ELSEVIER

Journal of Chromatography B, 761 (2001) 261–266

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Validated methods for direct determination of hydroquinone glucuronide and sulfate in human urine after oral intake of bearberry leaf extract by capillary zone electrophoresis

Ingmar Glöckl[a,b], Gottfried Blaschke[b], Markus Veit[c,*]

[a]*Forschungsvereinigung der Arzneimittel-Hersteller e.V., Kranzweiherweg 10, D-53489 Sinzig, Germany*
[b]*Institute of Pharmaceutical Chemistry, University of Münster, Hittorfstrasse 58–62, D-48149 Münster, Germany*
[c]*Zentralinstitut Arzneimittelforschung GmbH, Kranzweiherweg 10, D-53489 Sinzig, Germany*[1]

Received 23 January 2001; received in revised form 22 June 2001; accepted 10 July 2001

Abstract

Bearberry leaf extracts are used in herbal medicinal products for the treatment of lower urinary tract infections. Two metabolites of the major phenolic constituent in the extract, arbutin (hydroquinone-1-*O*-β-D-glucoside), must be assumed to be precursors of the active disinfectant principle hydroquinone. In order to assay the renal elimination of these two metabolites, i.e., hydroquinone conjugates with glucuronic and sulfuric acid, two separate capillary electrophoresis methods have been developed. Both methods were validated according to the criteria for validation of pharmaceutical bioanalytical methods as drafted by the US Department of Health and Human Services, 1998. As there is little sample preparation necessary, both methods are very suitable for urine analysis with large sample numbers as frequently coming up in the course of pharmaceutical bioavailability, bioequivalence and pharmacokinetic studies. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Hydroquinone glucuronide; Hydroquinone sulfate

1. Introduction

Herbal medicinal products (HMPs) containing extracts from bearberry leaves [*Arctostaphylos uva-ursi* (L.) Sprengel] are used as disinfectants in therapy of lower urinary tract infections. As active principle, the constituent of interest is arbutin (hydroquinone-1-*O*-β-D-glucoside; Fig. 1). After oral administration of HMPs containing bearberry leaf

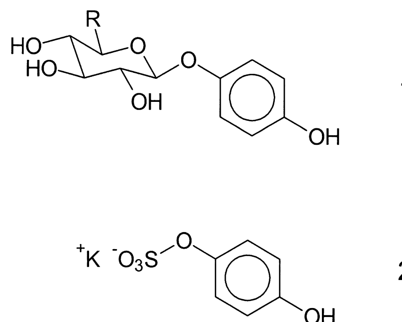

Fig. 1. Formula of arbutin (1, R=CH_2OH) and hydroquinone glucuronide (1, R=COOH) and hydroquinone sulfate potassium salt (2).

*Corresponding author. Tel.: +49-2642-9837-31; fax: +49-2642-9837-40.

E-mail address: markus.veit@za-sinzig.de (M. Veit).

[1]Address where the work was carried out.

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(01)00322-X

extracts no genuine arbutin was renally excreted but conjugates of hydroquinone with glucuronic and sulfuric acid were found as main urinary metabolites [1,2]. Both metabolites seem to be precursors of free hydroquinone liberated in the lower urinary tract. This hydrolysis may be catalyzed by human or bacterial enzymes but is described to occur also non-enzymatically. Free hydroquinone is discussed to be responsible for efficacy of HMPs containing bearberry leaf extracts. In previous studies on renal excretion of hydroquinone conjugates these compounds have only been assayed after enzymatic hydrolysis but no method for direct determination of conjugates with discrimination to free hydroquinone in urine has been published yet, to the best of our knowledge. Chromatographic methods frequently failed to separate the very polar analytes. Therefore the aim was applying a reliable capillary electrophoresis (CE) method for separation which should avoid any sample preparations in order to be suitable for urine analysis with large sample numbers as frequently coming up in the course of pharmaceutical bioavailability, bioequivalence and pharmacokinetic studies.

2. Experimental

2.1. Materials

Sodium tetraborate, sodium benzoate, 2-hydroxyethyl-salicylate, hydroquinone, magnesium chloride and Tris-buffer were obtained from Sigma–Aldrich (Steinheim, Germany). Ascorbic acid and sodium hydroxide were from Merck (Darmstadt, Germany). Acetonitrile, acetone and acetic acid were from Roth (Karlsruhe, Germany) and hydrochloric acid (1 mol l^{-1}) from Riedel-de Haen (Seelze, Germany). All reagents were of analytical-reagent grade. Aqueous solutions were prepared in deionized water.

