ELSEVIER

Journal of Chromatography B, 731 (1999) 323–334

JOURNAL OF CHROMATOGRAPHY B

Determination of urinary 18β-glycyrrhetinic acid by gas chromatography and its clinical application in man

C.P.F. Guillaume[a], J.C. van der Molen[a], M.N. Kerstens[b,*], R.P.F. Dullaart[b], B.G. Wolthers[a]

[a]*Department of Clinical Chemistry, University Hospital Groningen, Postbus 30001, 9700 RB Groningen, The Netherlands*
[b]*Department of Endocrinology, University Hospital Groningen, Postbus 30001, 9700 RB Groningen, The Netherlands*

Received 3 March 1999; received in revised form 17 May 1999; accepted 27 May 1999

Abstract

A sensitive and quantitative gas chromatographic assay for the determination of 18β-glycyrrhetinic acid (18β-GA), the main metabolite of glycyrrhizin after oral licorice consumption in human urine, has been developed and validated. For the extraction of 18β-GA from urine two Sep-Pak C_{18} extractions, hydrolysis with *Helix pomatia* and three liquid–liquid extractions were performed, using 18α-glycyrrhetinic acid (18α-GA) as internal standard. Both 18β-GA and internal standard were converted into their pentafluorobenzyl-ester/trimethylsilyl-ether derivatives and detected by flame ionization detection using a WCOT-fused-silica capillary column. Good quality control data were obtained in precision and accuracy tests. The detection limit of the gas chromatographic method was 10 μg/l with a urine volume of 10 ml. A detection limit of 3 μg/l was obtained by performing GC–MS. The GC method was used to monitor the urinary excretion of 18β-GA after licorice consumption by two healthy volunteers and a patient suspected of licorice abuse. Furthermore, it was shown that this GC assay enables to detect other metabolites related to licorice consumption.

Keywords: Licorice; 18β-Glycyrrhetinic acid

1. Introduction

The consumption of licorice is very popular in many European countries. The main ingredient of licorice is glycyrrhizin from the roots of the plant *Glycyrrhiza glabra* L. [1,2]. Glycyrrhizin is hydrolyzed to its principal metabolite 18β-glycyrrhetinic acid (18β-GA) by intestinal bacteria after oral licorice consumption [3]. After absorption from the gut, 18β-GA is metabolized in the liver to 18β-GA-monoglucuronide, which is predominantly excreted in the faeces. The urinary excretion of 18β-GA-monoglucuronide is considered to be less than 1% of the dose administered [4].

Overconsumption of glycyrrhizin results in mineralocorticoid-like side effects such as salt retention and hypokalaemic hypertension [5]. In severe cases, rhabdomyolysis, renal failure, cardiac arythmias and even death have been described [6–10]. The 18β-GA compound of licorice is responsible for these toxic

*Corresponding author. Tel.: +31-50-3616-161; fax: +31-50-3619-308.
E-mail address: m.n.kerstens@int.azg.nl (M.N. Kerstens)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00239-X

effects. The minimal toxic amount of glycyrrhizin consumption has not been determined precisely, but has been estimated to be about 100 mg of glycyrrhizin per day, which corresponds to 50 g of licorice with a glycyrrhizin content of 0.2% (w/w) [7]. There is a widespread use of glycyrrhizin in many food products, because of its sweet taste and pleasant smell. Apart from licorice, glycyrrhizin is present in several other food products like ice cream, chewing gum, tobacco and candies [2,10–12]. Besides, glycyrrhizin is also used as a herbal remedy [1,2]. Moreover, subjects may tend to deny or be unaware of glycyrrhizin intake [5,13]. Therefore, there is demand for a clinically applicable quantitative method to confirm definitely suspected licorice overconsumption.

Several methods, including enzyme immunoassay, gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC), HPLC–electrospray MS, have been developed for the determination of 18β-GA [14–27]. These methods deal with the isolation and quantification of 18β-GA in serum, bile or urine from volunteers or experimental animals. Moreover, these studies mostly describe the excretion of 18β-GA after intravenous or oral intake of therapeutic doses of (pure) glycyrrhizin, which is customary in traditional Japanese or Chinese medicine [14–25,28,29]. Contrary to the increasing pharmacological and clinical interest in 18β-GA, limited data exist on the applications of the developed methods in clinical practice, i.e., on the urinary excretion of 18β-GA after oral consumption of popular glycyrrhizin-containing confectioneries.

The aim of this study is to describe a sensitive GC method to detect excreted 18β-GA in urine after oral consumption of original Dutch confectionery licorice.

2. Experimental

2.1. Chemicals

18β-Glycyrrhetinic acid (18β-GA), 18α-glycyrrhetinic acid (18α-GA, internal standard) were obtained from Sigma (St. Louis, MO, USA). Derivatizing agents used were pentafluorobenzyl bromide (PFBB) from Sigma and *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) from Fluka (Buchs, Switzerland). Dimethylformamide was from Pierce (Rockford, IL, USA). Sodium chloride and diethyl ether (Merck, Darmstadt, Germany), triethylamine (Merck–Schuchardt, Hohenbrunn, Germany) were of analytical grade. Methanol and *n*-heptane (Rathburns, Walkerburn, UK) were HPLC-grade and used as purchased. Acetonitrile was HPLC-grade (Rathburns), but was dried on excess sodium sulfate (Merck) before use. Ultrapure water and aqueous solutions of acetate buffer (1 mol/l, pH 4.8), hydrochloric acid (0.1, 1 and 6 mol/l) and sodium hydroxide (1 and 4 mol/l) were obtained from the University Hospital Pharmacy (Groningen, The Netherlands). *Helix pomatia* juice was obtained from BioSepra (France).

Blank human control urine used for the preparation of calibration standards and validation standards were obtained from healthy volunteers. Blank human urine was defined as urine, freshly obtained from one subject who was instructed not to use any licorice or glycyrrhizin containing products for at least three months.

