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Validated method for the determination of hydroquinone in human urine by high-performance liquid chromatography–coulometric-array detection

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Abstract

The paper describes the computer aided method development and validation for the determination of hydroquinone in human urine from a clinical study on renal excretion of hydroquinone metabolites and the release of free hydroquinone in the urinary tract in order to evaluate the proposed urine disinfecting concept. The presented method uses high-performance liquid chromatography on reversed-phase material with a polar endcapping (Aqua-C_{18}, 250×4.6 mm). Selective and sensitive determination (LOQ=12.5 ng on-column) of the target compound was achieved by electrochemical array detection (CoulArray). Gradient and parameter optimization were supported by DryLab software in order to minimize efforts of the expensive and time-consuming method development. Specificity and selectivity were carried out by separation experiments involving the prodrug arbutin and the metabolites hydroquinone, hydroquinone glucuronide, and hydroquinone sulfate, respectively. Hydroquinone glucuronide reference standard was obtained from in vitro glucuronidation in a rat liver microsomes assay. The method was validated according to the criteria for validation of pharmaceutical bioanalytical methods as drafted by the US Department of Health and Human Services, 1998.

Keywords: Electrochemical detection; DryLab; Hydroquinone; Arbutin

1. Introduction

Bearberry [*Arctostaphylos uva-ursi* (L.) Sprengel] leaf extracts and respective formulations are used in herbal medicine as lower urinary tract disinfectants. Main indication areas are infections as uncomplicated cystitis often related to catheter usage, when antibiotic treatment is not considered essential or secondary treatment after application of antibiotics [1–4]. There are monographs provided from the European Pharmacopoeia [5] and the European Scientific Cooperative on Phytotherapy ESCOP [6] as well as a standard marketing authorization in Germany for respective products [7]. Hydroquinone glucuronide and sulfate are discussed as systemic metabolites in man after oral administration of the genuine bearberry leaf constituent arbutin (hydroquinone-1-*O*-β-D-glucopyranoside) [8,9]. Literature data suggest the metabolism of arbutin to hydroquinone glucuronide; a possible biotransformation of arbutin to hydroquinone sulfate is, however, discussed controversially [1,10,11]. Free hydroquinone

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is believed to be the active principle released renally from hydroquinone glucuronide and sulfate at the target site. Its disinfectant activities have been demonstrated by in vitro experiments only. Alkalized urine containing hydroquinone and hydroquinone glucuronide has shown to exhibit antibacterial activities [10,11].

Evaluation of the availability of potentially bioactive plant constituents occurring in food or herbal medicinal products at the target site is a major prerequisite for the interpretation of in vitro pharmacological testing. Therefore there is a general great demand on reliable selective and sensitive bioanalytical methods for assaying trace levels of genuine plant constituents and their metabolites at the specific target sides.

In order to prove the proposed concept of efficacy of bearberry leaf extracts and respective formulations, the aim of our study was the development of a robust, selective, and sufficiently sensitive method for urine analysis. Hydroquinone, its sulfate and glucuronide conjugates as the described systemic metabolites were analyzed in urine and water in comparison to blank urine matrix by high-performance liquid chromatography (HPLC) using a octadecyl silane stationary phase with a polar endcapping. As electrochemical detection was described to be superior to UV–Vis and fluorescence spectroscopy for determination of polyphenols in trace levels [12–14], very sensitive determination was achieved by using a multi channel electrochemical detector (CoulArray). This electrode coulometric detector is based on the measuring principle of the potentiostatic amperometry. In amperometry compounds which are subject to a potential difference become excited and undergo a molecular rearrangement with the loss (oxidation) or gain (reduction) of electrons. Faraday's law relates the response of the detector to the quantity of analytes undergoing an oxidation or reduction. As the detector has 12 electrodes in series set incrementally to different potentials a chromatogram is obtained at each potential. The plot of the 12 chromatograms – called a hydrodynamic voltammogram – is a function of the electrochemical thermodynamics and kinetic behavior of the analyte of interest. Similar to the UV–Vis spectra the hydrodynamic voltammogram can be used for peak identification.

