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Selective extraction of quercetrin in vegetable drugs and urine by off-line coupling of boronic acid affinity chromatography and high-performance liquid chromatography

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Abstract

Quercetrin, quercetin and chlorogenic acid were measured in urine or in drugs by combination of boronic acid affinity chromatography and HPLC. Simple reversed-phase HPLC with UV detection was used to determine quercetrin in five different *Solidago virgaurea* drugs. For determination of quercetrin in human urine immobilized boronic acid was applied for sample pretreatment. This procedure leads to a determination limit of 0.01 μg/ml with a recovery rate of 95.3%. The first results using this method for quercetrin pharmacokinetics are presented.

1. Introduction

Quercetrin and its aglycon quercetin are ubiquitously occurring flavonoids in the plant world and a common component in human diet [1]. They are effective components of some widely spread medicinal plants like *Solidago virgaurea*, *Ginkgo biloba* [2] or *Calendula officinalis* [3]. *Solidago virgaurea* has been used in urology since ages because of its diuretic, spasmolytic and antiphlogistic properties [2]. Quercetin itself is discussed to be anticarcinogenic [4], antiviral [5], antiulceric [6] and mutagenic [7,8]. Fig. 1 shows the structural formulas of quercetrin, quercetin, rutin, chlorogenic acid, caffeic acid, khellin and visnagin.

Different methods as HPLC [1,3,9], MS [10], GC–MS [11], capillary electrophoresis [12], LC–MS [13] and affinity chromatography [14] are used for flavonoid analysis. These methods cannot be used for either quantitative quercetrin–quercetin determination or for the analysis of urine samples.

Busch et al. chose the mutagenic activity of quercetrin for recovery experiments of quercetin in urine using the Ames-test with *Salmonella typhimurium* strains [15]. In spite of the extensive analytic procedure, recovery of quercetin was not better than 29–79%. Gugler and Dengler described a fluorometric method for determination of quercetin in urine and plasma [16]. This method, using tetraphenyldiboroxide as chelating reagent needs extensive liquid extraction techniques and leads to a recovery rate of about 80%.

Phenylboronic acid at higher pH has a strong

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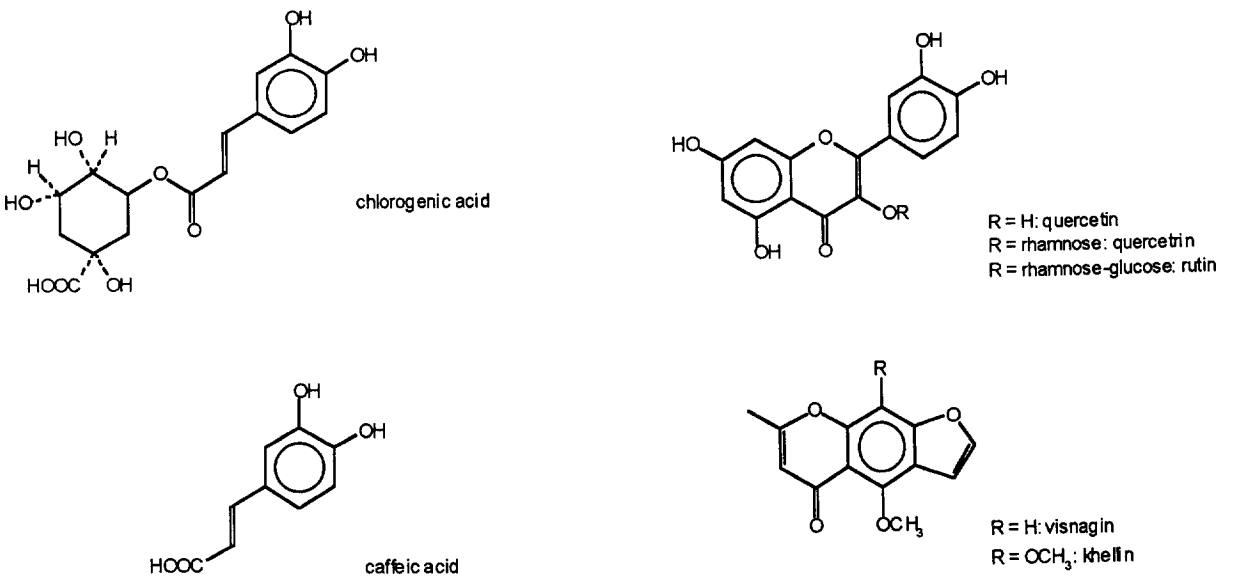

