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Determination of glycyrrhizic acid and 18-β-glycyrrhetinic acid in biological fluids by micellar electrokinetic chromatography

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Abstract

A micellar electrokinetic chromatographic technique for determining glycyrrhizic acid (GZA) and 18β-glycyrrhetinic acid (GRA) in human plasma and urine was developed. Sample clean-up and analyte concentration were carried out by solid-phase extraction (with C_{18} sorbent). The buffer solution used for MEKC contained 20 m*M* sodium dihydrogenphosphate, 20 m*M* sodium tetraborate, 20 m*M* tetrabutylammonium bromide and 50 m*M* sodium dodecylsulphate, at a pH of 8.7. Good linearities for both GZA and GRA in plasma and urine were obtained. The recoveries of the method were in the range 86.5 to 107%. The detection limits for GZA in urine (0.5 ml) and plasma (1 ml) were 1.6 μg ml^{-1} and 0.8 μg ml^{-1}, respectively, while for GRA in urine (0.5 ml) and plasma (1 ml) were 2 μg ml^{-1} and 1 μg ml^{-1}, respectively.

Keywords: Glycyrrhizic acid; Glycyrrhetinic acid; Methyltestosterone

1. Introduction

Glycyrrhizic acid (glycyrrhizin) (GZA) is the main constituent of licorice (*Glycyrrhiza glabra*) which is one of the most commonly used herbal drugs in traditional Chinese prescriptions. It is primarily used as a demulcent and sweetener. Its pharmacological properties include anti-viral, anti-inflammatory and anti-oxidative activities [1–3]. There have been some reports that GZA has cancer chemopreventive functions [4–7] and that it has been used clinically in patients with AIDS [8]. GZA is also used as an additive in some foods, and toothpaste. 18β-Glycyrrhetinic acid (GRA) is the major metabolite of GZA. The structures of GZA and GRA are shown in Fig. 1 which also depicts the structure of methyltestosterone (MT), used as the internal standard in this study.

Existing methods for the determination of GZA and GRA in biological fluids include thin-layer chromatography [9,10], enzyme immunoassay [11] and radioimmunoassay [12], gas chromatography (GC) [13,14] and high-performance liquid chromatography (HPLC) [15–22]. Most studies have been performed by HPLC [23]. A survey of the analytical literature indicated that a method for determining GZA and GRA in human plasma and urine by capillary electrophoresis has yet to be developed, although the technique has been used for determining the two compounds in traditional Chinese medicinal

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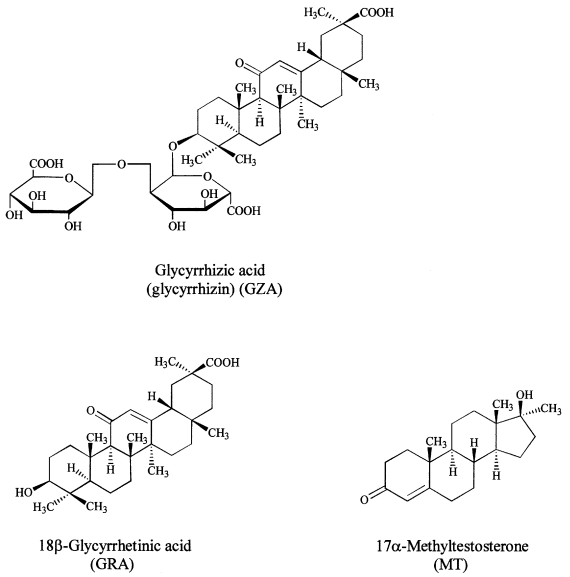

Fig. 1. The structures of GZA, GRA and MT.

preparations [24]. In this paper, we report the determination of GZA and GRA in human plasma and urine by micellar electrokinetic chromatography (MEKC) after sample preparation by cartridge-based C_{18} solid-phase extraction (SPE).

2. Experimental

2.1. Equipment and conditions

MEKC experiments were carried out on a PRINCE system equipped with an autosampler (Prince, Emmen, The Netherlands), and a Lauerlabs (Emmen, The Netherlands) UV detector. A 65 cm (effective length 51 cm)×50 μm I.D. uncoated capillary (CElect-FS50, Supelco, Bellefonte, PA, USA) was used for separations. Detection wavelength was 254 nm. Data acquisition and analysis was carried out on a Shimadzu (Tokyo, Japan) C-R6A integrator. Operating conditions were as follows: sampling time of 50 mbar for 0.2 min (hydrostatic injection); applied voltage of 20 kV; ambient temperature (22–24°C).

