IMPROVEMENT OF SELECTIVITY AND SENSITIVITY BY COLUMN SWITCHING IN THE DETERMINATION OF GLYCYRRHIZIN AND GLYCYRRHETIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive method is described for the simultaneous determination of glycyrrhizin and glycyrrhetic acid in human plasma. Quantitation is by high-performance liquid chromatography using ion-pair chromatography on a reversed-phase column. Variable-wavelength ultraviolet detection is employed. For the extraction of both compounds from plasma, a new solid-phase ion-pair extraction procedure using octadecylsilane columns was developed. Because of the strong forces involved in the protein binding of glycyrrhizin, quantitative extraction of this compound from plasma was possible only after an alkaline pH shift. A considerable improvement in selectivity and sensitivity was obtained by automated column switching involving on-line preseparation of the solid-phase extract on a short precolumn and chromatographic analysis of a heart-cut from the precolumn eluate. The limit of detection of both glycyrrhizin and glycyrrhetic acid was 0.1 mg/l in 0.5 ml of plasma. From a preliminary study in human volunteers, it was concluded that glycyrrhetic acid rather than glycyrrhizin is preferred in a study in human volunteers to assess the zero effect level of glycyrrhizin.

INTRODUCTION

Glycyrrhizin, the glycoside of glycyrrhetic acid, is a natural compound with an intensely sweet taste. It is extracted from the roots of *Glycyrrhiza glabra* L. which contains 6–14% of glycyrrhizin. In addition to its use as a sweetening agent in many food and luxury products such as liquorice, chocolates, beer, liquor and chewing

tobacco, glycyrrhizin has been used as a medicine in the treatment of ulcus ventriculi. However, serious side effects including headache, oedema, body weight increase and serious disturbances of body-electrolyte balances related to glycyrrhizin ingestion were observed either after daily consumption of amounts as low as 40 g of liquorice or in clinical use. Therefore, in our Institute a study was started in human volunteers in order to assess the zero effect level of glycyrrhizin. In parallel with this study, the human pharmacokinetics was studied of glycyrrhizin and glycyrrhetic acid, which is the main metabolite of glycyrrhizin and which is rapidly formed from the parent drug.

For this purpose a method was developed for the simultaneous determination of glycyrrhizin and glycyrrhetic acid in human plasma, which was suitable for application to the large series of samples which are commonly involved in pharmacokinetic studies. Few methods have been published on the determination of glycyrrhizin and glycyrrhetic acid in plasma. A very sensitive method involves gas chromatography–mass spectrometry with selected-ion monitoring after formation of the corresponding methyl and trimethylsilyl esters of the two compounds[1]. An insensitive high-performance liquid chromatographic (HPLC) method was reported by which the two compounds were determined during 1–36 h in plasma of rats to which 500 mg glycyrrhizin/kg body weight had orally been administered[2]. Pretreatment of the samples was by protein precipitation with ammonium hydroxide–ethanol. Separation was by reversed-phase chromatography and gradient elution. An HPLC method using anion-exchange chromatography with gradient elution after protein precipitation with methanol was reported which was sensitive enough to determine the concentrations of glycyrrhizin and glycyrrhetic acid in plasma during 10 to 240 min after oral administration of 500 mg glycyrrhizin/kg body weight[3]. A second HPLC method was reported by this group using adsorption chromatography after protein precipitation of plasma or tissue homogenates which could determine glycyrrhizin and glycyrrhetic acid in body fluid and tissues of rats during 1–3 h after oral administration of 100 mg glycyrrhizin/kg body weight[4]. In these three HPLC methods, UV detection at 245–254 nm was employed. None of the HPLC methods was sensitive enough for application in our study in which glycyrrhizin was to be administered in oral doses of up to 800 mg glycyrrhizin daily (10–15 mg/kg body weight). Therefore, a new method was developed with special emphasis on the sample pretreatment.

For the extraction of both compounds from plasma an ion-pair solid-phase extraction procedure was developed using octadecylsilane columns. Quantitation was performed by HPLC with UV detection at 258 nm using ion-pair chromatography on a reversed-phase column. Automated column switching was applied to analyze only a heart-cut from the eluate obtained from a short precolumn on which the solid-phase extract was preseparated.