As hydroquinone glucuronide was not commercially available as a standard it was biosynthesized in vitro by rat liver microsomes. Formation of the biosynthesized product was controlled by a glucuronidase assay. The presented method could be validated for a range of hydroquinone concentrations from 250 to 1000 ng ml^{-1} which corresponds to the range reported for pharmacological activities [8,15].

The developed method was used for the determination of hydroquinone in urine in a clinical study on the renal excretion of hydroquinone metabolites after oral administration of bearberry leaf extract. Data of this study will be published elsewhere.

2. Experimental

2.1. Materials

Hydroquinone (*p*-dihydroxybenzene, $C_6H_6O_2$, M_r=110.1, purity 99.9%) was obtained from Fluka (Seelze, Germany). Arbutin (hydroquinone-1-*O*-β-D-glucopyranoside, $C_{12}H_{16}O_7$, M_r=272.2, purity 91.2%) was isolated from bearberry leaves using 2-propanol (kindly provided by Geiger, Ellwangen, Germany). Both compounds were certificated as natural product reference substance (NPRS) at the Zentralinstitut Arzneimittelforschung (Sinzig, Germany). Hydroquinone monosulfate potassium salt ($KC_6H_5O_2SO_3$, M_r=228.3, purity 98.0%) was supplied from Tokyo Chemical Industry (Tokyo, Japan). Identity and purity of all reference standards was determined by quantitative nuclear magnetic resonance (NMR) according to Pauli [16].

85% aqueous orthophosphoric acid, 36% aqueous hydrochloric acid, acetic acid, sodium hydroxide, ascorbic acid, acetonitrile, acetone and methanol (gradient grade) were purchased from Merck (Darmstadt, Germany). Sodium dihydrogenphosphate ($NaH_2PO_4 \cdot H_2O$, M_r=138.0 ACS reagent), β-glucuronidase (EC 3.2.1.31), uridine-5′-diphosphoglucuronic acid (UDPGA) and 2-amino-2-hydroxymethyl-1.3-propanediol-hydrochloride buffer, pH 7.8 ($C_4H_{11}NO_3 \cdot HCl$, M_r=157.6, Tris-buffer) were obtained from Sigma–Aldrich (Deisenhofen, Germany). Magnesium chloride ($MgCl_2 \cdot 6H_2O$, M_r= 203.3, ACS reagent) was obtained from Grüssig (Wahrenholz, Germany).

2.2. HPLC–CoulArray system

The HPLC equipment was supplied by Environmental Sciences (ESA, Chelmsford, MA, USA) and consisted of an autosampler Model 542 (set to a temperature of 15°C, injection volume 50 μl), a solvent delivery system Model 582, a column-thermostat ''Mistral'' type 880 (set to a temperature of 25°C), and a coulometric array-detector CoulArray. The 12 cells of the electrochemical detector were set up from 75 to 900 mV in fixed increments of 75 mV. Hydroquinone determination was carried out at 225 mV as the dominant channel (Fig. 1). For separation a column (250×4.6 mm I.D., 5 μm) packed with C_{18}-Aqua (octadecyl silane with polar endcapping) prepacked by Phenomenex (Aschaffenburg, Germany) was used.

The solvents (all of gradient grade) were 0.02 *M* aqueous solutions of sodium dihydrogenphosphate adjusted to pH 3.4 (A) and a mixture of methanol–pH 1.4 adjusted 0.1 *M* aqueous solution of sodium dihydrogenphosphate (4:1, v/v) (B). pH adjustment was carried out by 85% aqueous orthophosphoric acid. The flow-rate was set to 0.8 ml min^{-1} and an isocratic profile at 15% B in A for 15 min was used for separation. Total analysis time was 35 min including 10 min column flushing and 10 min equilibration time.