To prevent sample carry-over, the capillary was flushed with the running buffer for 40 s under pressure (2000 bar) and then a voltage of 20 kV was applied for 20 s before injection.

2.2. Materials and reagents

Glycyrrhizic acid ammonium salt (95% purity) and 17α-methyltestosterone (MT) (99% purity) were purchased from Fluka (Buchs, Switzerland). 18β-glycyrrhetinic acid (analytical-reagent grade) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Anhydrous sodium tetraborate, sodium dihydrogenphosphate (NaH_2PO_4), sodium dodecyl sulphate (SDS), tetrabutylammonium bromide (TBAB), sodium hydroxide, urea, Sudan III and methanol were purchased from Fluka, Sigma or Aldrich (Milwaukee, WI, USA). All solutions were prepared with water purified on a NANOpure (Barnstead, Dubuque, IA, USA) system. Methanol was of HPLC grade, and the other chemicals were of analytical-reagent grade.

Standard solutions of GRA and MT (used as internal standard) were prepared by dissolving in methanol, while that of GZA was prepared in water (each at 1 mg ml^{-1}). All solutions were stored in the dark at 4°C; they were stable for four months.

The electrolyte was made up of 20 m*M* NaH_2PO_4, 20 m*M* sodium tetraborate, 20 m*M* TBAB and 50 m*M* SDS. The pH was 8.7.

2.3. Sample preparation

SPE cartridges packed with silica-bonded C_{18} groups (200 mg packing weight, 3-ml reservoir volume) were obtained from Varian (Sunnyvale, CA, USA), and were preconditioned sequentially with 2.5 ml of methanol and 2.5 ml of 0.2 m*M* TBAB by using a Supelco vacuum manifold.

To 0.5 ml urine sample or 1 ml plasma sample, 0.4 g urea, 0.1 ml of 0.5 *M* sodium hydroxide, 10 μl of the MT internal standard and 0.2 ml of 0.5 *M* TBAB were added. The solution was vortex-mixed for 1 min to ensure homogeneity, and was then passed through the previously conditioned SPE cartridge at a rate of ca. 1 ml min^{-1}. After washing the cartridge with 2.5 ml of 5 m*M* TBAB and 2.5 ml of methanol–water (30:70, v/v), the compounds of interest were eluted into a test tube with 2 ml of methanol. The eluate was dried at room temperature

with the aid of a stream of nitrogen, and reconstituted as the analytical sample with 0.2–0.3 ml of the buffer (which was also used as the MEKC background electrolyte).

2.4. Calibration curve

The calibration curve was prepared from drug-free urine or plasma spiked with GZA and GRA at five concentrations ranging from 40–200 μg ml^{-1} (GRA) and 20–160 μg ml^{-1} (GZA). The spiked samples were extracted as described.

2.5. Recovery tests

Blank urine or plasma samples were spiked with three different amounts each of GZA (covering the range 40–200 μg ml^{-1}), and GRA (20–160 μg ml^{-1}). The samples were prepared in triplicate and extracted as previously described.

3. Results and discussion

3.1. The role of TBAB

In our procedure, TBAB was used both in sample preparation and MEKC separation. TBAB was used to form ion pairs with the (deprotonated) GZA and GRA anions before the actual SPE operation. In this way, the ion pairs were retained on the cartridge while other interfering materials were eluted by the methanol–water.

The role of tetraalkyammonium (TAA) salts in the running buffer for MEKC has been studied [25]. The addition of TBAB makes the separation mechanism more complex than either capillary zone electrophoresis or MEKC without such TAA salts, because two separation mechanisms may be involved. It is likely that TBAB combined with the anionic SDS micelle as a counterion (in competition with the sodium cation), thus altering the characteristics of the micelle. The anionic solutes could also form ion pairs with TBAB. These ion pairs would partition between the micellar and the aqueous phases, as would the free anionic solutes.