Fig. 1. Structural formulas of quercetrin, quercetin, rutin, chlorogenic acid, caffeic acid, khellin and visnagin.

affinity to vicinal 1,2-diol groups. This affinity is used for separation of flavonoids from plants [14] and in standard methods for determination of catecholamines in urine and serum [17,18].

In view of the pharmacokinetic properties of quercetrin we combined affinity chromatography and HPLC to an effective analytic method.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile of HPLC-grade were obtained from J.T. Baker (Deventer, Netherlands). Hydrochloric acid (25%, p.a.), disodiumhydrogenphosphate (Na_2HPO_4, p.a.), sodium azide (NaN_3, p.a.), phosphoric acid Suprapur (85%), sodium hydroxide Suprapur (30%) and sodium hydroxide pellets p.a. were purchased from Merck, Germany. Affinity chromatography gel Affi-Gel 601 was manufactured by BIO-RAD (Richmond, CA, USA). Water of HPLC-grade was taken from a Millipore Milli-Q plus ultra-pure water system and filtered through a 0.2-mm filter (Schleicher & Schüll, Germany). Quercetrin dihydrate, quercetin dihydrate, khellin and chlorogenic acid were obtained from Roth, Karlsruhe, Germany. Caffeic acid was purchased from Fluka (Buchs, Switzerland). Visnagin was obtained from Aldrich, Germany.

Urol-S, Urol-mono and solidago extracts were kindly provided by Hoyer (Neuss, Germany). Urodyn film tablets and Urodyn drops were kindly provided by Bionorica (Neumarkt, Germany). Solidagoren-N drops were produced by Klein (Zell-Harmersbach, Germany).

2.2. Solidago drugs

We included five solidago drugs in our investigation. Two of them (Urol-S and Urol-mono) were capsules which contain several oils and waxes, sugars, glycerol, and iron oxide as major components among the extracts. Urol-mono contains only solidago, whereas Urol-S also contains *Ammi visnaga* and *Taraxacum officinale* extracts. Urodyn drops and Solidagoren-N are ethanolic solutions of solidago extracts. Urodyn drops contain only mint oil and saccharin as additional components, whereas Solidagoren-N contains potentilla and *Equisetum arvense* extracts in addition to solidago extracts. Finally, the film tablet form of Urodyn contains major components similar to Urol-mono.

Urol-S, Urol-mono, Urodyn film tablets,

Urodyn drops and Solidagoren-N drops subsequently will be referred to as drug 1, drug 2, drug 3, drug 4 and drug 5, respectively.

2.3. Sample preparation

Urine

Spontaneous urine (60 ml) of a healthy male subject was adjusted to a pH 8.5 with NaOH. For the determination of the recovery rate and determination limit, different samples of this urine were spiked to quercetrin levels of 0.01 μg/ml, 1.13 mg/ml and 1.5 μg/ml. The urine was then filtered through a folded filter (Schleicher & Schüll, Germany) into a 50-ml measuring flask.

Solidago virgaurea extracts

About 10 mg extract was weighed into a 50-ml measuring flask filled with water–methanol (80:20, v/v). The solution was stirred for 5 min and then filtered through a 0.2-mm cellulose acetate filter (Sartorius, Germany). The solution was diluted 10 to 20 fold with eluent A.