MATERIALS AND METHODS*

Apparatus

The HPLC analyses were performed by a fully automated system comprised of

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a simple solvent delivery system (Pump 1, Model 9208; Kipp Analytica, Emmen, The Netherlands), from which the damping units were removed to decrease the dead volume for faster solvent switching, and a high-quality solvent delivery system (Pump 2, Spectroflow SF 400; Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) in combination with a sample processor (WISP 710B; Waters Associates, Milford, MA, U.S.A.) and a column thermostat (Model SpH 99; Spark Holland, Emmen, The Netherlands). Solvent-stream switching was controlled and carried out by a time-programmable multiport stream-switch unit including two high-pressure two-position six-port valves and one low-pressure six-position solvent selection valve (MUST-IET, Spark Holland). Detection was performed with a variable-wavelength UV detector (Spectroflow 773, Kratos Analytical Instruments) operated at 258 nm. The detector signal was recorded and integrated via an external-input board by the integrator part of a dual-channel gas chromatograph (Model 5880 A; Hewlett-Packard, Waldbronn, F.R.G.). The settings of the detector and integrator corresponded to a sensitivity of 0.01 a.u.f.s. Both the switching unit and integrator were controlled by the autosampler via its external contact closure junctions.

A 15 mm × 3.2 mm I.D. precolumn was used, packed with 7-μm octadecylsilane (NewGuard RP-18; Brownlee Labs., Santa Clara, CA, U.S.A.). A 150 mm × 4.6 mm I.D. analytical column was used which was slurry-packed in our laboratory with 5-μm Hypersil ODS (Shandon Southern, Runcorn, U.K.). Two 75 mm × 2.1 mm I.D. columns packed with 40-μm octadecylsilane (Chrompack International, Middelburg, The Netherlands) were used respectively as an HPLC-solvent saturation column and as a guard column for the analytical column. All columns were mounted in the column oven, the temperature of which was maintained at 50°C.

The HPLC eluent (mobile phase) was a mixture of methanol and 0.02 M phosphate buffer pH 7.2 (0.77 g of sodium dihydrogenphosphate monohydrate and 5.16 g of disodium hydrogenphosphate dodecahydrate per 1 of water) (80:20, v/v). Cetrimonium bromide was added to the eluent in a concentration of 0.0033 M. The mobile-phase flow-rate was 1.0 ml/min. Solvent 1, which was used to load the precolumn with the sample, consisted of methanol, water and 0.02 M phosphate buffer pH 7.2 (55:20:25, v/v/v) with 0.0033 M cetrimonium bromide. Solvent 2, which was used to clean the precolumn, consisted of methanol and 0.02 M phosphate buffer pH 7.2 (90:10, v/v) with 0.0033 M cetrimonium bromide. The flow-rate of either solvent was 1 ml/min.

The solid-phase extractions were performed using 1-ml reversed-phase octadecylsilane (ODS) extraction columns (J. T. Baker Chemicals, Deventer, The Netherlands) fitted to a Baker-10 SPE extraction manifold.

Reagents and reference solutions

Reagents used were methanol (Lichrosolv), ethanol, sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dodecahydrate, anhydrous sodium carbonate, sodium hydroxide and cetrimonium bromide (all of analytical grade; E. Merck, Darmstadt, F.R.G.). Water was distilled in glass from alkaline permanganate solution. Glycyrrhizin (glycyrrhizic acid ammonium salt) and glycyrrhetic acid (18-β-glycyrrhetic acid) were obtained from Fluka (Buchs, Switzerland). Stock solutions of glycyrrhizin (1000 mg/l) and glycyrrhetic acid (1000 mg/l) were prepared in glass test-tubes in ethanol. These solutions were stable for several months

at $+4°C$. From these stock solutions, working solutions of 5 mg/l of both compounds were freshly prepared monthly in polypropylene test-tubes in methanol–water (1:1, v/v). Aliquots of these working solutions were used to prepare calibration standards.