2.2. Instrumentation and chromatographic conditions

2.2.1. Gas chromatography

GC was carried out on a Chrompack wall-coated open tubular (WCOT)-fused-silica column with stationary phase CP-Sil5 CB (25 m×0.32 mm I.D.). The column was housed in a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a carousel automatic solid sampler and a flame ionization detection (FID) system. The complete analysis including the subsequent data processing was computer-controlled using ChromPerfect for Windows (Justice Innovations, Mountain View, CA, USA). Three-μl aliquots of samples were injected splitless with an oven temperature of 120°C. Following an initial period of 1 min, the oven temperature was raised to 290°C at a rate of 30°C/min. The temperature was then programmed to 325°C at a rate of 2.5°C/min for a second isothermal period of 5 min. Helium was the carrier gas at a flow-rate of 1 ml/min.

2.2.2. Gas chromatography–mass spectrometry

Some samples were also measured by GC–MS according to an automated procedure. The samples were splitless injected at 200°C on a Model 5890 Hewlett-Packard gas chromatograph using a similar column as described above. Helium was the carrier gas at a flow-rate of 1 ml/min. The samples were analyzed during a temperature programmed run (200–320°C) at a rate of 30°C/min. Mass analysis was performed on a Micromass 70-250S mass spectrometer (Manchester, UK) in the electron impact (EI) mode scanning range *m/z* 100–750 or selected ion monitoring (SIM) at *m/z* 483.

2.3. Preparation of stock solutions, calibration standards and validation standards

Primary stock solution of 18β-GA (100 mg/l) was prepared in methanol and used for the preparation of calibration standards and validation standards. The subsequent working solutions (1 and 10 ng/μl) were prepared by dilution with methanol. The internal standard (18α-glycyrrhetinic acid) was prepared and diluted in methanol (10 ng/μl). All solutions were stored at 4°C when not in use.

Calibration standards and validation standards were prepared by adding appropriate amounts of 18β-GA working solutions to 10 ml of blank human urine. These standards were prepared just before use.

2.4. Sample preparation

Unknown samples to be analyzed, calibration standards and validation standards were treated in the same way.

2.4.1. Urine processing

Urine samples were adjusted to pH 5–6 by 4 mol/l NaOH solution or 6 mol/l HCl solution. The sample was centrifuged for 1 min. Aliquots of 10 ml of supernatant urine were fortified with 1000 ng internal standard. The urine was added in small portions to the internal standard solution. After transferring each portion the mixture was vortexed (2300 rpm) for 15 s. The extraction of 18β-GA and the internal standard was carried out similar to the method of extraction of steroid conjugates described by Shackleton employing Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) [17]. First each cartridge was preconditioned with 5 ml methanol and 5 ml distilled water, respectively. The urine sample was then applied to the cartridge. 18β-GA and the internal standard were retained by the cartridge, letting water and salts pass through. The cartridge was washed with 2.5 ml distilled water, and 18β-GA and the internal standard were recovered by elution with 5 ml methanol. The mixture was evaporated at 40°C under N_2 and reconstituted in 2 ml distilled water, 200 μl of 1 mol/l acetate buffer and 50 μl *Helix pomatia* juice. This mixture was hydrolyzed for 18 h at 37°C. Thereafter, the sample was diluted with 2 ml water, applied once more to a Sep-Pak C_{18} cartridge and finally recovered by elution with 5 ml methanol.

2.4.2. Liquid–liquid extractions

Liquid–liquid extraction I: The resulting eluate of methanol was evaporated to dryness at 40°C under N_2 and redissolved in 1 ml of 1 mol/l NaOH. The alkaline aqueous layer was washed with 4 ml of *n*-heptane by vortexing for 30 s at 2300 rpm.

Liquid–liquid extraction II: After removal of the *n*-heptane layer, the pH of the aqueous layer was adjusted to pH 1 with a solution of 6 mol/l HCl. After saturating with 0.7 g of sodium chloride the acidic aqueous layer was extracted with 4 ml diethyl ether by vortexing for 90 s.

Liquid–liquid extraction III: The diethyl ether layer was isolated and evaporated to dryness at 40°C under N_2 in glass tubes. To the residue 4 ml *n*-heptane was added. After shaking for 60 s and sonication for 30 min, the *n*-heptane layer was washed with 1 ml of 0.1 mol/l HCl for 30 s. The *n*-heptane layer was then isolated and evaporated to dryness at 40°C under N_2.

2.4.3. Derivatization

The residue was mixed with 40 μl triethylamine and 100 μl of a mixture of PFBB and acetonitrile (1:9, v/v). After incubating for 30 min at 40°C and evaporating to dryness at 40°C under N_2, the residue was reconstituted in 4 ml *n*-heptane and extracted with 1 ml of 1 mol/l HCl. The isolated *n*-heptane layer was evaporated to dryness at 40°C under N_2. To the residue was added 200 μl of BSTFA and 40 μl dimethylformamide. The mixture was incubated

for 60 min at 90°C. Then the reaction mixture was mixed with 4 ml of *n*-heptane and washed with 1 ml of 0.1 mol/l HCl. The isolated *n*-heptane layer was washed once again with 1 ml water. After isolating the *n*-heptane layer and subsequent evaporating to dryness at 40°C under N_2, the residue was reconstituted in 40 μl *n*-heptane and about 25 μl was transferred to an autosampler GC microvial.

2.4.4. Calculation of the 18β-GA concentration

Concentrations of 18β-GA were calculated from the calibration curve using peak-area ratios of 18β-GA and internal standard.

2.5. Validation

2.5.1. Extraction efficiency

The extraction efficiency (absolute recovery) of 18β-GA through the extraction procedure, including the Sep-Pak C_{18} extractions and the liquid–liquid extractions, was determined using 10-ml aliquots of blank human urine spiked with 1000 ng 18β-GA. This urine sample was processed without addition of the internal standard. The internal standard was added in an amount of 1000 ng immediately after the extraction procedure, but prior to derivatization. The analytical results, obtained for these samples, were compared to those in which both 18β-GA and internal standard were added to urine before performing the Sep-Pak C_{18} extractions and the liquid–liquid extractions.