Drug 5 and drug 4 drops

An amount of 500 μl was dissolved in 50 ml water–methanol (80:20, v/v). The solution was diluted 500 to 5000 fold and directly used for HPLC analysis.

Drug 3 tablets

A tablet was weighed into a 50-ml measuring flask filled with water–methanol (80:20, v/v) and the solution stirred for 2 h. The cloudy liquid was filtered through a 0.2-mm cellulose acetate filter (Sartorius) and diluted 1:500 for HPLC analysis.

Drug 1 and drug 2 capsules

Capsules were shaken on a vortex-mixer for about 10 s. Then the capsule was cut open lengthwise with a scalpel. A slide was put on a balance. About 100 mg of the combined content of five capsules was transferred to the slide with a 1-ml pipette equipped with a cutoff pipette point. The slide was transferred into a 50-ml beaker filled with about 20–30 ml water–methanol (80:20, v/v). It was stirred for 10 min. The beaker content was added to a 50-ml measuring flask and filled to 50 ml with water–methanol. The liquid was filtered through a 0.2-mm cellulose acetate filter (Sartorius) and diluted 1:3 for the determination of quercetin and 1:30 for the determination of quercetrin.

2.4. High-performance liquid chromatography

The chromatographic system consists of two Knauer HPLC pumps Model 64 connected to a Knauer dynamic mixing chamber and a Rheodyne 7125 sample injector with a 20-μl sample loop (Knauer, Berlin, Germany). The sample loop was filled with a SGE 500-μl syringe (Alltech, Germany). For UV detection, a Spectra Physics UV-2000 detector is used (Thermo Separation Group, Germany). Absorbance was measured at 254 nm and 0.002–0.0005 AUFS. Data were collected on a PC running Knauer HPLC-software version 2.1a. Substances were quantified via peak area and external calibration curves, which were performed every day.

A 250 × 4.6 mm I.D. steel column filled with Eurospher 100 C_{18} (5 μm) (Knauer) was used for reversed-phase chromatography. Either gradient or isocratic elution was applied. Eluent A was methanol–water–85% phosphoric acid (450:550:2, v/v/v) and eluent B was acetonitrile–water–methanol (400:100:100, v/v/v). Isocratic elution was carried out with 90% eluent A and 10% eluent B at a flow-rate of 1 ml/min. For gradient elution (1 ml/min) eluent A was changed from 90% to 60% in 10 min, then left unchanged for 2 min and finally taken back to 90% in 3 min. Urine extracts, solidago extracts and drugs 2–5 were measured with isocratic, and drug 1 with gradient elution. Eluents were degassed with helium.

2.5. Affinity chromatography

An amount of 275 mg BIO-RAD Affi-Gel 601 was filled in a 100 × 10 mm BIO-RAD glassy column equipped with a polyethylene 2-way tap. The gel was equilibrated for 24 h in 0.1 *M* phosphate buffer adjusted to pH 8.5. For preventing bacteria proliferation the buffer was conserved with 0.01 *M* NaN_3.

For separating quercetrin, 50 ml prepared urine was rinsed through the column with a Braun perfusor Model VI at a flow-rate of 30 ml/h. The column was then washed with phosphate buffer (0.1 *M*, pH 8.5) containing 20% (v/v) methanol for 2 h at the mentioned flow-rate. For eluting quercetrin a water–methanol solution (80:20, v/v) was prepared and adjusted to pH 2 with 25% HCl p.a. Elution was carried out with the Braun perfusor at a flow-rate of 3 ml/h into a 50-ml measuring flask. The eluate was used for HPLC analysis without further treatment. Elution was done at night immediately before HPLC analysis to prevent deglycolization of the flavonoid.

3. Results and discussion

Solidago virgaurea extracts are used in a multitude of drugs. Quercetrin, rutin and chlorogenic acid are assumed components of these extracts. We could find these compounds in ethanolic solidago extracts; in addition we could also identify caffeic acid in these extracts. All substances were identified by retention time, spiking and by their UV spectra, which were taken during the run.