All pipetting and transfer of samples and solutions was with variable pipettes using disposable polypropylene tips (Gilson France, Villiers-le-Bel, France).

Sample pretreatment

An aliquot of 0.5 ml of plasma was transferred to a 1.5-ml polypropylene reaction vial (Eppendorf, Hamburg, F.R.G.). To the sample, 350 μl pf 0.028 M cetrimonium bromide in 0.15 M sodium hydroxide were added. The mixture was vigorously (2500 rpm) vortexed for 1 min. After 15 min of equilibration, the mixture was vortexed again for 1 min. The total sample was processed as described in solid-phase extraction.

Solid-phase extraction

An ODS extraction column was fitted to the Baker-10 SPE extraction manifold. Preceding the extraction, the column was pretreated consecutively twice with 1 ml of methanol, once with 1 ml of water, twice with 1 ml of 0.0055 M cetrimonium bromide in methanol and once with 1 ml of 0.0055 M cetrimonium bromide in 0.005 M sodium hydroxide. In order to obtain a high and reproducible extraction recovery, all fluids should pass slowly through the column and intrusion of air in the column, *e.g.*, caused by vacuum suction, was strictly prevented.

The pretreated sample was placed on top of the SPE column. The eluate was discarded. Water- and methanol-soluble plasma components were removed by washing the column consecutively with 1 ml of 0.0055 M cetrimonium bromide in 0.1 M sodium carbonate (with a final pH of 10.9) and 1 ml of water. The major part of the residual water was removed by passing 50 μl of methanol through the column. The drugs were eluted with 500 μl of methanol into a 1.5-ml polypropylene reaction vial (Eppendorf) which was placed under the column. The methanol content in the eluate was evaporated under a gentle stream of nitrogen at 40°C until a final sample volume of about 50–100 μl. The final sample volume was determined by weighing of the reaction vial. An aliquot of 200 μl methanol–water (1:1, v/v) was added to the residue and the mixture was vortexed for 30 s, placed in an ultrasonic bath for 5 min and vortexed for 10 s. The final extract was transferred to a 0.4-ml polypropylene micro insert (reaction vial for the Beckman Ultra Micro System of which the cap and the upper 6 mm was cut off) for the WISP sample vial. An aliquot of 100 μl was taken for HPLC analysis.

Oral administration of glycyrrhizin to human volunteers

The concentrations of glycyrrhizin and glycyrrhetic acid were determined in plasma samples obtained from a selection of five healthy volunteers to which glycyrrhizin was administered in daily oral doses of either 400 or 800 mg. Blood samples were collected at 0.5, 1, 2, 4, 8 and 24 h after the first administration and five times further during the next 4 weeks within which glycyrrhizin was daily administered. Samples were collected just before ingestion of the respective daily dose. In addition, blank blood samples were collected from each volunteer before the first glycyrrhizin administration. The blood samples were collected in heparinized tubes, centrifuged and the plasma separated and stored at $-25°C$ until analysis.

RESULTS

Figs. 1–3 show the positions of the two high-pressure two-position six-port valves A and B and the low-pressure six-position solvent selection valve. The thick lines indicate the respective solvent stream which is relevant during a particular step for the transport of the analytes through the chromatographic system. The thin lines indicate the other solvent streams which are present. The solvent streams during the analytical process are determined by the positions of solvent switch A and the solvent selection valve.

Fig. 1 (step 1) shows the preconditioning of the precolumn during 3 min with solvent 1, a sample-loading solvent of low elution strength, and the loading of the sample onto the precolumn with this solvent during 4 min (time of injection is time 0). Fig. 2 (step 2) shows the transport of a heart-cut from the precolumn to the analytical column. This is effected by passing the HPLC eluent over the precolumn. After 5.5 min from the time of injection, the total amounts of glycyrrhizin and glycyrrhetic acid have passed quantitatively to the analytical column. Fig. 3 (step 3) shows the switch settings during separation of the two compounds from the still present plasma components on the analytical column during 5.5 to 12 min after injection. During this time the precolumn is flushed with solvent 2 of high elution strength.