In the same way, the extraction efficiencies for the liquid–liquid extractions were determined. For this purpose blank human urine was put through the Sep-Pak C_{18} extractions including *Helix pomatia* hydrolysis. Then the urine extract was fortified with 1000 ng 18β-GA prior to the particular liquid–liquid extraction of interest. Immediately after the particular extraction step the internal standard (1000 ng) was added to the pre-extraction-spiked urine followed by subsequent derivatization. The investigated extraction steps were studied in triplicate.

2.5.2. Linearity

Separate calibration standards were prepared in triplicate by spiking 10-ml aliquots of blank human urine with 18β-GA in amounts of 0, 100, 200, 500, 700, 1000, 1500 and 2000 ng in addition with 1000 ng internal standard. The blank standard spiked with internal standard was not included in the calculation of the calibration curve. The calibration curve was constructed, using unweighted linear regression analysis, from the peak-area ratios of 18β-GA and internal standard (y) versus the concentration (μg/l) of 18β-GA in the calibration standards (x).

2.5.3. Accuracy and precision

The precision and accuracy within a run were determined using validation standards of blank human urine (10-ml aliquots) fortified with 1000 ng internal standard and 18β-GA in amounts of 300, 600 and 1200 ng. Five separate validation standards were prepared for each concentration.

Another validation standard consisted of blank human urine (10-ml aliquots) spiked with 1000 ng internal standard and 1000 ng 18β-GA. Eight separate validation standards were prepared in duplicate or triplicate, extracted and assayed over a period of three months in order to assess the between-run precision and accuracy.

2.6. Clinical application

The urinary excretion of 18β-GA was investigated after consumption of 500 g of Dutch confectionery licorice by a volunteer. The amount was consumed within 4 h. Every urine excretion was collected separately for several days after starting licorice consumption. The content of 18β-GA in each urine sample was measured in duplicate.

In another volunteer, the urinary excretion of 18β-GA was also studied after a daily consumption of 40 g of Dutch confectionery licorice during five days. Every urine sample was collected separately during these five days. The collection of urine samples was continued for the next five days; two separate urine samples were collected arbitrarily on days 6, 8 and 10. The content of 18β-GA in each urine sample was investigated in duplicate. Urinary creatinine was measured on a Mega Multi-Test analyzer (Merck) in each collected sample of this volunteer.

A urine sample of a 33-year-old woman being suspected of licorice abuse was analyzed in duplicate according to the procedure as described above. This patient was evaluated elsewhere for hypokalaemic

hypertension with suppressed plasma levels of renin activity and aldosterone.

3. Results and discussion

3.1. Derivatization

Derivatization reactions used in this GC assay included the esterification of the C30-carboxylic groups of 18α-GA and 18β-GA to their pentafluorobenzyl esters (PFBB) using pentafluorobenzyl bromide followed by subsequent etherification of the C3-hydroxy groups by *N*,*O*-bis(trimethylsilyl)trifluoroacetamide yielding their trimethylsilyl (TMS) ethers.

In other GC–MS studies the carboxylic group of 18β-GA was converted to its methyl ester using diazomethane, whereas the C3-hydroxy group was derivatized to its trimethylsilyl ether [15–17]. We experienced that using acetylchloride in methanol or diazomethane as methylation agents did not only yield the desired derivative but often also led to side-products that deteriorated the GC assay (data not shown). Therefore, the esterification was performed with pentafluorobenzyl bromide in this GC assay.

3.2. Clean-up and chromatography

The usefulness of extending Sep-Pak C_{18} extractions and hydrolysis with three liquid–liquid extractions prior to both derivatization steps was investigated in the first set of experiments. Fig. 1 shows the resulting chromatogram after performing only both Sep-Pak C_{18} extractions including hydrolysis and subsequent derivatizations. This chromatogram shows contaminating peaks near the main peaks of 18β-GA and the internal standard 18α-GA. The performance of the NaOH/*n*-heptane extraction and the HCl/diethyl ether extraction preceding the derivatization reactions resulted in a similar chromatogram as depicted in Fig. 1. Following the complete analytical procedure including the third liquid–liquid extraction, a considerable clean-up of the chromatograms was reached as depicted in Fig. 2. This figure shows representative chromatograms after assaying only blank urine (Fig. 2a), blank urine fortified with 18β-GA (Fig. 2b) and also urine obtained after oral licorice consumption by a volunteer (Fig. 2c). Fig. 2b shows completely isolated

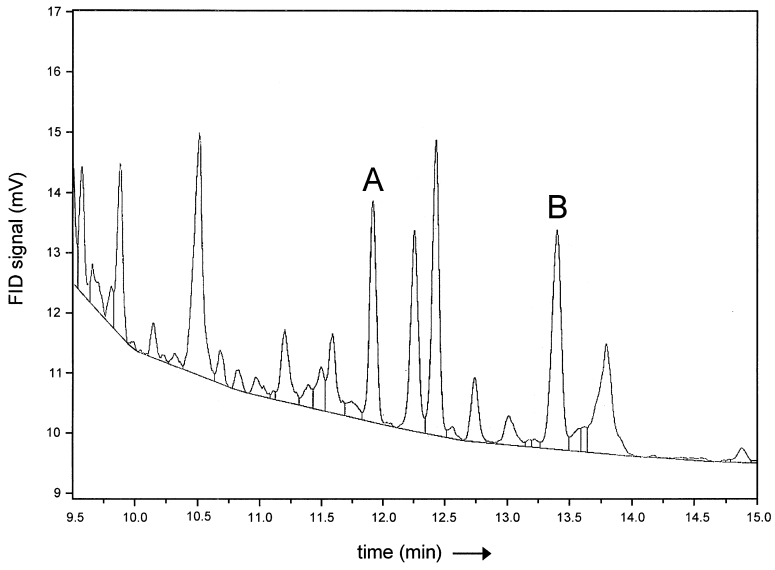

Fig. 1. Blank human urine sample spiked with 18β-GA and internal standard both at a concentration of 100 μg/l. The gas chromatogram represents the result after performing both Sep-Pak C_{18} extractions including hydrolysis by *Helix pomatia* and subsequent derivatizations. Peaks A and B correspond to 18β-GA and internal standard (18α-GA), respectively. Other peaks originate from endogenous substituents.