Uridine-5-diphospho-glucuronic acid (UDP-GA) and β-glucuronidase (EC 3.2.1.31) were from Sigma–Aldrich.

Hydroquinone monosulfate potassium salt were purchased from Chemos (Regenstauf, Germany). Hydroquinone-1-β-D-*O*-glucoside (arbutin) was isolated from bearberry leaves. For bioavailability studies a preparation of a commercial bearberry leaf extract (starting material for Arctuvan™ film tablets) was used.

Identity of reference compounds arbutin and hydroquinone monosulfate potassium salt was proven by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Purity assignment was carried out by means of quantitative NMR [3]. ^{1}H-NMR spectra were recorded with a Bruker AMX300 spectrometer operating at a basic frequency of 300 MHz, for quantitation using solvents signals as reference [CD_3OD, $\delta=3.31$ ppm, for arbutin and dimethylsulfoxide (DMSO), $\delta=2.49$ ppm, for hydroquinone sulfate] for intercalibration with etacrynic acid CRS (Chemical Reference Substance of the European Pharmacopoeia) used as standard in the respective solvents.

2.2. Sample preparation

Urine collection in studies was performed at predefined sampling intervals after administration of bearberry leaf extract formulations. For method development and validation urine was collected at about 4 h after oral intake of a formulation containing bearberry leaf extract, assigned as approximate t_{max} for urine excretion of the two target analytes. The urine samples were stabilized with ascorbic acid provided in the sampling flasks yielding concentrations of about 0.5% (m/v) depending on amount of urine collected. The samples were either analyzed immediately or stored frozen at −20°C prior to analysis.

Turbid samples due to protein precipitation were centrifuged at 11 500 *g* (UEC Micro 14/B; Uni-Equip, Martinsried, Germany) for 5 min. No further sample preparation was necessary.

2.3. Instrumentation and method description

Method development and assays were carried out on a Bio-Rad Biofocus 3000 system (Munich, Germany). The system was equipped with a fast scanning UV detector and liquid cooling system for the capillary. Autosampler and capillary were thermostated at 20°C. Detection wavelength for both meth-

ods was 200 nm. Samples were injected by pressure (4 p.s.i. s; 1 p.s.i. = 8694.76 Pa). Length of bare fused-silica capillaries were 50 cm (45.4 cm to detector), capillary I.D.s were 50 μm. Prior to analysis capillary was preconditioned once for 30 min with 0.1 mol l^{-1} sodium hydroxide. Between each run capillaries were rinsed for 5 min with 0.1 mol l^{-1} sodium hydroxide and for 2 min with running buffer.

Hydroquinone glucuronide was separated in capillaries purchased from BGB (Schlossböckelheim, Germany) with 25 kV voltage. As running buffer 400 mmol l^{-1} borate was used, adjusted with sodium hydroxide to pH 9.80. 2-Hydroxyethyl-salicylate used as internal standard at a concentration of about 50 μg ml^{-1}.

Hydroquinone sulfate was separated in capillaries purchased from and Agilent Technologies (Waldbronn, Germany). Detection was performed with a 150 μm bubble cell detection window. Applied voltage was 30 kV. 200 mmol l^{-1} borate (pH 9.35) was used as separation buffer. The concentration of sodium benzoate used as internal standard was 100 μg ml^{-1}. pH adjustment of buffer solutions was performed with a pH meter; pH 540 GLP (WTW; Weilheim, Germany).

2.4. Preparation of microsomes

Microsomes were obtained from rat livers at the University of Münster (the protocol for animal experiments was approved and permit was issued by Bezirksregierung Münster, Az. 23.0835.1.0, G1/98, 17.01.00).

Preparation was performed in a buffer containing 0.25 mol l^{-1} D-(+)-sucrose, 5 mmol l^{-1} Tris–HCl and 0.5 mmol l^{-1} EDTA at pH 7.4. After homogenization of rat livers tissue using Potter–Elvehjem homogenizer the suspensions were diluted to 38% (m/v) with buffer and centrifuged for 15 min at 10 000 *g*. The supernatant was removed, stored in ice and replaced by fresh buffer. The renewed suspension was again centrifuged for 15 min at 10 000 *g*. After ultra centrifugation of the combined supernatant fractions for 90 min at 100 000 *g* the sediment pellet (microsomes) were resuspended in buffer and frozen at −20°C prior to incubation experiments [4].