Switch B is used for a regular check of the performance of the precolumn with respect to the retention of glycyrrhizin and glycyrrhetic acid and to its efficiency. If, in step 1, switch B is in the second position, the eluate of the precolumn is distributed to the detector inlet. In this way the elution profile of the two compounds over the precolumn only can be obtained. Fig. 4 shows a chromatogram of glycyrrhizin and glycyrrhetic acid obtained with these switch positions. The two compounds are not separated on the precolumn, which is convenient for effecting a sharp heart-cut from

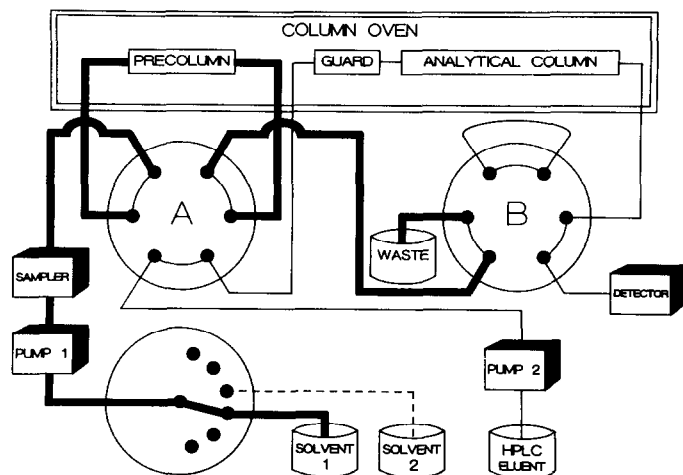STEP 1: PRECONDITIONING AND SAMPLE RETENTION ON PRECOLUMN (0–4.00 MIN)

Fig. 1. Column switching scheme for the first step including column preconditioning and loading of the precolumn with the sample. The thick lines show the solvent stream which is relevant to the transport of the analytes through the chromatographic system.

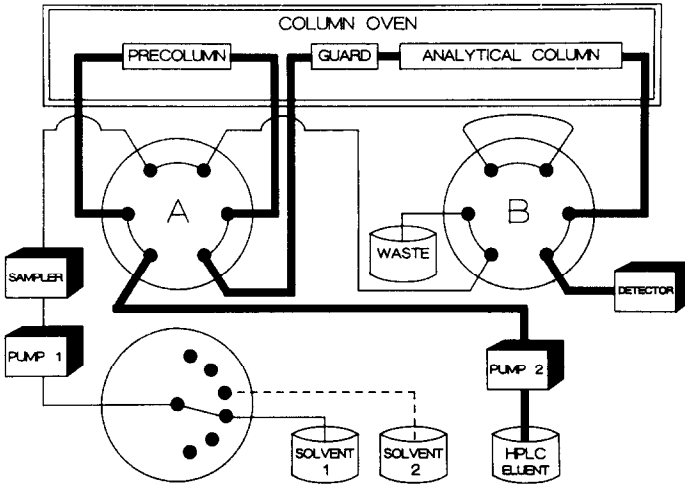STEP 2: HEART CUT FROM PRECOLUMN TO ANALYTICAL COLUMN (4.00-5.50 MIN)

Fig. 2. Column switching scheme for the second step including transport of a heart-cut of the precolumn eluate to the analytical column. The thick lines show the solvent stream which is relevant to the transport of the analytes through the chromatographic system.

the precolumn eluate. The elution of the two compounds from the precolumn starts at about 4.5 min. On the basis of this elution profile, the solvent switch A is switched after 4.00 min.