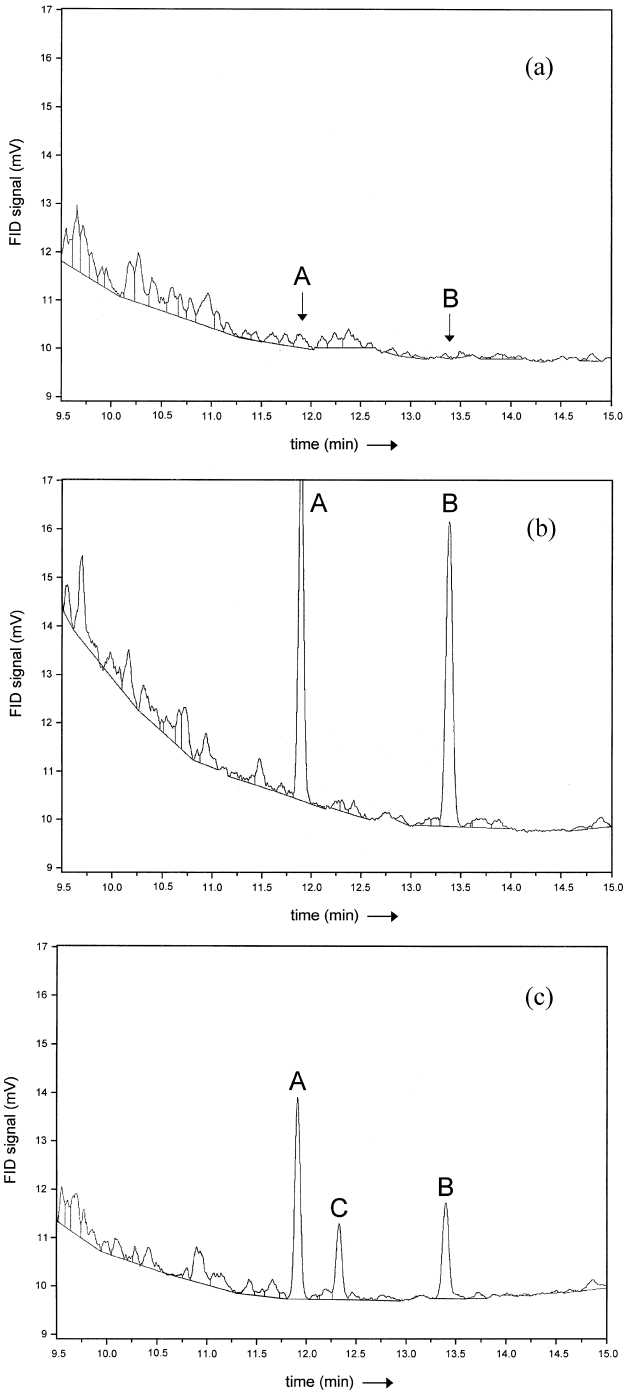

Fig. 2. (a) Gas chromatogram of a blank human urine sample that has been cleaned up according to the complete GC assay procedure, including all liquid–liquid extractions. (b) Gas chromatogram of 18β-GA in a spiked blank human urine sample, containing 100 μg/l of both 18β-GA and internal standard. Peaks A and B are 18β-GA and internal standard (18α-GA), respectively. (c) Urine sample obtained from a healthy volunteer after consumption of 500 g of licorice. The chromatogram shows the presence of 18β-GA (peak A) and internal standard (peak B). Peak C is a metabolite that accompanies the excretion of 18β-GA.

peaks of 18β-GA and the internal standard, whereas no peaks are detectable if only blank urine has been worked up through the procedure (Fig. 2a). As a result, the presented part of the chromatogram is very selective for the presence of 18β-GA and shows no endogenous compounds interfering with the detection of 18β-GA or internal standard. The importance of substituting the diethyl ether layer for *n*-heptane is also emphasized in Fig. 2c, which shows the results after licorice consumption by a volunteer. This chromatogram shows an additional peak between the peaks of 18β-GA and the internal standard, related to licorice consumption, because of its absence in the chromatograms of Fig. 2a and b. Therefore, performing the diethyl ether/*n*-heptane substitution has the added advantage of detecting other metabolites or side products of 18β-GA in the chromatogram.

Much effort was invested in developing an extensive clean-up procedure which eliminated interfering endogenous substances. For this purpose it was taken into account, that, depending on pH, glycyrrhetinic acid is both of lipophilic nature by its steroid-like skeleton and of hydrophilic nature by its carboxylic acid moiety. The strongly apolar impurities are removed by *n*-heptane/NaOH extraction, glycyrrhetinic acid remaining in the alkaline aqueous phase as sodium salt. The isolated alkaline aqueous phase is acidified by excess HCl prior to the subsequent HCl/diethyl ether extraction, making possible the extraction of un-ionized glycyrrhetinic acid into the diethyl ether layer. Here, the solvent phase is still yellow–brown colored, indicating the presence of contaminants. Because of these contaminating components, giving rise to chromatogram as shown in Fig. 1, diethyl ether was evaporated and substituted for the more apolar *n*-heptane. We observed, that, after evaporating the diethyl ether, glycyrrhetinic acid sticked to the glassware together with a brownish film of endogenous compounds and urine pigments. Glycyrrhetinic acid was extracted back into *n*-heptane by mixing vigorously and additional ultrasound treatment, leaving the brownish film of contaminants including urine pigments adsorbed to the glassware. This apolar shift results in a optimal isolation of glycyrrhetinic acid from less apolar components, as illustrated by the chromatograms in Fig. 2.