3.2. Optimization of the buffer system

In capillary electrophoresis, the buffer system plays an important role in the separation. In order to obtain good separation and sensitive detection at the same time, several random trials based on univariate optimization were carried out. Finally, a buffer which contained 20 m*M* sodium tetraborate, 20 m*M* NaH_2PO_4, 50 m*M* SDS and 20 m*M* TBAB (at pH 8.7) was selected as the background electrolyte. A typical chromatogram for urine sample obtained by this buffer system under a voltage of 20 kV is shown in Fig. 2. Trace methanol was used to determine the electroosmotic flow. Sudan III was used as the marker of the micelle velocity to ensure that GRA could be separated within the separation range of MEKC. However, methanol and Sudan III were not added in the subsequent analysis of GZA and GRA. Fig. 3 shows a typical chromatogram for a plasma

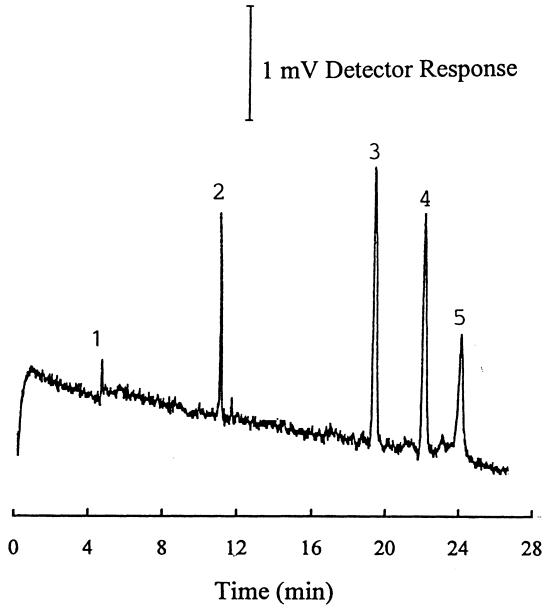

Fig. 2. Chromatogram of urine sample spiked with GZA (8.6 μg ml^{-1}), GRA (5.0 μg ml^{-1}) and MT (5.0 μg ml^{-1}). Peaks: 1=methanol; 2=GZA; 3=MT; 4=GRA; 5=Sudan III. Sample solvent: CE running buffer. CE conditions as in Section 2.1.

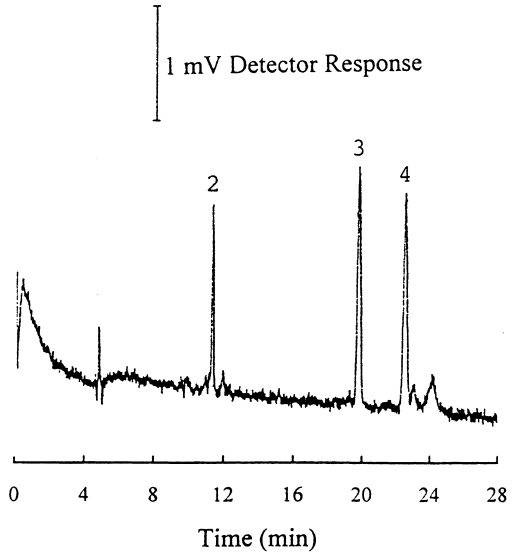

Fig. 3. Chromatogram of plasma sample spiked with GZA (4.3 μg ml^{-1}), GRA (2.5 μg ml^{-1}) and MT (2.5 μg ml^{-1}). Peaks: 2=GZA; 3=MT; 4=GRA. Sample solvent: CE running buffer. CE conditions as in Section 2.1.

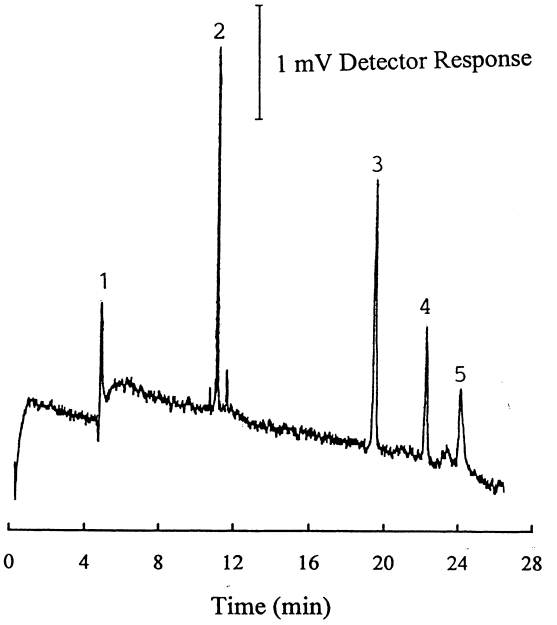

Fig. 4. of urine sample spiked with GZA (8.6 μg ml^{-1}), GRA (5.0 μg ml^{-1}) and MT (5.0 μg ml^{-1}). Peaks: 1=methanol; 2=GZA; 3=MT; 4=GRA; 5=Sudan III. Sample solvent: methanol–water (90:10, v/v). CE conditions as in Fig. 2.

sample. Experiments indicated that no interfering components were present in blank urine (0.5 ml) and plasma (1 ml) after SPE.