2.5. Preparation of hydroquinone glucuronide

As hydroquinone glucuronide was not commercially available as a reference substance it was obtained by means of enzymatic synthesis from hydroquinone with rat liver microsomes.

A 3.57-mg amount of hydroquinone was dissolved in 25 ml of a 50 mmol l^{-1} Tris–5 mmol l^{-1} $MgCl_2$ buffer, adjusted to pH 7.80 using HCl. A 31.5-mg amount of UDP-GA was dissolved in 5 ml of the buffer. A 700-μl volume of the hydroquinone solution and 100 μl of the UDP-GA solution were mixed with 200 μl of suspension of microsomes and incubated for 4 h at 37°C under a nitrogen atmosphere [5]. The reaction was stopped and microsomes were precipitated with acetonitrile prior to centrifugation. Acetonitrile in the collected supernatant was removed under reduced pressure. The identity of the hydroquinone glucuronide in the resulting solution was confirmed by comparison of CE separations before and after enzymatic cleavage of reaction product hydroquinone glucuronide with β-glucuronidase to hydroquinone (see Section 2.6). About 4 h after oral administration of a preparation containing bearberry leaf extract, the hydroquinone glucuronide peak was assigned in volunteer urine by spiking the sample with hydroquinone glucuronide containing solution obtained from the rat liver microsomes. Comigration revealed identity of the hydroquinone glucuronide peak in volunteer urine.

2.6. Enzymatic cleavage of hydroquinone glucuronide

A 10-μl volume of 1% (m/v) ascorbic acid, 70 μl of 0.58 mol l^{-1} acetic acid and 30 μl of β-glucuronidase were added to 500 μl of the solution of hydroquinone glucuronide obtained from the rat liver microsomes (see Section 2.5). The reaction was stopped after 1 h at 37°C by addition of 600 μl acetone. The supernatant was used for further CE analysis after centrifugation.

2.7. Method development

As both target analytes were ionic compounds and separation by high-performance liquid chromatography (HPLC) failed CE appeared to be a suitable

separation technique. In urine matrix however it was not possible to separate hydroquinone glucuronide and hydroquinone sulfate in a single run due to comigration of genuine urine compounds with target analytes. For this reason two different methods were developed, applying a borate buffer system.

In order to improve the separation of target analytes from interfering genuine substances in human urine, pH and ionic strength were optimized for separation. Increased concentration of borate improved peak shape and separation of analytes, despite decreased borate concentration resulted in shorter run times. Higher pH resulted in increased electroosmotic flow and therefore decreased run times. By varying these parameters optimum conditions were found as follows: for hydroquinone glucuronide at pH 9.80 and 400 m*M* borate with 25 kV, 20°C thermostated. For hydroquinone sulfate at pH 9.35 and 200 m*M* borate with 30 kV, 20°C thermostated.

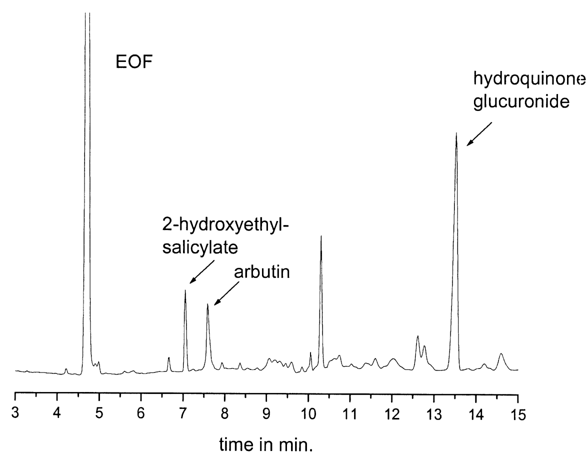

Fig. 2. Exemplary electropherogram of hydroquinone glucuronide assay in urine, after administration of bearberry leaf extract, spiked with internal standard 2-hydroxyethyl-salicylate and arbutin for calibration. Method: 400 mmol l^{-1} borate, pH 9.80; 25 kV; injection, 4 p.s.i. s; detection at 200 nm; temperature, 20°C; capillary, 50 cm (45.4 cm to detector)×50 μm I.D.