3.3. Mass spectrometry

Fig. 3 shows the typical electron impact ionization mass spectrum. A mass-to-charge m/z 722 is observed corresponding to the molecular ion $[M]^+$ of derivatized glycyrrhetinic acid. Moreover, two major fragments are observed, having masses of m/z 593 $[M-129]^+$ and m/z 483 $[M-239]^+$ respectively. Itoh presented in his study, using methyl-TMS glycyrrhetinic acid, fragments having a mass spectrum consistent with that shown in Fig. 3, considering the difference in mass of 166 between the methyl ester and the pentafluorobenzyl ester [16]. Thus fragment m/z 593 (compatible with m/z 427 in the spectrum of the methyl ester) is assigned to A-ring cleavage including the loss of TMS group, whereas fragment m/z 483 (compatible with m/z 317 in the spectrum of the methyl ester) is assigned to additional B-ring cleavage [16]. Both fragments are depicted by dotted lines in the structural formula of the derivatized glycyrrhetinic acid (Fig. 3). The m/z 483 fragment was used for identification in SIM, because of its more sensitive signal compared to the m/z 593 fragment or the molecular ion (m/z 722). Although the ions m/z 593 and m/z 722 can be monitored as an additional check, it was found that monitoring these ions did not yield more information, i.e., these ions gave the same quantitative result. Consequently, SIM was carried out at m/z 483 (data not shown). Fig. 4 shows the representative GC–MS-SIM chromatograms at m/z 483 of a blank urine sample spiked with 18β-GA and internal standard and a urine sample obtained following oral licorice consumption. A comparison of Fig. 4a and b with the corresponding GC chromatograms in Fig. 2 indicates that the peaks of 18β-GA and internal standard, including the possible metabolite, are indeed related to glycyrrhetinic acid.

3.4. Extraction efficiency

Table 1 summarizes the extraction efficiencies of the investigated extraction steps. The mean extraction efficiency of the overall clean-up procedure including the Sep-Pak C_{18} procedures, the hydrolysis by *Helix pomatia* and all liquid–liquid extractions was found to be $32.1 \pm 6.2\%$ ($n=3$). Nevertheless, we were able to quantitate 18β-GA with acceptable

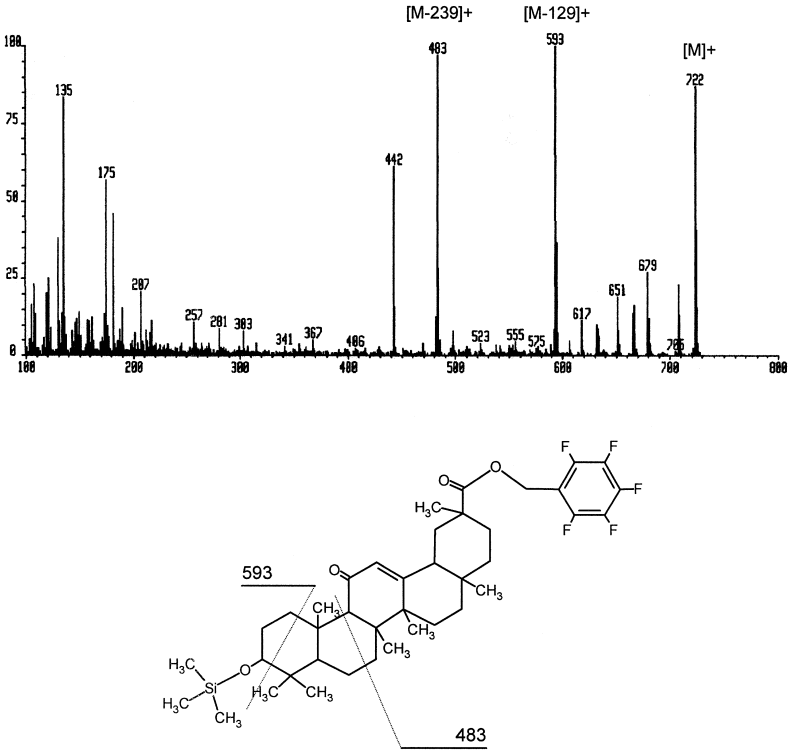

Fig. 3. Typical electron impact mass spectrum of 18β-GA presents a spiked blank urine sample. *m*/*z* 722 corresponds to the molecular ion. According to Itoh et al. the fragments *m*/*z* 593 and *m*/*z* 483 are obtained by cleavage of the A ring including the TMS ether (*m*/*z* 593) and by subsequent B-ring cleavage (*m*/*z* 483) as depicted in the structural formula of the derivatized 18β-GA [16].

accuracy and precision. As can be seen from Table 1, the extraction efficiencies after performing the NaOH/*n*-heptane extraction or the NaOH/*n*-heptane extraction and the subsequent HCl/diethyl ether extraction are all more than 90%. We found that repeating the HCl/diethyl ether extraction resulted in an additional yield of only 6.1±1.6% (n=3) and thus did not substantially improve its extraction efficiency. Remarkably, a low recovery (50.9±2.6%) was found when, in addition to the NaOH/*n*-heptane extraction and the HCl/diethyl ether extraction, diethyl ether was evaporated to dryness and reconstituted with *n*-heptane. Adsorption to the tube resulting in incomplete reconstitution of 18β-GA is an important contribution to this low extraction efficiency. A second *n*-heptane extraction was not effective, because of its additional yield of only 2.9±3.3% (n=3). Therefore, ultrasound treatment has been carried out, which slightly improved the average extraction efficiency (data not shown) [30]. Comparing the extraction efficiency of the overall clean-up procedure (32.1±6.2%) on the one hand and the extraction efficiency of only the liquid–liquid extractions (50.9±2.6%) on the other hand, the performance of the Sep-Pak C_{18} procedures and the hydrolysis by *Helix pomatia* contributes to an additional loss of 18β-GA.