Development of the solvent profile for chromatographic separation was carried out by computer assistance using the DryLab software.

2.3. Validation parameters and procedures

Method validation included tests on specificity, calibration curve, precision, accuracy, and stability. For validation purposes a homogenous urine matrix [17] was mixed using equal urine aliquots of three male and three female volunteers, respectively, containing 0.5% L-(+)-ascorbic acid (Merck). All volunteers gave their informed written consent. The resulting urine matrix was used for the determination of limit of quantification (LOQ), calibration curve, precision, accuracy, and stability.

2.4. Urine sample preparation from volunteers

The relative systemic availability of hydroquinone derivates from various formulations containing arbutin was investigated in a study with healthy

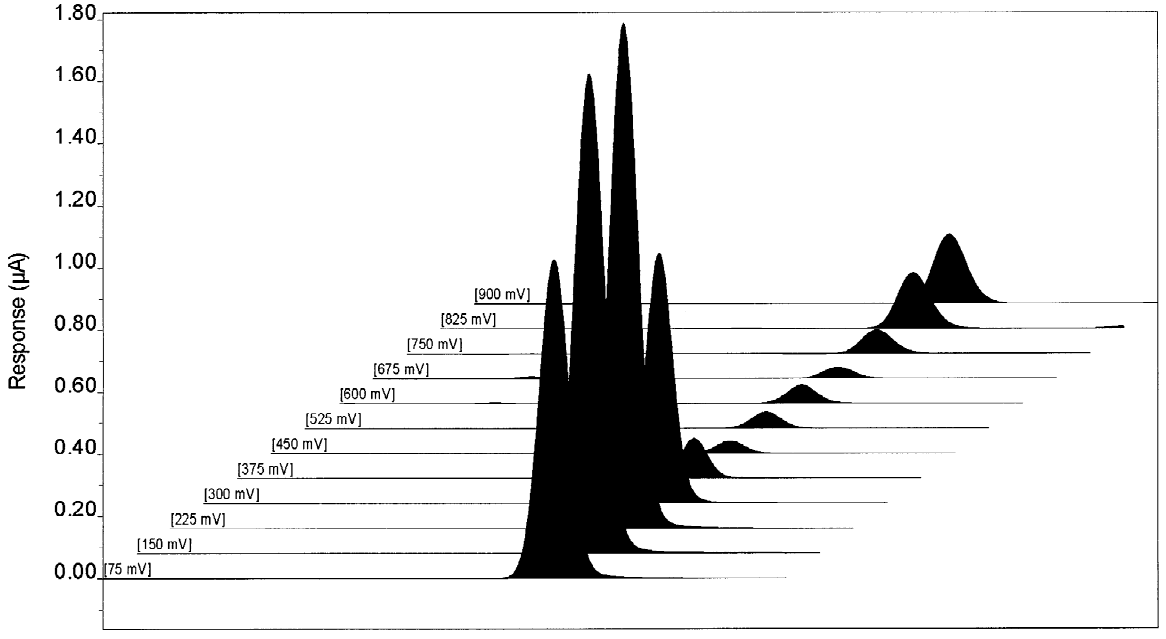

Fig. 1. Voltammogram (75–900 mV, fixed increment of 75 mV) of hydroquinone in urine matrix with dominant channel 225 mV.

volunteers. During the study subjects were kept on a xanthine and arbutin free diet to avoid interference with other dietary phenolic compounds. During the study any co-medication were omitted. The study was approved by an independent Medical Ethical Review Committee.

After consumption of bearberry leaf extract urine was collected in intervals of 4 h. In order to avoid oxidation, collection container were preloaded with 1 g ascorbic acid (Merck). Due to the fact that the extent of the renal hydroquinone conjugates cleavage is correlated to the pH of urine [15], no post-collection pH-adjustment of urine samples was done. For HPLC–CoulArray analysis urine samples were filtered using 0.22-μm nylon membrane filters.

