reach the linear elimination phase within the 1.5 hours of the sampling time (**Figure 4D**). After application of 150 µg iohexol, the linear elimination phase was reached already after 15 min and the detected mass signals were within the validated concentration range (**Figure 4F**).

The influence of iohexol on immediate early or early regulated gene expression in thyroid and renal cortex was tested in order to detect cellular responses to exposure of these tissues to the given iohexol concentrations. Tissue samples were analyzed for mRNA expression profiles after iohexol i.v. application at four longitudinal time points i.e. 15, 30, 60 and 90 min. Induction factor of relative mRNA expression levels of genes that are commonly up-regulated by cellular response to stress conditions were analyzed by RTPCR. Several genes showed consistently a dose dependent up-regulation upon iohexol exposure particularly in thyroidal tissue when the expression was related to non-induced baseline levels. Genes like Ptgs2, Srf, Smad7, Jun, showed increased mRNA levels at and above an iohexol dosage of 1.5 mg per animal (app. 0.5 mg/kg) compared to the lower dosage of 0.15 mg per animal (app. 50 µg/kg) (**Figure 5**). This induction of mRNA expression occurs rapidly after iohexol application of 1.5 mg per animal, since the increased mRNA levels were already detectable at the 15 min tissue sampling time point for most of the analyzed genes. These up-regulated genes belong to

a group of genes which are induced by cellular activation in response to stress signals. Furthermore, other transcription factors and injury response genes have been induced at 1.5 mg per animal in thyroid, i.e. F3 (tissue factor), Dusp1, Nupr1, Nra4, Cyp24a1, Egr1, Timp-1 (**data not shown**) to a similar extent as shown for the genes presented by **Figure 5**. Of the mentioned genes, only Smad 7 was weakly induced in renal cortex (**data not shown**).

The calculated GFR in rats after an iohexol application rate of 150 µg was 1.38 ± 0.54 ml/min per 100 g body weight and it was comparable with the GFR in WIS rats described in the literature [26]. The GFR in rats after iohexol application of 15 mg and 1.5 mg was not calculated since the linear elimination phase was not reached within the 1.5 hours of the sampling time.

DISCUSSION

Iohexol is a commonly used reagent for accurate GFR measurement in order to estimate kidney function. The LC-ESI-MS method established and validated in this study is appropriate to quantify low concentrations of iohexol in serum. This LC-ESI-MS-based method is characterised by a simple and robust sample preparation, short separation time and low limit of quantification. Furthermore, the method is selective, accurate and precise for the quantification of iohexol.

The validation revealed a wide linear concentration range of 50 pg-40 ng. The inter- and intra-day precisions were determined as a range of 2.7 %-12.1 %. The inter- and intra-day accuracies were between 91.2 % and 98.7 %. The validated mean recovery rate was 100 % and allowed further reduction of the dose and sample volume.

The sample preparation includes one-step protein removal by centrifugation using 3 kDa filter device. Only 100 µl serum are needed for iohexol quantification. The current method has been successfully applied for reliable quantification of iohexol in blood of rats at different

concentration levels for proving the concept. Even after injection of 150 µg iohexol into rats, the concentration in serum was quantifiable above the LLOQ of 50 pg. In addition, less blood volume is necessary for iohexol quantification, which is highly relevant in CKD patients suffering from anemia[27]. Moreover, due to a decreased iohexol application dose the linear elimination phase of iohexol resulted in a decreased follow-up period, whereby time for treatment could be significantly reduced and thus the quality of patient life could be improved.

The application of 15 mg to rats equals approximately the iohexol application dose currently used for the GFR measurements in humans. Therefore, the application dose of iohexol could be now decreased to one hundredth of the application dose used presently for the GFR determination in humans. In detail, currently 3235 mg of iohexol are administered to humans for GFR determination in the clinical routine. Using the method described in the current study, the iohexol amount could be reduced to approximately 30 mg. This considerably reduced dose may lower the risk of contrast medium associated adverse effects. Contrast media induced effects have been reported for iohexol in preclinical study settings[19, 20, 28].

These findings are in line with the results of the current study demonstrating an induction of stress-response genes like Jun, Srf, Smad7, Ptgs2 in a dose-dependent manner as shown in **Figure 5**. Further genes of cellular activation which are reported to be induced by stress or tissue damage like Dusp1, Cyp24a1, Egr1, Timp-1, Nupr1, and F3 (tissue factor) were up-regulated to a similar extent after exposure of the thyroid to the given iohexol concentrations too (**data not shown**).