3.5. Linearity and sensitivity

The GC method showed good linearity in the range 10–200 μg/l. The linear regression line was calculated as $y=0.0079x+0.057$ with $R^2=0.9949$. The background noise slightly differs between different blank urine samples. For this reason the limit of quantitation for the GC method appeared to be 10 to 20 μg/l by inspecting each chromatogram visually and considering a signal-to-noise ratio>3:1.

Contrary to the GC assay, GC–MS analysis was found to be only linear in the calibration range

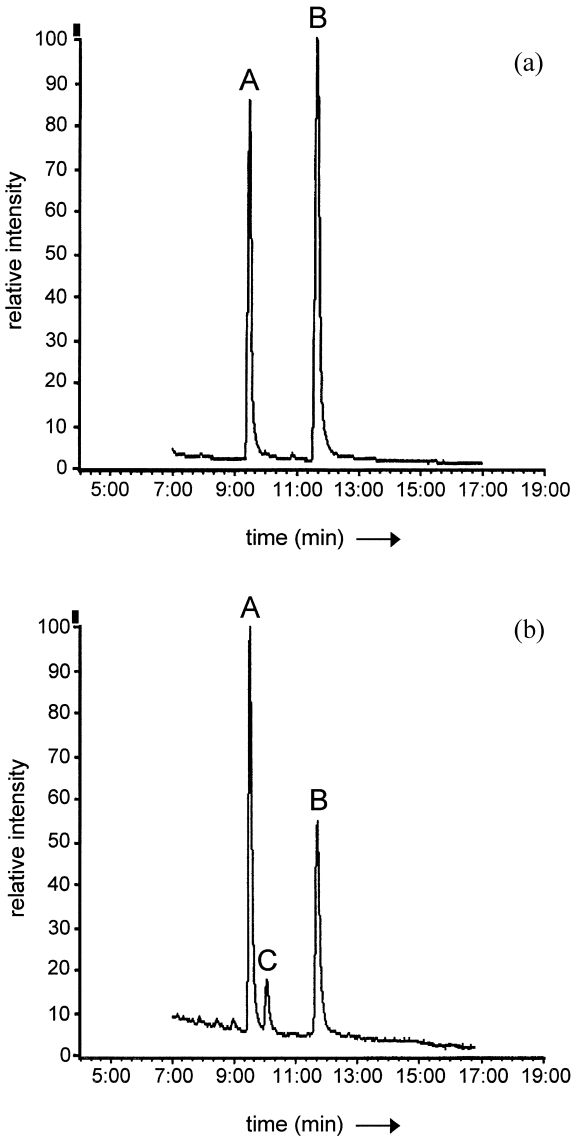

Fig. 4. GC–MS-SIM chromatograms (ion at m/z 483 was monitored) of 18β-GA in spiked blank urine (panel a) and in urine after licorice consumption by a human volunteer (panel b). Peaks A and B are 18β-GA and internal standard, respectively. In (b), peak C represents the metabolite that is seen after licorice consumption.

50–150 μg/l. No relationship was found between peak area and concentration at concentrations lower than 50 μg/l. Therefore, quantitation by GC–MS was not feasible and the GC assay was selected for further analysis of clinical samples. However, a lower limit of detection of the GC–MS analysis was demonstrated to be 3 μg/l. Thus, if a weak signal in the GC chromatogram is obtained, the presence of 18β-GA in a sample can be proved using this lower detection limit of the GC–MS analysis.

3.6. Accuracy and precision

Data of the assay performance are presented in Table 2. The values for within-run precision (expressed as relative standard deviation) and between-run precision were both less than 10% in the concentration range used, representing an acceptable repeatability of the GC assay. The within-run accuracy (expressed as percentage relative recovery) ranged from 100.3% to 113.7% (Table 2), which is also acceptable. Between-run precision and accuracy were determined in eight assays over a period of three months. The between-run accuracy was found to be 104.2%, which is also acceptable in view of the purpose of this assay.

3.7. Clinical application

The GC assay has been successfully applied in the quantitation of 18β-GA in urine of two human volunteers after oral consumption of licorice. Table 3 summarizes the urinary excretion data of 18β-GA after a single oral consumption of 500 g of licorice. Fig. 5 shows the excretion profile after a daily oral consumption of 40 g of licorice during 5 days. The concentration data of 18β-GA in Fig. 5 are corrected for the creatinine content in each urine sample eliminating the problem of different sample volumes.

Both Fig. 5 and Table 3 show the excellent applicability of the GC method in monitoring the urinary excretion of 18β-GA. Even after a daily intake of 40 g of licorice it is quantified easily.

It took approximately 8 h before 18β-GA was firstly detected in urine, when 500 g of licorice were consumed (Table 3). Following the first oral administration of 40 g of licorice, it took 9 h before first detection of 18β-GA was possible. The delay in urinary excretion can be explained by intestinal bacterial hydrolysis of glycyrrhizin into 18β-GA and subsequent absorption from the gut [3].

Remarkably, the excretion of 18β-GA can still be detected by the GC assay after more than 48 h after the single consumption of licorice (Table 3) and for

Table 1
Extraction efficiencies of the investigated three liquid–liquid extractions and of the complete procedure

Extraction step(s)	Recovery (mean±SD) (%)
NaOH/*n*-heptane extraction	>99
NaOH/*n*-heptane extraction+HCl/diethyl ether extraction	93.1±3.4
NaOH/*n*-heptane extraction+HCl/diethyl ether extraction+diethyl ether/*n*-heptane substitution	50.9±2.6
Overall procedure, including Sep-Pak C_{18} extractions, hydrolysis by *Helix pomatia* and all liquid–liquid extractions	32.1±6.2

Table 2
Accuracy and precision data for the GC analysis of 18β-GA

Nominal concentration (μg/l)	Precision (%)	Accuracy (mean±SD) (%)	*n*
Within-run variation			
30	7.8	100.3±7.9	5
60	5.0	109.3±5.5	5
120	4.3	113.7±4.9	5
Between-run variation			
100	6.0	104.2±6.6	8

at least three days after stopping consumption of licorice, when it has been consumed repeatedly for 5 days (Fig. 5). This phenomenon of extended excretion and the changeable course of the excretion profile are explained by enterohepatic cycling [3].