2.8. Quantitation

For quantitation peak areas normalized according to the migration times were used.

3. Results and discussion

3.1. Assay of hydroquinone glucuronide

Hydroquinone glucuronide was synthesized for peak identification purposes but was not available in sufficient amounts for calibration. Therefore the assay in human urine was carried out using calibration data of hydroquinone glucoside (arbutin). The exemplary electropherogram of the analysis of hydroquinone glucuronide in human volunteer urine after administration of a bearberry leaf extract is shown in Fig. 2.

3.1.1. Specificity

Specificity of the method could be demonstrated by comparison of electropherograms of blank samples from six volunteers (under ambient diet and no intake of arbutin) with electropherograms of respective urine spiked with internal standard and hydroquinone glucuronide and electropherograms of urine samples from the same volunteers after oral administration of a preparation containing bearberry leaf extract.

3.1.2. Linearity

Linearity was demonstrated in a concentration range of 36.73 to 1836.55 μmol l^{-1} arbutin (10 to 500 μg ml^{-1}). Samples spiked with arbutin at nine concentration levels containing 2-hydroxyethyl-salicylate (50 μg ml^{-1}) as internal standard were analyzed in duplicate. Resulting ratios of corrected peak areas were evaluated by linear regression analysis ($R^2=0.9972$, $y=0.0138x+0.1404$).

3.1.3. Limit of quantitation (LOQ)

The LOQ was set to 73.46 μmol l^{-1} (20 μg ml^{-1}) corresponding to the spiked sample with the lowest hydroquinone glucoside concentration meeting the acceptance criteria for bioanalytical methods validation (precision and accuracy ≤15% over the working range, ≤20% at LOQ [5]).

3.1.4. Precision and accuracy

Precision and accuracy were determined on 3 different days. On each day precision and accuracy were calculated at different concentration levels of arbutin: the lowest (73.46 μmol l^{-1}; 20 μg ml^{-1}), a

median (367.31 μmol l^{-1}; 100 μg ml^{-1}) and at the highest (1836.55 μmol l^{-1}; 500 μg ml^{-1}) of the specified working range. The precision of the method was characterized as the relative standard deviation (RSD) of obtained mean values ($n=6$ at each concentration level). The precision was determined as 1.93 to 5.16% for the highest, as 1.77 to 4.91% for the median and as 2.82 to 3.85% for the lowest concentration level representing the LOQ. Accuracy was calculated as +2.46 to +4.26% for the highest, +0.76 to +2.48% for median and as +2.41 to +4.09% for the lowest concentration level representing the LOQ.

3.1.5. Stability

Stability tests of hydroquinone glucuronide in urine samples during thaw–freeze cycles as well as of hydroquinone sulfate, hydroquinone glucoside and internal standards during autosampler storage were established. No degradation of target analytes could be observed.

3.2. Hydroquinone sulfate

The exemplary electropherogram of the analysis of hydroquinone sulfate in human volunteer urine after administration of a bearberry leaf extract is shown in Fig. 3.

3.2.1. Specificity

Specificity of the method could be demonstrated in analogy to Section 3.1.1.

3.2.2. Linearity

Linearity was demonstrated in the concentration range of 43.81 to 2190.48 μmol l^{-1} hydroquinone sulfate potassium salt (10 to 500 μg ml^{-1}). Samples spiked with hydroquinone sulfate (potassium salt) at six concentration levels containing 100 μg ml^{-1} sodium benzoate as internal standard were analyzed in duplicate. Resulting ratios of corrected peak areas were evaluated by linear regression analysis ($R^2=1$, $y=0.0046x+0.0257$).

3.2.3. LOQ

The LOQ was set to 87.62 μmol l^{-1} (20 μg ml^{-1}) corresponding to the spiked sample with the lowest hydroquinone sulfate concentration meeting the acceptance criteria for bioanalytical methods validation (precision and accuracy ≤15% over the working range, ≤20% at LOQ [5]).