Based on these findings, it is proposed to evaluate the GFR determination in CKD patients by application of significantly reduced iohexol dosages and quantification of iohexol by LC-ESI-MS detection.

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FIGURE LEGENDS

Figure 1: **A**: Molecular structure of iohexol

B: Representative positive ESI MS mass spectrum of iohexol

C: Representative positive ESI MS^2 mass spectrum of iohexol (parent ion: m/z 821.8)

D: Representative positive ESI MS^3 mass spectrum of iohexol (transmission: m/z 821.8 -> 803.8)

Figure 2: **A**: The molecular structure of iothalamic acid, used as internal standard

B: Representative positive ESI MS mass spectrum of iothalamic acid (internal standard)

C: Representative positive ESI MS^2 mass spectrum of iothalamic acid (internal standard) (parent ion: m/z 614.7)

D: Representative positive ESI MS^3 mass spectrum of iothalamic acid (internal standard) (transmission: m/z 614.7 -> 583.6)

Figure 3: **A**: Overview of the preparation steps for the iohexol quantification

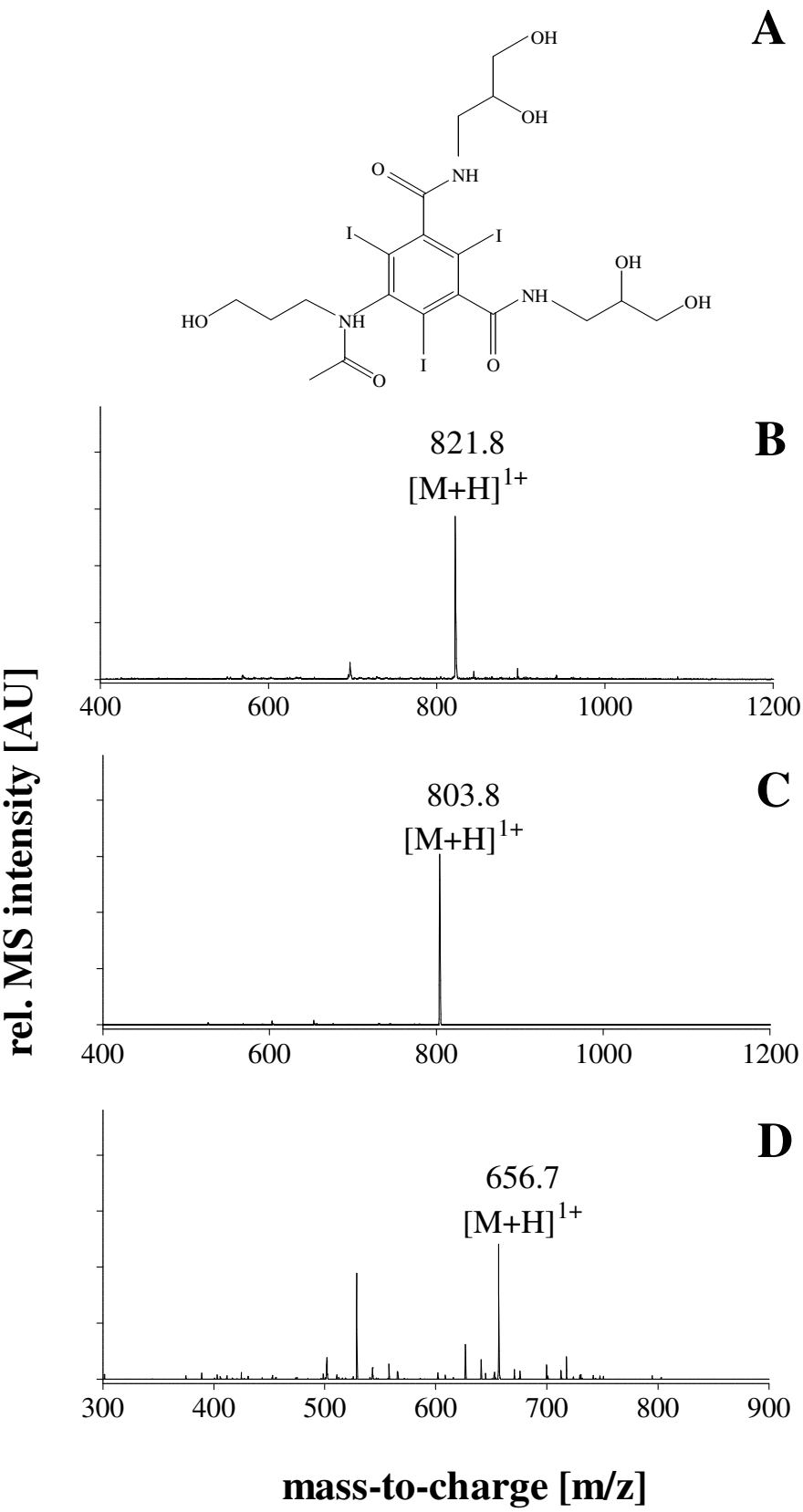
B: Representative extracted ion chromatograms of iohexol (m/z range: 656.7 ± 0.2 Da) and iothalamic acid (m/z range: 455.7 ± 0.2 Da)