After single consumption of 500 g of licorice urinary 18β-GA concentrations were 155 μg/l or lower, apart from peak levels 225 μg/l and 380 μg/l, which were measured in two samples obtained in the first 18 h (Table 3). Apart from a few reports, most previously described assays showed detection limits ranging from 10 μg/l up to 2500 μg/l [16–29]. It appears that the majority of these concentrations depicted in Table 3, are below or near the detection limits which are usually found in other reported assays on 18β-GA. Taken the urinary 18β-GA concentrations as shown in Table 3 into consideration, a detection limit of 10 μg/l seems of clinical relevance to demonstrate licorice abuse.

From Fig. 2c it is apparent that urinary excretion of 18β-GA is accompanied with an unidentified metabolite. Apart from hepatic metabolism, 18β-GA is also metabolized by intestinal flora, resulting in 3α-epimers of 18β-GA [31,32]. Moreover, different compounds related to glycyrrhizin are found to be present in the roots of the plant *Glycyrrhiza Glabra* L., which may also be the case in confectionery licorice [33]. An interesting phenomenon is the presence of more, additional fully resolved peaks, close to the peak of 18β-GA in the chromatogram of

Table 3
Urinary excretion data of 18β-GA following a single oral consumption of 500 g of licorice by a human volunteer

Time of collection (h)	Concentration of 18β-GA in urine (μg/l)
0	0
0–7.7	227
7.7–8.4	66
8.4–17.8	378
17.8–27	154
27–30	91
30–33.5	27
33.5–45	89
45–53	10
53–64	0

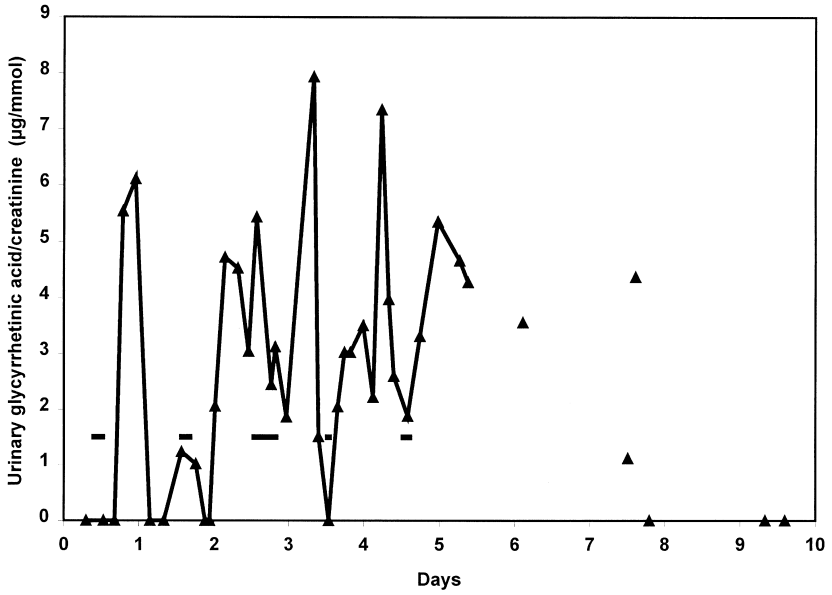

Fig. 5. Urinary excretion of 18β-GA (µg/l), normalized to urinary creatinine (mmol/l), as a function of time following the oral administration of 40 g of licorice by a volunteer. The horizontal bars show the periods during which licorice is consumed. After discontinuing licorice consumption on day 5, additional urine samples are collected on days 6, 8 and 10.

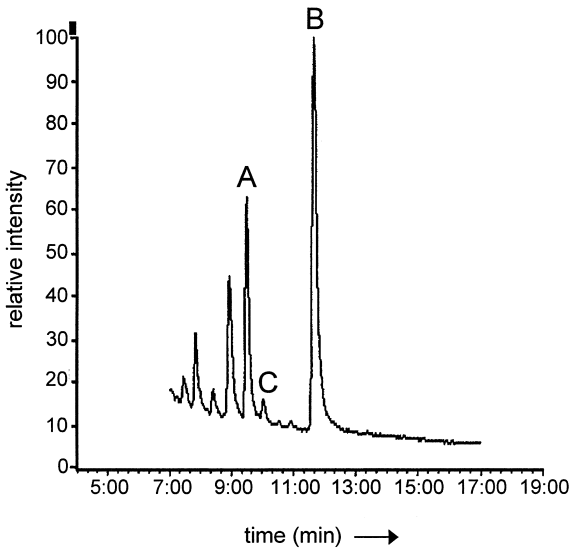

Fig. 6. GC–MS chromatogram (SIM at m/z 483) representing the urine obtained from the 33-year-old patient who was suspected of licorice abuse. A 18β-GA concentration of 13 µg/mmol creatinine (73 µg/l) was measured. Besides metabolite peak C, 18β-GA (peak A) and internal standard (peak B) are accompanied by more metabolites, related to glycyrrhetinic acid.

a 33-year-old patient suspected of licorice abuse. These peaks were identified as being also related to 18β-GA by SIM at m/z 483 (Fig. 6). In addition, more peaks were seen in chromatograms of the volunteer on the fifth day of licorice consumption, indicating that the appearance of more peaks could possibly be connected to prolonged licorice consumption. The usefulness of detecting these additional peaks with regard to licorice abuse still remains to be investigated, although this presence supports the diagnosis of licorice abuse.