Fig. 2 shows a typical chromatogram of a solidago extract. The chromatogram shows that quercetrin and chlorogenic acid are the major components of solidago extracts. We examined solidago extracts from different seasons and found different levels of quercetrin and chlorogenic acid throughout the year.

The content of quercetrin in the extracts varied from 2.5% to 4.6%; 1.4 to 2.6% of the whole extract contains chlorogenic acid. Of the flavonoid content, which is certified by the manufacturer (photometrically determined), about 40% is found to be due to quercetrin. There is no clear correlation between quercetrin or chlorogenic acid content and season. In contrast, extracts with a higher quercetrin content also seem

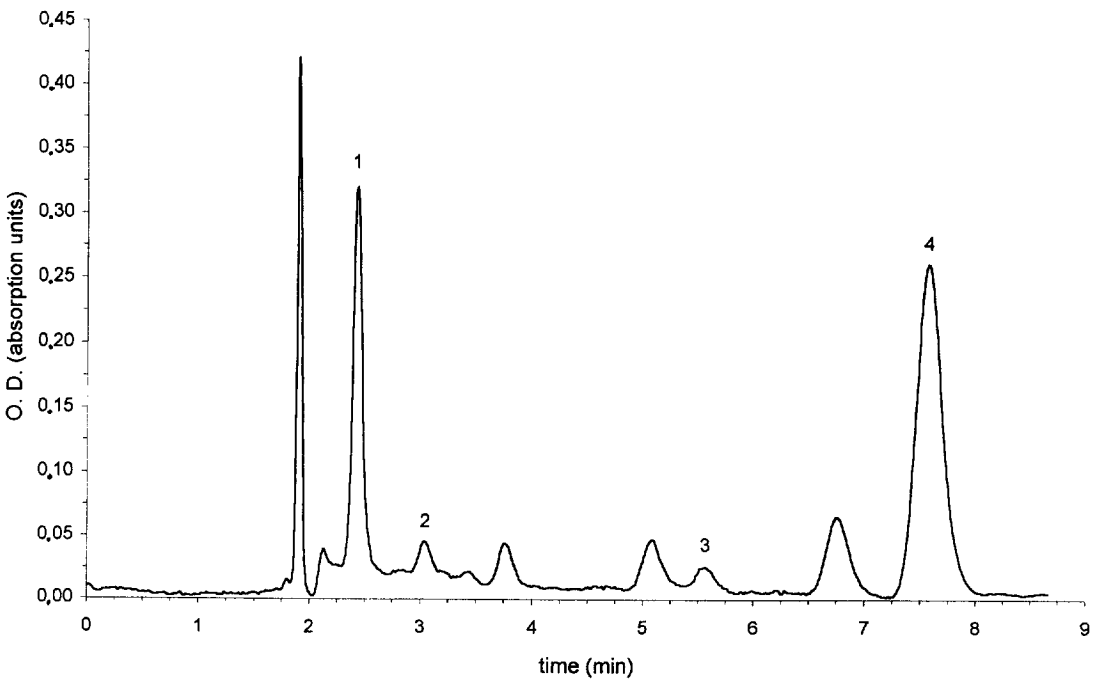

Fig. 2. Chromatogram of an ethanolic *Solidago virgaurea* extract (12.2 μg/ml extract). 1 = Chlorogenic acid; 2 = caffeic acid; 3 = rutin; 4 = quercetrin.

Table 1
Quercetrin content and (recommended) daily quercetrin intake of five commercial drugs

Drug No.	Quercetrin content	Recommended intake	Daily quercetrin intake (mg)
1	3.6 mg/capsule	6 capsules	21.6
2	13.2 mg/capsule	6–8 capsules	79.2–105.6
3	16.4 mg/tablet	6 tablets	98.4
4	2.5 mg/ml	200 drops (7.2 ml)	18
5	2.1 mg/ml	90 drops (2.6 ml)	5.5

to have a higher chlorogenic acid content (ratio about 1.8:1).