2.5. Rat liver microsomes glucuronyl-transferase assay [18]

In order to get an authentic reference compound, hydroquinone was glucuronidated using rat liver microsomes. The experiment was approved by the district authorities of the City of Münster (file: 23.0835.1.0–G1/98), Germany.

An aliquot of 200 μl of an aqueous suspension of rat liver microsomes stimulated by phenobarbital was added to 800 μl of an aqueous 50 m*M* 2-amino-2-hydroxymethyl-1.3-propanediol-(Tris) hydrochloride buffer (pH 7.8) containing 0.91 nmol hydroquinone, 5.0 μmol magnesium chloride, and 1.1 nmol UDPGA. The mixture was incubated at 37°C under a nitrogen atmosphere for 4 h. The enzyme reaction was stopped by adding 2.0 ml of acetonitrile. For separation of denatured protein the reaction cocktail was centrifuged at about 8000 *g* for 10 min. The glucuronidation rate was about 70%.

2.6. β-Glucuronidase assay

In order to check the identity of the enzymatically generated reference standard hydroquinone glucuronide, a 500 μl aliquot of the rat liver microsomes assay supernatant was mixed with 10 μl of a 1.0% aqueous solution of ascorbic acid and adjusted to pH 5.0 using 0.58 *M* aqueous acetic acid. This reaction cocktail was incubated with 30 μl β-glucuronidase for 60 min at 37°C. Enzymatic reaction was stopped by 600 μl acetone. After centrifugation at about 8000 *g* for 10 min the resulting supernatant was analyzed by HPLC–CoulArray.

Fig. 2 presents chromatograms of the rat liver microsomes assay before and after enzyme treatment which show the decrease of hydroquinone glucuronide and the increase of hydroquinone after β-glucuronidase assay. Assay analysis was carried out by the described HPLC–CoulArray system. Due to assay–matrix interferences with the target analyte a distinct isocratic separation with 7% solvent B in A was developed (Fig. 2).

3. Results

3.1. Computer aided method development

Gradient development was carried out by computer assistance using the DryLab software. Three linear gradient separations from 0% B in A to 100% B in A in 20, 40 and 60 min, respectively, involving the reference standards of hydroquinone glucuronide, hydroquinone sulfate, hydroquinone, and arbutin were used as input data for the DryLab software. As a result of software calculations not a gradient but an isocratic separation method in a range of 10 to 20% B in A turned out to give reasonable and robust resolution of the desired peaks.

In a second DryLab experiment four isocratic separations of the spiked rat liver microsomes assay (containing hydroquinone glucuronide and hydroquinone, arbutin and hydroquinone sulfate) at 7, 15, 22 and 30% B in A were performed. A solvent composition of 10% B in A was calculated for optimal peak resolution of the four compounds (Fig. 3). However, due to interferences caused by peaks of the urine matrix with the target compound the isocratic profile had to be adjusted to 15% B in A. This solvent system turned out to be ideal for analyzing the target compound, hydroquinone, as it showed no significant interferences with urine matrix components.

3.2. Selectivity

Chromatograms of the 10 blank urine samples (five female, five male) were compared with a chromatogram derived from a urine matrix sample