4. Conclusion

A sensitive GC method has been developed for the quantitative analysis of 18β-GA in urine. The additional liquid–liquid extractions offer increased clean-up of the chromatograms. Especially the performance of the diethyl ether/*n*-heptane extraction has proved to be very successful in excluding interfering endogenous substances from the GC chromatograms. Therefore, the assay is very selective for the detection of 18β-GA in urine. Although the diethyl ether/*n*-heptane substitution is a critical step in the

clean-up procedure, the GC assay has been found to be reliable, accurate and precise in the view of its purpose. Analysis of the samples of the volunteers shows the good applicability of the presented GC assay in following the urinary excretion after ingesting different amounts of licorice. Moreover, the described GC assay shows its usefulness in observing the urinary excretion of metabolites related to 18β-GA.

Acknowledgements

We thank Rutger-Patrick van Rijswijk and Jeanine Kamps of the University of Groningen for their cooperation in our clinical study. Dr. A.R. Hermus, University Hospital Nijmegen, The Netherlands, provided urine of the patient suspected of licorice abuse.

References

[1] E.A. Spinks, G.R. Fenwick, Food Addit. Contam. 7 (1990) 769.
[2] D. Martinetz, Z. Ärztl. Fortbild. 88 (1994) 261.
[3] S. Takeda, K. Ishihara, Y. Wakui, S. Amagaya, M. Maruno, T. Akao, K. Kobashi, J. Pharm. Pharmacol. 48 (1996) 902.
[4] S. Krähenbühl, F. Hasler, B.M. Frey, F.J. Frey, R. Brenneisen, R. Krapf, J. Clin. Endocrinol. Metab. 78 (1994) 581.
[5] J. Heikens, E. Fliers, E. Endert, M. Ackermans, G. van Montfrans, Neth. J. Med. 47 (1995) 230.
[6] L. Luchon, A. Meyrier, F. Paillard, Néphrologie 14 (1993) 177.
[7] F.C. Størmer, R. Reistad, J. Alexander, Fd. Chem. Toxic. 31 (1993) 303.
[8] A. van der Zwan, Clin. Neurol. Neurosurg. 95 (1993) 35.
[9] A. Chubachi, H. Wakui, K. Asakura, S. Nishimura, Y. Nakamoto, A.B. Miura, Int. Med. 31 (1992) 708.
[10] H.T. Heidemann, E. Kreuzfelder, Klin. Wochenschr. 61 (1983) 303.
[11] J.D. Blachley, J.P. Knochel, New Engl. J. Med. 302 (1980) 784.
[12] M. Rosseel, D. Schoors, Lancet 341 (1993) 175.
[13] P.C. White, T. Mune, A.K. Agarwal, Endocr. Rev. 18 (1997) 135.
[14] M. Kanaoka, S. Yano, H. Kato, Chem. Pharm. Bull. 36 (1988) 8.
[15] R.V. Farese, E.G. Biglieri, C.H.L. Shackleton, I. Irony, R. Gomez-Fontes, New Engl. J. Med. 325 (1991) 1223.
[16] M. Itoh, N. Asakawa, Y. Hashimoto, M. Ishibashi, H. Miyazaki, Yakugaku Zasshi 105 (1985) 1150.
[17] C.H.L. Shackleton, J. Steroid Biochem. Molec. Biol. 45 (1993) 127.
[18] G. Groot, R. Koops, E.A. Hogendoorn, C.E. Goewie, T.J.F. Savelkoul, P. van Vloten, J. Chromatogr. 456 (1988) 71.
[19] K.-I. Abe, A. Suzuki, H. Katayama, Y. Tatsumi, E. Yumioka, J. Chromatogr. B 653 (1994) 112.
[20] Y. Yamamura, J. Kawakami, T. Santa, H. Kotaki, K. Uchino, Y. Sawada, T. Iga, J. Chromatogr. 567 (1991) 151.
[21] F. Hasler, R. Krapf, R. Brenneisen, D. Bourquin, S. Krähenbühl, J. Chromatogr. 620 (1993) 73.
[22] T.-H. Tsai, C. Chen, J. Chromatogr. 567 (1991) 405.
[23] S. Takeda, H. Ono, Y. Wakui, A. Asami, Y. Matsuzaki, H. Sasaki, M. Aburada, E. Hosoya, J. Chromatogr. 530 (1990) 447.
[24] X.Y. Zhang, R.J. Wu, J. Chen, D.K. An, J. Chromatogr. 495 (1989) 343.
[25] M.A. Raggi, F. Maffei, F. Bugamelli, G. Cantelli Forti, Pharmazie 42 (1994) 269.
[26] P. Wang, S.F. Li, K.H. Lee, J. Chromatogr. A 811 (1998) 219.
[27] J. Brown-Thomas, R.G. Christensen, R. Rieger, W. Malone, W.E. May, J. Chromatogr. 568 (1991) 232.
[28] G. Cantelli-Forti, F. Maffei, P. Hrelia, F. Bugamelli, M. Bernardi, P. D'Intino, M. Maranesi, M.A. Raggi, Environ. Health. Persp. 102 (1994) 65.
[29] K. Terasawa, M. Bandoh, H. Tosa, J. Hirate, J. Pharmacobio-Dyn. 9 (1986) 95.
[30] R.K. Müller, in: Toxicological Analysis, Verlag Gesundheit, Berlin, 1991, p. 65.
[31] T. Akao, M. Aoyama, T. Akao, M. Hattori, Y. Imai, T. Namba, Y. Tezuka, T. Kikuchi, K. Kobashi, Biochem. Pharmacol. 40 (1990) 291.
[32] M. Hattori, T. Sakamoto, K. Kobashi, T. Namba, Planta Med. 48 (1983) 38.
[33] J. Lutomski, Pharm. Unserer Zeit 12 (1983) 49.