As solidago extracts are often used in vegetable drugs we examined some of these drugs to obtain basic data for further pharmaceutical studies. We determined the quercetrin content of all five drugs. The results and the daily quercetrin intake (calculated from the recommended drug intake and the quercetrin content) are presented in Table 1. Drugs containing dried solidago extract have a 2 to 5 fold higher quercetrin content than drugs composed of liquid extracts. We determined the quercetrin content of drug 5 to be four times higher than was declared by the manufacturer.

The chromatograms of drugs 2–5 are similar to the pure solidago extract and very similar to each other. Fig. 3 shows a typical chromatogram of drugs 2–5. The gradient elution applied for drug 1 made it possible to determine (baseline resolved) the quercetin content of drug 1 (retention time 9 min) as well as the furochromones khellin (retention time 11 min 40 s) and visnagin content (retention time 14 min) from *Ammi*

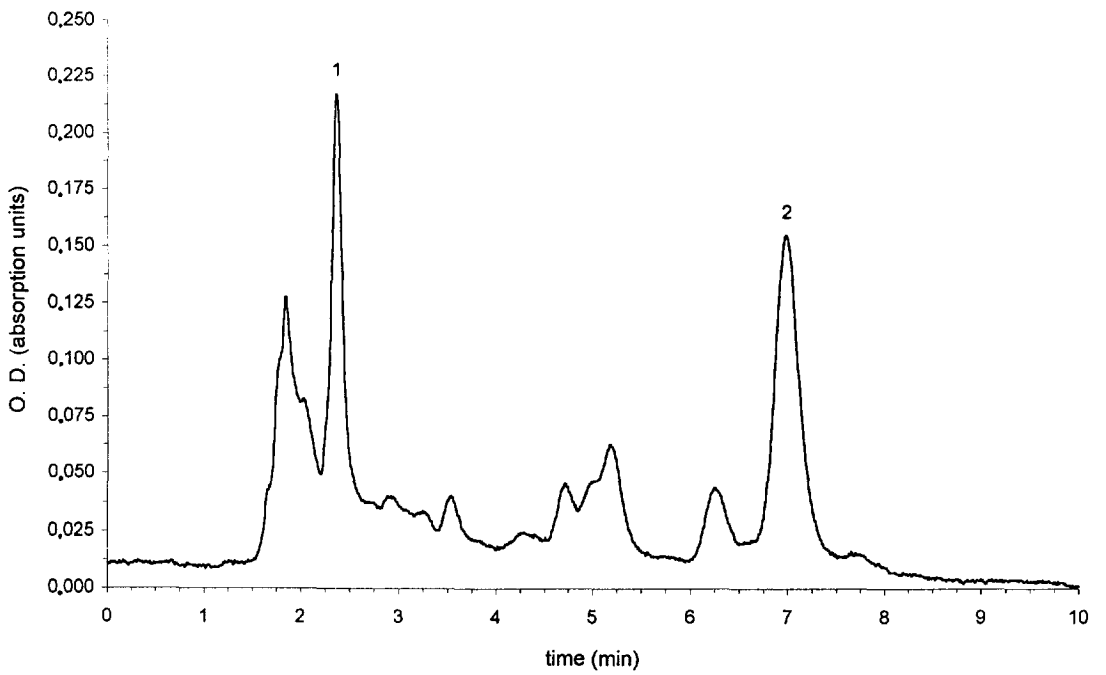

Fig. 3. Chromatogram of drug 2 capsule content (35.1 μg/ml). 1 = Chlorogenic acid; 2 = quercetrin.

visnaga. Because of the high vasodilatoric activity of khellin and visnagin, this gradient chromatographic system may be of interest for quality control of khella drugs, particularly because other HPLC applications lack separation speed [19] or need extensive sample preparation [20].