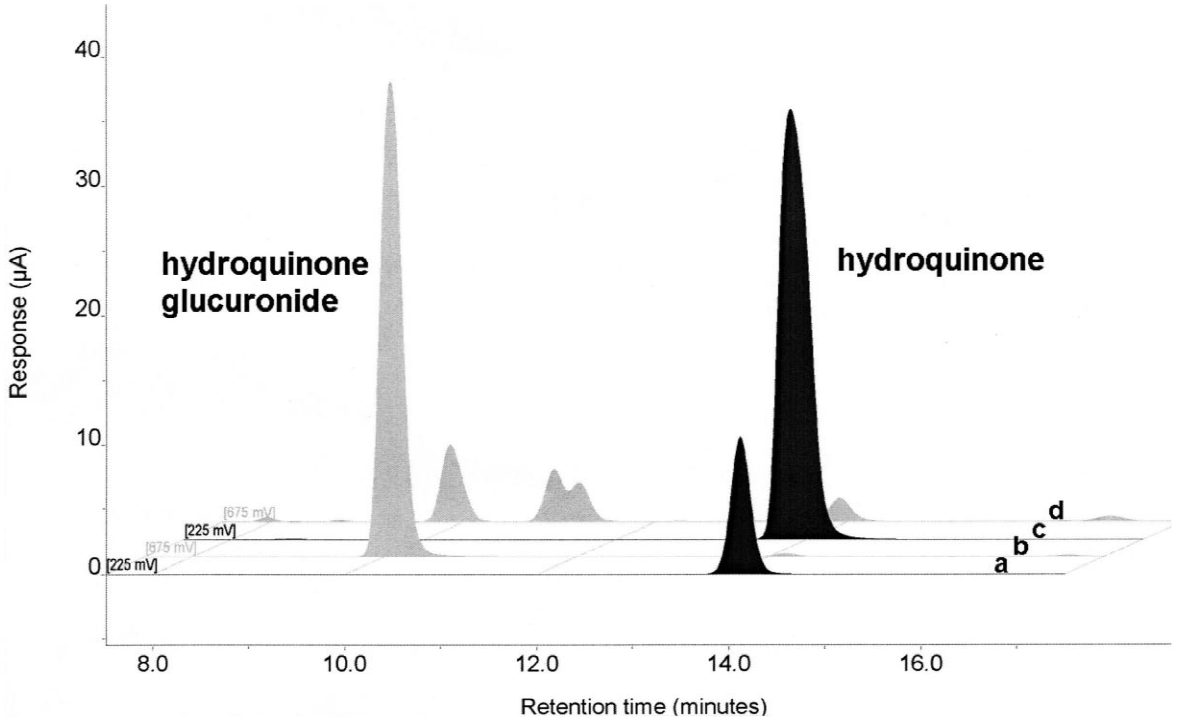

Fig. 2. Chromatograms of the rat liver microsomes assay before (a, b) and after (c, d) glucuronidase treatment (at 7% B in A, isocratic).

spiked with 500 ng ml^{-1} hydroquinone (2×LOQ) and tested for peak interferences. No significant interferences of a matrix compound peak with the hydroquinone peak could be observed.

In the course of method development selectivity for hydroquinone oxidation products was checked. Benzoquinone (t_R=17.1 min at 15% B in A) showed no significant interference with components in the urine matrix but LOQ was estimated to be about 5 μg ml^{-1} (150 mV). Therefore electrochemical detection was not considered to be suitable for very sensitive detection of benzoquinone as compared to UV detection (LOD=32 ng ml^{-1}, at 245 nm). Stability testing revealed no oxidation or degradation of hydroquinone in urine.

3.3. Limit of quantification

For LOQ a hydroquinone concentration meeting the requirements for precision and accuracy generally accepted in bioanalytical analysis was chosen [17]. As the lower limit for the quantification of hydroquinone needed to be in the 300 ng ml^{-1} range, a LOQ of a hydroquinone concentration of 250 ng ml^{-1} or 12.5 ng on-column, respectively, in urine matrix was validated. Precision at LOQ was determined as a relative standard deviation (RSD) of 2.9% accuracy was determined as 7.39% deviation from nominal value.

3.4. Calibration curve

Linear correlation of electrochemical signal and of hydroquinone concentration was shown up to 5 μg ml^{-1}. A calibration curve was prepared by spiking 490 μl urine matrix with 10 μl of various hydroquinone solutions resulting in hydroquinone concentrations of 0.25, 0.375, 0.5, 0.625, 0.75, and 1.0 μg ml^{-1}, respectively. Solutions were analyzed in duplicate at each concentration level. The calibration function was $y=23.809x+0.0974$ and a correlation coefficient of 0.9996 was calculated.