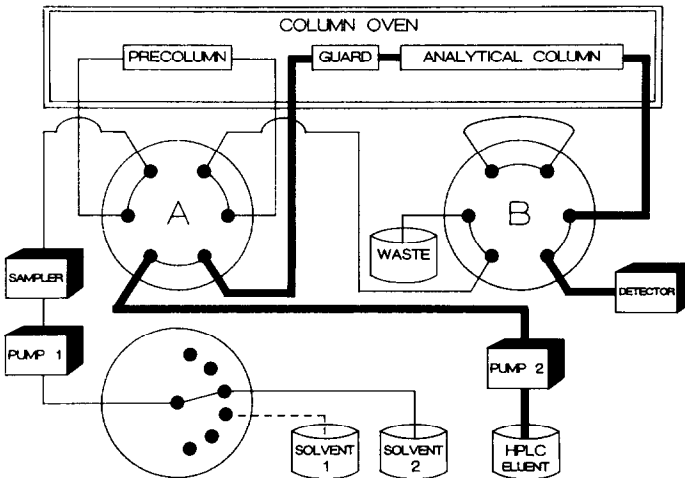STEP 3: SEPARATION ON ANALYTICAL COLUMN (5.50-12.00 MIN)

Fig. 3. Column switching scheme for the third step including separation of the analytes on the analytical column and simultaneous flushing of the precolumn. The thick lines show the solvent stream which is relevant for the transport of the analytes through the chromatographic system.
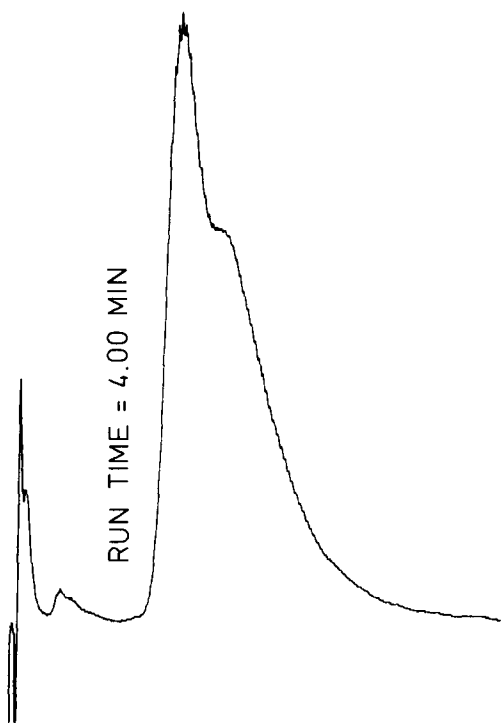
Fig. 4. Elution profile of glycyrrhizin and glycyrrhetic acid from the precolumn. Positions of switch A and solvent selection switch as in Fig. 1. Switch B is in the second position connecting the precolumn eluate to the detector inlet.
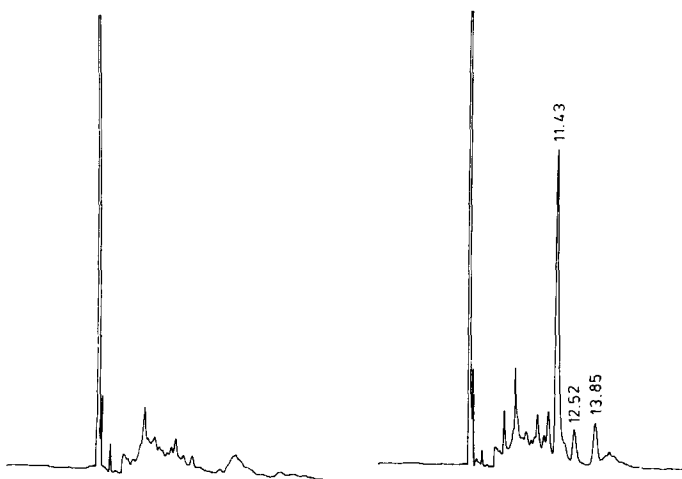

Fig. 5. Chromatogram of an extract of 0.5 ml of blank human plasma (left) and of an extract of 0.5 ml plasma from a volunteer to whom 800 mg of glycyrrhizin (corresponding to 100 g of liquorice) were orally administered (right). Retention times: glycyrrhizin, 9.95 min; glycyrrhetic acid; 11.43 min; the peaks at 12.52 min and 13.85 min are probably due to other (unidentified) glycyrrhizin metabolites.