C: Linear responses in iohexol/iothalamic acid area ratio versus iohexol amount in samples

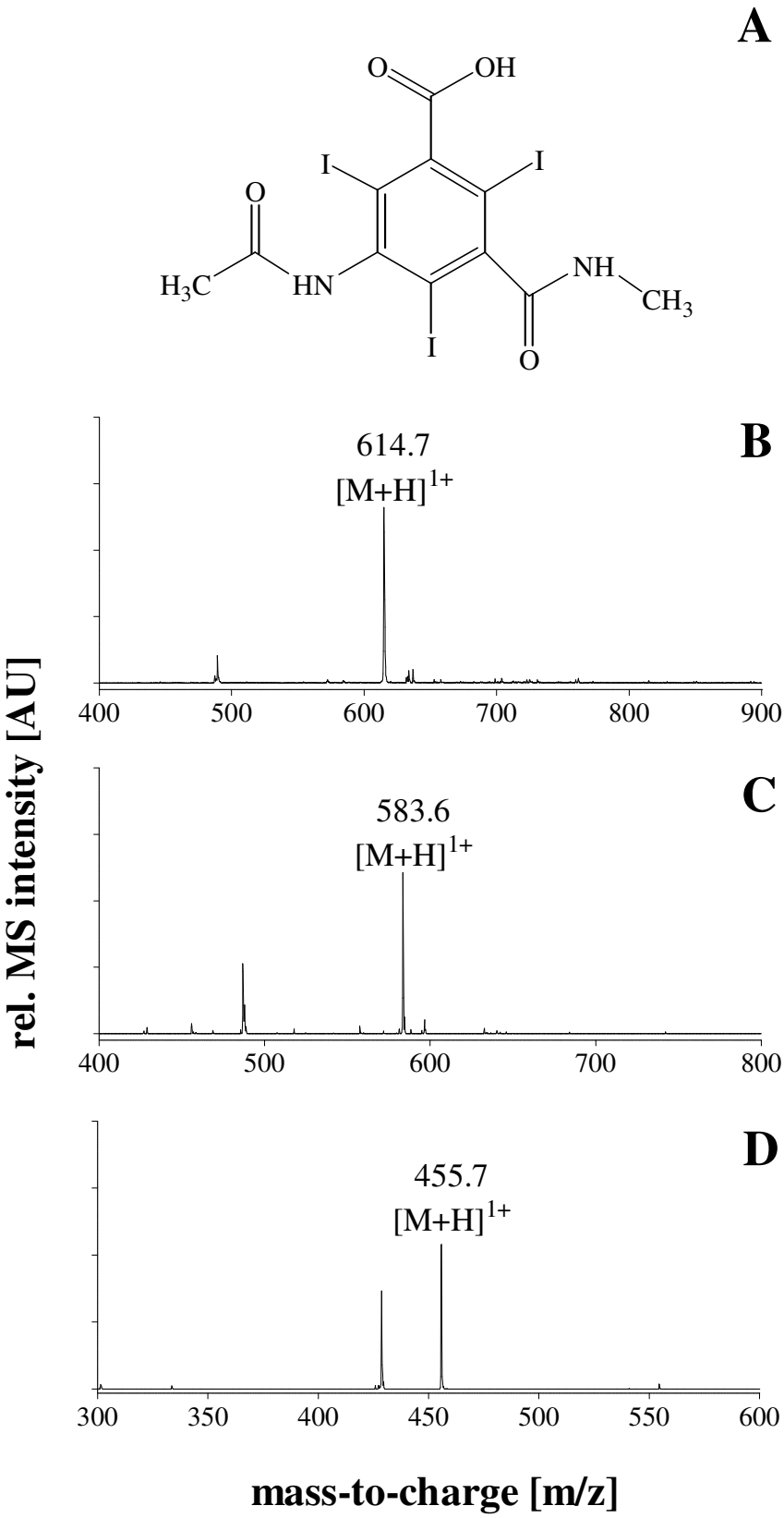
Figure 4: Time-dependent extracted ion chromatograms of iohexol (m/z range: 656.7 ± 0.2 Da) in serum following single intravenous injection of **A**: 15 mg; **B**: 1.5 mg; **C**: 150 µg iohexol to male HsdRCCHan:WIS rats (n = 3 per time point and dosage.) at different points after application (15, 30, 60, 90 min) and corresponding mean serum-concentration time profile of iohexol in rat serum following single intravenous injection of **C**: 15 mg; **D**: 1.5 mg; **E**: 150 µg iohexol.