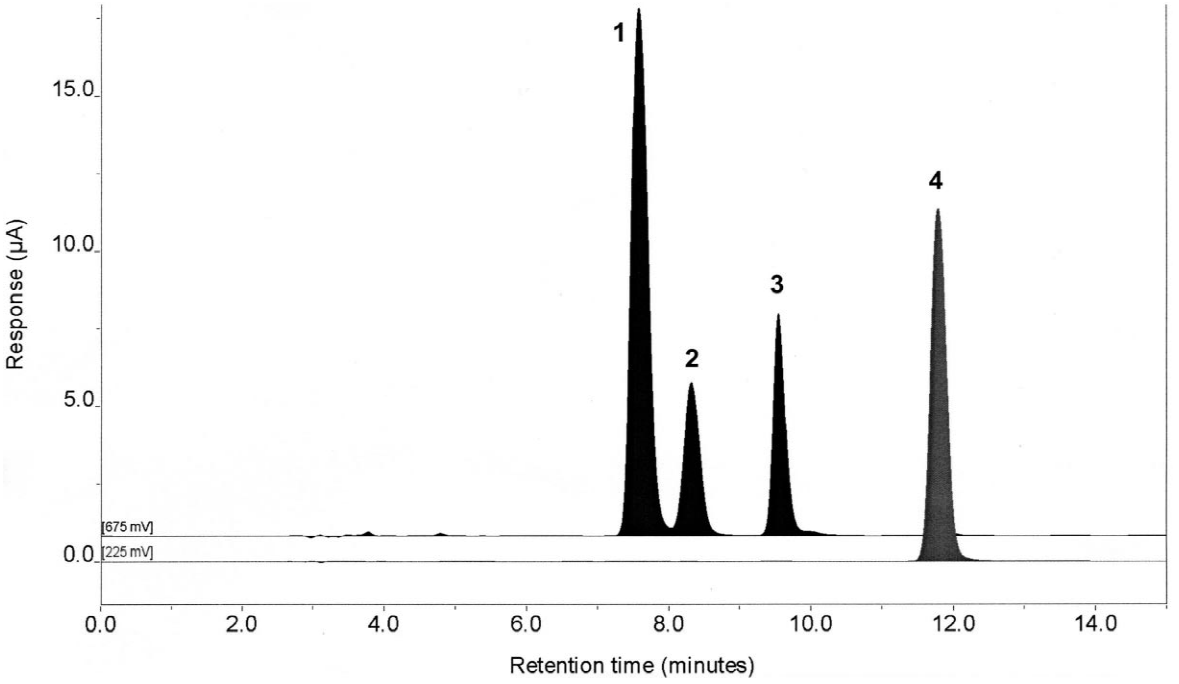

Fig. 3. Authentic chromatogram of the rat liver microsomes assay containing hydroquinone glucuronide (1) and hydroquinone (4) spiked with arbutin (2) and hydroquinone sulfate (3) (at 10% B in A, isocratic) – simultaneous electrochemical detection at 675 mV (1, 2, 3) and 225 mV (4).

3.5. Precision [17]

Precision was demonstrated by multiple injections ($n=6$) of hydroquinone solutions in urine matrix at concentration levels of 0.25, 0.5, and 0.75 μg ml^{-1}, respectively, covering the range of expected hydroquinone concentrations. Determinations were repeated twice on different days. Precision data were subdivided into within-day precision and reproducibility. In order to calculate reproducibility, data from 3 validation days had to be corrected for weighting differences. Reproducibility testing involved in addition to different days different analysts and different solvent batches. Data obtained are presented in Table 1.