The elution profiles of glycyrrhizin and glycyrrhetic acid from the used octadecylsilane extraction column were determined by passing aqueous reference solutions through the column. Subsequently, the compounds were eluted in consecutive fractions of 200 μl methanol and the concentrations were determined in each fraction. Based on these elution profiles it was assessed that the washing and elution volumes described provided a maximum elution of the compounds. By comparing the recovered amounts with directly injected reference solutions, the recoveries were calculated as 97% for glycyrrhizin and 89% for glycyrrhetic acid.

The extraction recoveries from plasma were determined from extracts ($n = 3$) of blank human plasma to which glycyrrhizin and glycyrrhetic acid had been added. The samples were carried through the extraction procedure and the extraction recoveries from plasma were determined by comparing the peak areas from the chromatograms obtained with those obtained after direct injection of the respective reference solutions in corresponding concentrations. The extraction recoveries ($\pm$ standard deviations) were $90.0 \pm 6.1\%$ for glycyrrhizin and $85.8 \pm 9.7\%$ for glycyrrhetic acid. The analytical pocedure was calibrated by construction of calibration lines based on series of calibration standards in human plasma, covering relevant concentration ranges.

The lowest detectable concentration of glycyrrhizin or glycyrrhetic acid was 100 μg/l.

Fig. 5 shows typical chromatograms of an extract of blank human plasma (left) and of a plasma extract from a volunteer on a daily oral dose of 800 mg of glycyrrhizin which corresponds to 100 g of liquorice (right). Glycyrrhizin and glycyrrhetic acid were eluted after 9.95 and 11.43 min, respectively. Only glycyrrhetic acid was detected in this plasma sample in a concentration of 1.5 mg/l.

DISCUSSION

The aim of the present study was the development of a method for the determination of glycyrrhizin and its main metabolite glycyrrhetic acid in human plasma. The method had to be applied in a preliminary study in human volunteers with the following objectives. The first objective was the assessment of a glycyrrhizin dose range for a subsequent extended study in human volunteers in which the zero-effect level of glycyrrhizin had to be assessed. The second objective was to obtain insight into the expected biological effects related to glycyrrhizin ingestion, to detect possible effects of gender and to obtain insight into the biological parameters relevant to an adequate registration and interpretation of these effects. The third objective was to obtain insight into the organization and administration of the main study. The final objective was to obtain insight into the pharmacokinetics of glycyrrhizin after single and repeated oral administration and to assess a parameter adequate for monitoring single and repeated glycyrrhizin ingestion. Because of its preliminary character, the study was restricted to eight male and eight female healthy human volunteers. The present report is restricted to the final objective.

Problems encountered during the development of the analytical procedure were in particular related to glycyrrhizin: its strong binding to plasma proteins, its acidic character and its instability above room temperature and at low pH values. These properties of glycyrrhizin excluded the development of a straightforward extraction

procedure including ion suppression at low pH and liquid–liquid or solid-phase extraction of the neutral compounds. The protein binding of glycyrrhizin could be cleaved only at high pH values. Since both glycyrrhizin and glycyrrhetic acid are ionized at this pH, extraction was carried out after formation of ion pairs of the compounds with cetrimonium bromide, a quaternary ammonium compound. Based on our experience with solid-phase extraction procedures[5–8], an ion-pair solid-phase extraction method was developed.

Because of the acidic character of both compounds, gas chromatographic determination was possible only after complex derivatization procedures[1]. For this reason and because of the thermal instability of glycyrrhizin, HPLC was chosen as a quantitation technique. A problem to be solved in the development of an HPLC method was the large difference in polarity between glycyrrhizin and glycyrrhetic acid. Consequently, simultaneous determination of these compounds was possible only by using ion-pair chromatography on a reversed-phase (ODS, octadecylsilane) column using cetrimonium bromide. Sensitive and selective determination of the two compounds by using selective detection techniques was impossible since the two compounds were not sensitive to fluorescence or electrochemical detection and only weakly sensitive to UV detection (absorption maximum at 248 nm).