For the precise determination of quercetrin in urine we developed the above-described method for separating quercetrin from other urinary components using affinity chromatography with immobilized phenylboronic acid. In preliminary investigations we found common solid-phase extraction on reversed-phase silica gel or XAD gel not satisfactory. Either recovery was poor (silica gel) or separation was too unspecific (XAD).

Although among Affi-Gel 601 other immobilized phenylboronic acid derivatives on silica gel basis are available and used for catecholamine analysis [18,21,22] we chose Affi-Gel 601, as because of the polyacryl backbone the stability at the desired pH value is higher. We found the affinity properties of the system Affi-Gel/flavonoids to be different from the catecholamine/silica gel system. Fast and strong binding of quercetrin and quercetin appears at a pH of 8.5 and a flow-rate of 30 ml/h. Higher flow-rates during the absorption or pH values of 8 or lower as described by Elliger and Rabin [14], who used acrylamide gel for separation but not for quantification of flavonoids, result in poor recovery rates of about 50–70%.

For the quantitative splitting of the flavonoid–boronic acid binding we used ordinary HCl adjusted to a pH of 2 containing 20% (v/v) methanol. Using Suprapur HCl as eluent considerably decreases the broad injection peaks in Fig. 4. Since the separation is still good enough, there is no point in using HCl of higher purity. The methanol in the washing buffer and the eluent reduces nonspecific adsorption on the acrylamide gel [14]. Again, the use of 0.5 *M* acetic acid eluent with a pH of 3 as described by Elliger and Rabin results in poor recovery rates (probably caused by too large elution volumes).

Sample preparation on Affi-Gel 601 and following HPLC results in the typical chromatogram are shown in Fig. 4. Quercetrin is eluted baseline resolved at 6:50. Peak identity was checked by retention time, spiking and UV spectra (taken during the run); chromatograms taken at 254 nm and 355 nm have a constant ratio at peak elution time indicating peak purity. No quercetin was found.

The determination limit of the HPLC system (five times standard deviation of baseline noise) was 0.01 μg/ml (254 nm, 0.0005 AUFS). The peak area increases linear with the amount of quercetrin in the range of from 0.01 μg/ml to 1.5 μg/ml. No significant change of slope or intercept was observed from day to day.

The recovery rate (mean of 10 samples) for the combined method (extraction and HPLC determination) was 95.3% (C.V. = 8.9%) at a quercetrin level of 0.01 μg/ml, 97.8% (C.V. = 3.3) at a quercetrin level of 1.13 μg/ml and 97.1% (C.V. = 2.4) at a quercetrin level of 1.5 μg/ml (sample to sample precision). The recovery rate of the 15th sample prepared on a single column was not significantly lower than the mean recovery rate of the first ten samples, so at least ten urine samples could be prepared on one column.

Extraction of a urine sample spiked to a quercetrin content of 1.13 μg/ml on 10 newly prepared affinity columns leads to a mean recovery rate of 97.3% with a C.V. of 4.4% (column to column precision).

Baba et al. [23], who applied TLC and GC–MS for analysis, could not find unchanged rutin or quercetin in urine after oral administration of rutin. It was interesting for us to determine if quercetrin could be found after drug intake. Eight healthy male subjects under standardized nutrition took six capsules of drug 2 for a period of four days and urine was collected during the next 24 h period. The resulting chromatogram of a 24 h urine sample is shown in Fig. 4. The quercetrin content of this sample was 0.031 mg/ml. With a urine volume of 1900 ml this is a 24 h excretion of 58.9 mg, corresponding to about 1/1000 of the amount taken in.

Although the presented method is sufficient for our special requirement, in view of other applications using boronic acid affinity chromatography the further automation of the sample preparation should be kept in mind.