3.3. Effect of sample solvent on detection sensitivity

It was found that the sample solvent has a great effect on the detection sensitivity of both GZA and GRA. Fig. 4 shows a chromatogram when methanol–water (90:10, v/v) was used to dissolve the residue after SPE. In comparison with the analysis whose chromatogram is shown in Fig. 2, whereby the electrophoretic running buffer was used to dissolve the residue, the sensitivity for GZA was nearly doubled but for GRA it decreased to half. This is probably due to the ion-pairs formed as stated earlier, and their interactions with the SDS micelles.

GZA dissolves easily in water and thus is more hydrophilic than GRA. The ion-pair formed by GZA and TBAB is not stable so that the interaction of the (deprotonated) GZA anion with the SDS micelle is the major separation mechanism in this case. If the sample media is methanol and water, the difference of the conductivity of the buffer and the sample media is large enough so that the sample stacking will happen. This increases the sensitivity for GZA.

As for GRA, the ion pair formed by it and TBAB is more stable than that formed by GZA. The interaction of the ion pair with the SDS micelles is the major separation mechanism in this case. If the sample is dissolved in the solvent system that contains a high concentration of methanol, the latter solvent in the sample plug will diffuse into the surrounding buffer solution, causing the breakdown of the micelle. As a result, it would take a longer time for the ion-pair to be incorporated into the SDS micelle. A significant dilution of the sample band and loss of detection sensitivity will consequently occur [26].

3.4. Linearity

Calibration curves were constructed as described in Section 2.4 with GZA-, GRA- and MT-free human plasma and urine. Linearity data for GZA and

Table 1
Calibration data for GZA and GRA (n=5)

Sample	Compound	Calibration curve[a]	Correlation coefficient (R^2)	Working range ($\mu g\ ml^{-1}$)
Plasma	GZA	$y=0.00360x+0.133$	0.9904	40–200
	GRA	$y=0.0104x-0.0151$	0.9983	20–160
Urine	GZA	$y=0.00787x+0.120$	0.9915	40–200
	GRA	$y=0.0211x+0.0356$	0.9946	20–160

[a] Where y is the peak height and x is the concentration of the compound.

GRA are listed in Table 1 which shows correlation coefficients (R^2) exceeding 0.99.

3.5. Recovery

The efficiency of the method was determined by comparing the amounts of GRA and GZA recovered from the spiked samples with those added. Recoveries ranged from ca. 87–107%, as shown in Table 2.

3.6. Precision and detection limits

Within-run and between-run (over three days) precision results (with R.S.D.s ranging from 2.6–7.8%) and detection limits (based on a signal-to-noise ratio of 3) for GZA (0.8 $\mu g\ ml^{-1}$ for plasma, and 1.6 $\mu g\ ml^{-1}$ for urine) GRA (1 $\mu g\ ml^{-1}$ for plasma, and 2 $\mu g\ ml^{-1}$ for urine) in spiked samples are given in Table 3.

4. Conclusions

A new method has been established for the analysis of glycyrrhizic acid and 18β-glycyrrhetinic acid in human plasma and urine by MEKC. Both compounds can be determined with acceptable recovery, accuracy and precision from human plasma and urine after SPE with C_{18}-packed cartridges. By combining SPE (extraction and concentration) with MEKC, comparable limits of detection with other methods were obtained. MEKC additionally has the advantage of simplicity, speed of analysis and relatively lower cost in contrast to, for example, HPLC [24]. Moreover, in the present work, all the components were eluted cleanly, ensuring no carryover to the subsequent analysis.

Table 2
Recovery data for GZA and GRA

Sample	Compound	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
Plasma	GZA	20.0	17.3	86.5
		80.0	72.1	90.1
		160.0	147.0	91.9
	GRA	40.0	38.2	95.5
		120.0	115.0	95.8
		200.0	204.0	102.0
Urine	GZA	20.0	18.1	90.5
		80.0	71.4	89.3
		160.0	149.0	93.1
	GRA	40.0	39.4	98.5
		120.0	128.0	107.0
		200.0	193.0	96.5

Table 3
Precision and detection limits for GZA and GRA

Sample	Compound	Within-run R.S.D. (%) (n=8)	Between-run R.S.D. (%) (n=3)	Detection limit ($\mu g\ ml^{-1}$)
Plasma (1 ml)	GZA	4.9	7.8	0.8
	GRA	3.3	4.5	1.0
Urine (0.5 ml)	GZA	4.5	5.7	1.6
	GRA	2.6	4.6	2.0

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