3.6. Accuracy [17]

Mean area values ($n=6$) of the within-day precision at the different concentration steps were used as ''true'' values, according to the US Food and Drug Administration (FDA) Draft Guideline for Bioanalytical Methods Validation for Human Studies. ''Actual values'' of peak areas could be calculated

Table 1
Data of within-day precision (determined on 3 different days), reproducibility and accuracy

Concentration levels of hydroquinone in urine matrix (μg ml^{-1})	RSD of within-day precision ($n=6$) (%)	RSD of reproducibility ($n=18$) (%)	Accuracy as RAD of ''actual value''[a] from ''true value''[a] ($n=6$) (%)
0.25	1.2–3.2	3.8	0.75–7.39
0.50	1.1–4.0	3.3	0.14–3.95
0.75	0.9–1.6	1.7	0.08–2.10

[a] Referring to Ref. [17].

by inserting the actual hydroquinone concentration value into the calibration curve. Accuracy was described by the percentage of relative deviation of the ''actual value'' from the ''true value''. Accuracy data are presented in Table 1 and meet the requirements as proposed by the draft document of the FDA.

3.7. Quality control samples

Quality control (QC) samples of urine matrix containing 500 ng ml^{-1} hydroquinone were analyzed occasionally but at least before each daily sample sequence. Accuracy of QC samples was within ±3.77% for a 20-day period of about 200 urine assays.

3.8. Stability

Stability testing of the analyte hydroquinone was subdivided into autosampler stability and freeze and thaw stability. Autosampler stability was monitored by replicate injection of a solution of 500 ng ml^{-1} hydroquinone in both purified water and urine matrix stored at 15°C in the autosampler tray. The initial value was determined by triplicate injection. Autosampler stability of hydroquinone was demonstrated for solutions of both purified water and urine matrix over a period of 23 h. Due to the content of ascorbic acid hydroquinone in urine matrix was stable for 48 h.

Hydroquinone was stable in urine matrix during four freeze and thaw cycles (recovery 101.1–99.7%). A freeze and thaw cycle included deep freezing of the desired sample at −80°C for at least 12 h (first cycle was 24 h) and defrosting at room temperature without external heating. The initial value was determined by a triplicate injection, experimental data were collected in duplicate.

4. Discussion

A reliable and robust HPLC assay was developed for hydroquinone determination in human urine. Solvent profile for selective hydroquinone determination in the complex urine matrix was created within 2 days by computer aided method development. The efforts of the expensive and time consuming method development step have been minimized to about 10 injections instead of the quite common ''trial and error'' procedure.

The Aqua stationary phase, used for reliable separation of urine samples, is an octadecyl silane material endcapped with a hydrophilic reagent, which avoid collapsing of the C_{18} chains under polar solvent conditions and ensure a high selectivity for polar compounds. This result, compared to stationary phases with common endcapping, in excellent peak shape and reproducible resolution of polar analytes from polar matrices.

As the target compound hydroquinone is a redox sensitive compound coulometric array detection was found to be an appropriate method for selective and highly sensitive determination of the target compound in urine matrix. In terms of sensitivity electrochemical detection of hydroquinone (LOQ=250 ng ml^{-1} at 225 mV) is superior to spectroscopical methods such as fluorescence spectroscopy [8] and UV–Vis spectroscopy [15].

The channel with a maximal potential (225 mV) was used for hydroquinone determination, simultaneously eleven other electrochemical potentials were monitored online. Voltammograms obtained from the signals in the different channels were used for peak identification.

Intra- and inter-day precision of the method developed were below RSD 4.1% and accuracy of the method was determined to be below 7.5% even at the LOQ level. Therefore the method was suitable for determination of hydroquinone in urine samples in an ongoing clinical study according to GLP/GCP standards. System suitability data obtained during continuous assays over a 20-day period were within 96 to 104% of nominal hydroquinone content and demonstrated suitable robustness of the HPLC–CoulArray system.

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