The method developed in this way included an ODS solid-phase ion-pair extraction procedure and ion-pair chromatography on an ODS column. In both procedures, cetrimonium bromide was used as an ion-pairing agent. UV detection was employed at 258 nm. However, because of a lack of selectivity, both for glycyrrhizin and for glycyrrhetic acid, the procedure was not sensitive enough to detect the compounds in plasma from healthy volunteers after oral ingestion of relevant doses (400 or 800 mg daily) of glycyrrhizin. A considerable improvement in selectivity was obtained by implementation of a stream-switch unit which permitted preseparation of the ion-pair solid-phase extracts on a short (15 mm × 3.2 mm) precolumn filled with 7-μm ODS particles. In contradistinction with the precolumns used in the majority of other published methods, the precolumn we used had an efficiency of 300–750 theoretical plates, which enabled the transport of a sharp "heart-cut", including only the relevant part of the precolumn eluate, to the analytical column. The resulting chromatograms showed a significant clean-up of the extracts resulting in a significant improvement in the selectivity and sensitivity of the procedure. The corresponding reduction in the limit of detection (about 100 μg/l for either compound) permitted the quantitation of glycyrrhizin and glycyrrhetic acid in this study.

A problem was posed by the instability of the precolumn. Although extracts rather than plasma samples were injected, the performance of the precolumn rapidly decreased as evidenced by a rapid decrease in the retention of glycyrrhetic acid and glycyrrhizin after a few sample injections. A total number of about 10–15 samples could be injected on the precolumn before an irreversible loss of retention necessitated its replacement. Obviously, the alkaline chromatographic conditions dictated by the simultaneous analysis of glycyrrhizin and glycyrrhetic acid caused the rapid pre-column deterioration, although the performance of the analytical column was hardly affected by similar chromatographic conditions. In order to check regularly the performance of the precolumn, after every five injections the elution profile of glycyrrhizin and glycyrrhetic acid was recorded. Since the performance of the precolumn is paramount with respect to the overall analytical recovery, the

column-switching configuration with two high-pressure switching valves described, which permits a convenient performance check, is recommended.

Fig. 5 shows a typical chromatogram of a sample extract from a volunteer given an oral dose of 800 mg glycyrrhizin. A similar concentration–time course was observed for glycyrrhizin and glycyrrhetic acid in both 400-mg and 800-mg dose groups: up to 4 h, neither glycyrrhizin nor glycyrrhetic acid was detectable in the plasma samples from either dose group. From 8 h after the first administration, glycyrrhetic acid was detected in all plasma samples ($>$ 100 μg/l). In the next weeks of glycyrrhizin administration a steady state concentration of glycyrrhetic acid in plasma was reached in each volunteer, of 0.5–1.7 mg/l. After terminating the oral glycyrrhizin administration, glycyrrhetic acid was still detectable in plasma during 2–4 days. Glycyrrhizin was not detected ($<$ 100 μg/l) in any of these plasma samples. This is in accordance with results of Sakiya *et al.*[3] who reported, after very high oral doses to rats (500 mg/kg glycyrrhizin), high glycyrrhetic acid concentrations in plasma up to 30 mg/l and only minor concentrations of glycyrrhizin. After bolus administration of glycyrrhizin into the portal vein, glycyrrhetic acid was not detected in plasma. On the basis of these results, they concluded that glycyrrhizin is not metabolized in the body to glycyrrhetic acid, but in the gastro-intestinal tract is converted into glycyrrhetic acid and predominantly absorbed as such.

In addition to the appearance of a glycyrrhetic acid peak at 11.43 min in the chromatograms, two other peaks were recorded at 12.52 and 13.85 min which were absent from the chromatograms of blank plasma from these volunteers and which were related in height to glycyrrhetic acid. Although these peaks were not further identified, it is suggested that they are due to additional metabolites of glycyrrhizin.

Based on our results it can be concluded that glycyrrhetic acid rather than glycyrrhizin is preferred for studies in human volunteers to assess the zero effect level of glycyrrhizin. Beside its potential use in monitoring concentration-related effects, determination of the glycyrrhetic acid concentration in plasma can be used to detect recent (up to 4 days) liquorice (or other glycyrrhizin-containing products) ingestion. Therefore, further optimization of the analytical procedure, especially with respect to chromatographic conditions which are less deleterious for the precolumn, will be directed primarily to the assay of glycyrrhetic acid in plasma.

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