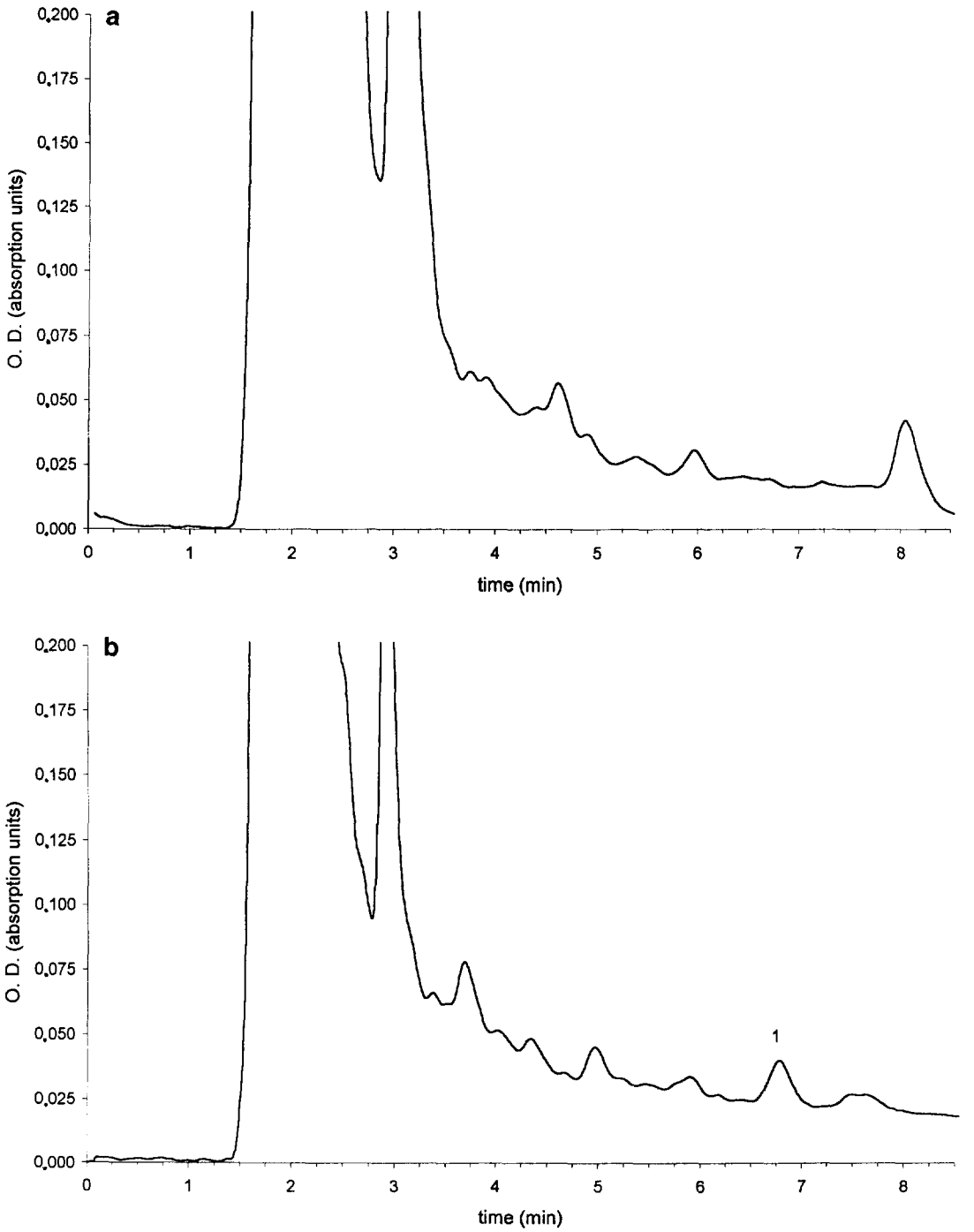

Fig. 4. (a) Chromatogram of blank 24 h urine sample. (b) Chromatogram of 24 h urine sample after intake of six capsules of drug 2 (eluent from Affi-Gel 601). 1 = Quercetrin.

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