Figure 5: Induction factor of relative mRNA expression of stress-response genes (**A**) Ptgs2, (**B**) SRF, (**C**) Smad7 and (**D**) Jun in rat thyroidal tissue after 15, 30, 60 and 90 min of i.v. iohexol application. Each bar represents the ratio of mean mRNA levels obtained from three thyroids per time point and dosage related to non-induced baseline levels of the respective genes.

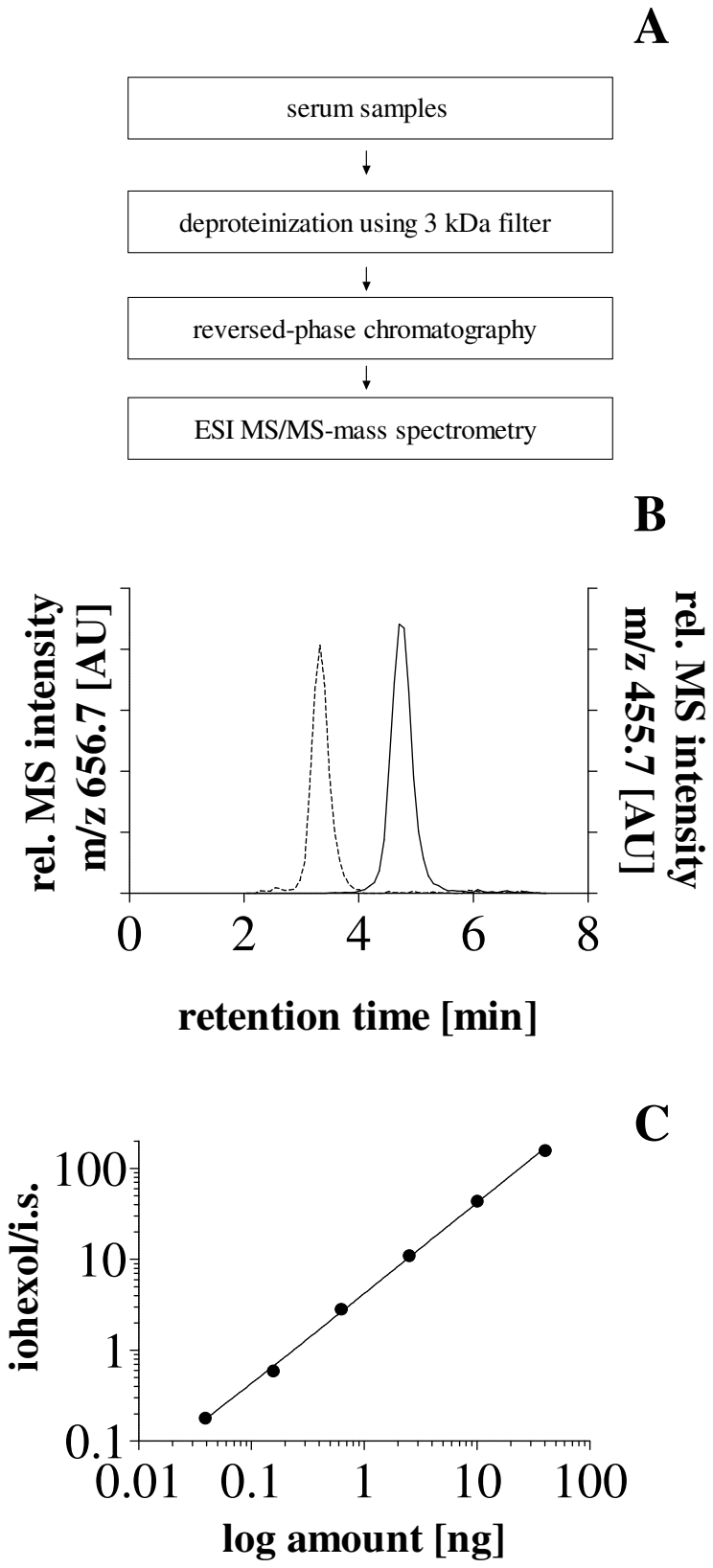
Schulz et al., Figure 1



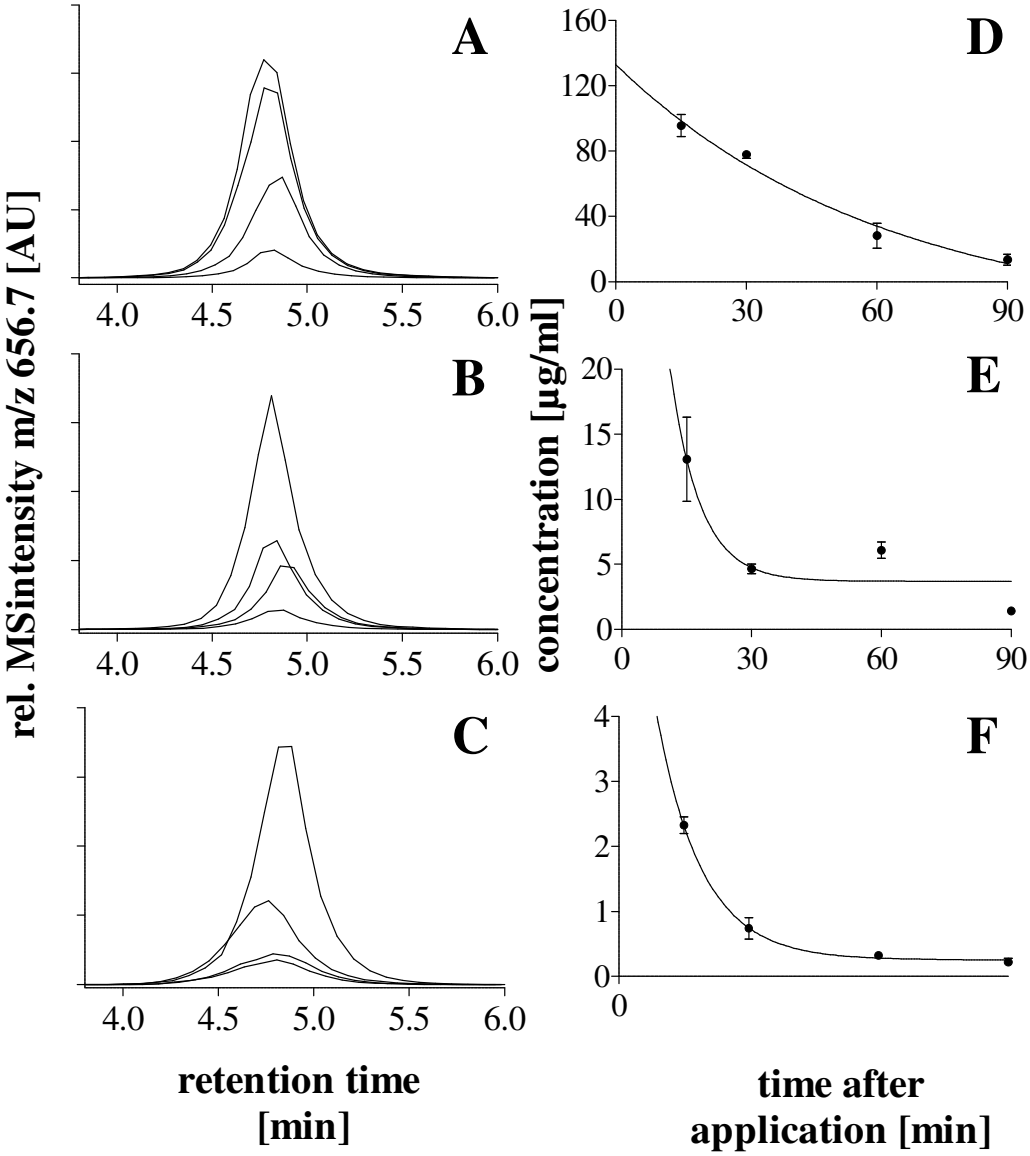
Schulz et al., Figure 2



Schulz et al., Figure 3



Schulz et al., Figure 4



Schulz et al., Figure 5

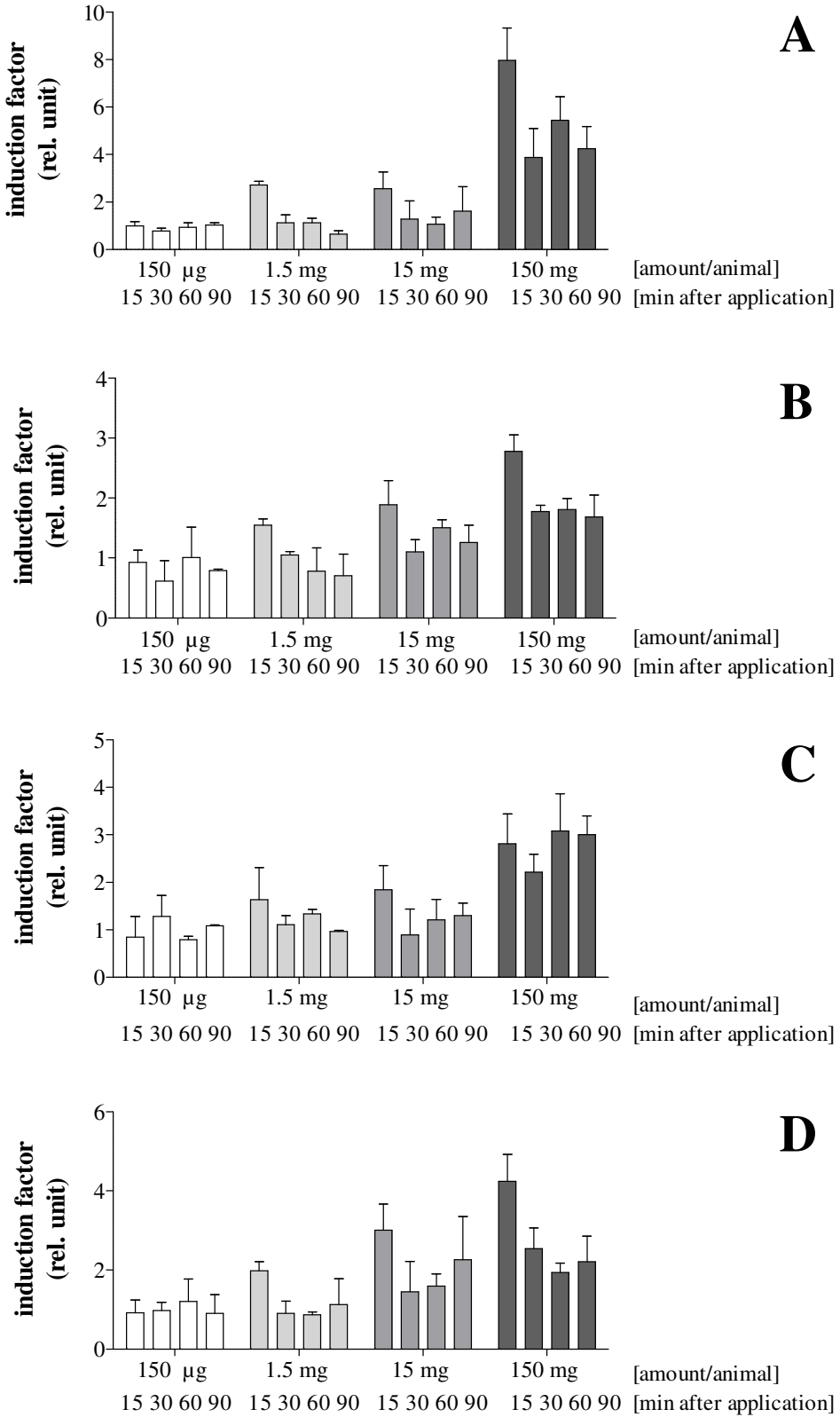


Table 1: Accuracy and precision of quality control samples

	QC	amount (pg)	accuracy (%)[1]	precision (%)[2]
Intra-day	LLOQ	50	98.7	2.7
	LQC	200	91.2	12.1
	MQC	2000	95.4	11.1
	HQC	20000	91.4	9.2
Inter-day	LLOQ	50	95.9	9.2
	LQC	200	93.9	7.6
	MQC	2000	96.7	7.7
	HQC	20000	97.7	8.8

[1]calculated as (mean determined amount/nominal amount×100)
[2]calculated as % CV. (SD./mean)×100