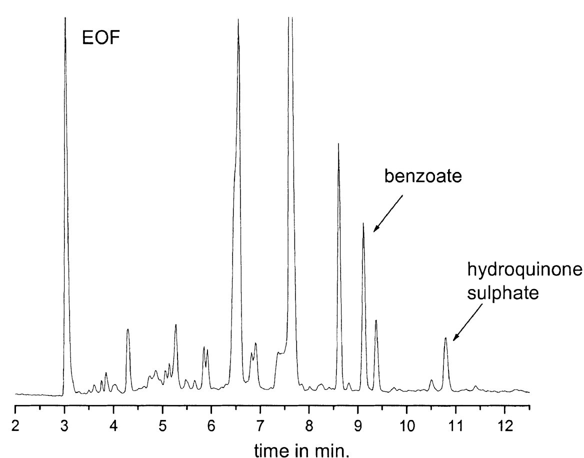

Fig. 3. Exemplary electropherogram of hydroquinone sulfate assay in urine after administration of bearberry leaf extract, spiked with benzoate as internal standard. Method: 200 mmol l^{-1} borate (pH 9.35); 30 kV; injection, 4 p.s.i. s; detection at 200 nm; temperature, 20°C; capillary, 50 cm (45.4 cm to detector)×50 μm I.D. (at detector 150 μm).

3.2.4. Precision and accuracy

Precision and accuracy were determined on 3 different days. On each day precision and accuracy were calculated at different concentration levels: the lowest (87.62 μmol l^{-1}), a median (438.10 μmol l^{-1}; 100 μg ml^{-1}) and at the highest (2190.48 μmol l^{-1}; 500 μg ml^{-1}) of the specified working range. The precision of the method was characterized as the RSD of obtained mean values ($n=6$ at each concentration). The precision was determined as 1.87 to 4.52% for the highest, as 4.94 to 7.98% for the median and as 6.83 to 11.37% for the lowest concentration level representing the LOQ.

Accuracy was calculated as −1.87 to +4.80% for the highest, −5.00 to +5.07% for median and as −6.47 to +14.91% for the lowest concentration level representing the LOQ.

3.2.5. Stability

Stability tests of hydroquinone sulfate in urine samples during thaw–freeze cycles as well as of hydroquinone sulfate and internal standard during

autosampler storage were established. No degradation of target analytes could be observed.

4. Determination of metabolites after oral administration of bearberry leaf extracts (film tablets) in human urine

As an example the determination of hydroquinone glucuronide and hydroquinone sulfate in urine of one volunteer is demonstrated. In the sampling interval 0–4 h 869 ml urine were collected after oral-intake of a single dose of a preparation containing 945 mg bear berry leaf extract (with 210 mg arbutin). Concentration of excreted hydroquinone glucuronide was determined as 224.5 μmol l^{-1} using calibration data of arbutin. Concentration of excreted hydroquinone sulfate were determined as 182.06 μmol l^{-1}. This is about half of the dose of the administered arbutin excreted as metabolites after 4 h. For sample electropherograms see Figs. 2 and 3.

5. Conclusions

CE has been shown to be a very useful separation technique for hydrophilic conjugates in bioanalysis of urine. A direct determination of the arbutin metabolites hydroquinone glucuronide and hydroquinone sulfate in human urine with two different methods was established. Both methods were validated according to international regulatory requirements [6]. Both methods were found to be appropriate for its intended use in bioavailability studies with herbal medicinal products containing bearberry leaf extracts. The respective results will be published elsewhere [7].

Acknowledgements

The financial support by ‘‘Stiftung Rheinland-Pfalz für Innovation’’ is gratefully acknowledged.

References

[1] C.P. Siegers, J.P. Siegers, R. Pentz, C. Bodinet, J. Freudenstein, Pharm. Pharmacol. Lett. 7 (1997) 90.
[2] D.H. Paper, J. Kohler, G. Franz, Pharmacol. Lett. 3 (1993) 63.
[3] G.F. Pauli, Phytochem. Anal. 11/12 (2000) 28.
[4] J. Rudolf, G. Blaschke, Enantiomer 4 (1999) 317.
[5] S. Michels, Ph.D. Thesis, University of Münster, Münster, 2000.
[6] Guidance (Draft) for Industry: Bioanalytical Methods Validation for Human Studies, US Department of Health and Human Servives, Rockville, MD, 1998, http://www.fda.gov/cder/guidance/2578dft.pdf.
[7] B. Brinkhaus, U. Patzak, G. Schindler, A. von Nieciecki, J. Wittig, I. Gloeckl, M. Veit, J. Clin. Pharmacol. (2001) submitted for publication.