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Simultaneous determination of the stereoisomers of guggulsterone in serum by high-performance liquid chromatography[1]

Niharika Verma, Shio Kumar Singh, Ram Chandra Gupta*

Pharmacokinetics and Metabolism Division, Central Drug Research Institute, Post Box 173, Lucknow 226 001, India

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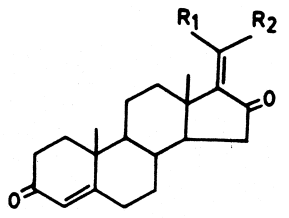
Abstract

Simultaneous separation of *E*- and *Z*-guggulsterone, which is the main ingredient of 'Guggulip', an ayurvedic drug, was accomplished by HPLC on a C_{18} column using methanol, acetonitrile, buffer and tetrahydrofuran as a mobile phase. The compounds were monitored at 248 nm on a photodiode array detector. The assay method was used for the simultaneous determination of stereoisomers (*E* and *Z*) of guggulsterone in spiked serum and dosed (50 mg/kg, p.o.) rats. The recoveries of *E*- and *Z*-isomers from serum samples were always greater than 90%. The calibration graph was linear over the range of 25–2500 ng/ml for *Z*- and *E*-isomers. Lowest quantitation limit of *Z*- and *E*-guggulsterones was 25 ng/ml. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Guggulsterone

1. Introduction

'Guggulip', an ayurvedic drug, is being marketed in India as hypolipidaemic agent [1]. It is derived from the plant *Commiphora mukul* [2] and contains several plant sterols, diterpenes, steroids, esters and higher alcohols [3]. The main ingredient of the drug is guggulsterone (**1**, Fig. 1; 4,17(20)-pregnadiene-3,16-dione) which possess marked cholesterol- and lipid-lowering activity [4–6]. It is also found to be efficacious in the treatment of rheumatoid arthritis, obesity and allied disorders [7]. Guggulsterone is currently being developed in this institute as new hypolipidaemic agent. Two different arrangements of CH_3 at C_{20} in three-dimensional space and the



1

	R1, R2	Compound	Absolute Configuration
1a	R1=H, R2=CH3	Guggulsterone	Z−
1b	R1=CH3, R2=H	Guggulsterone	E−

Fig. 1. The chemical structure of guggulsterone (**1**), and *Z*-isomer (**1a**) and *E*-isomer (**1b**).

*Corresponding author

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hindered rotation about the carbon–carbon double bond at C_{17} and C_{20} classifies the guggulsterone into *Z*-[4,17(20)-*cis*-pregnadiene-3,16-dione, (**1a**)] and *E*-[4,17(20)-*trans*-pregnadiene-3,16-dione, (**1b**)] stereoisomers. There are several reasons for the interest in the two isomers of (**1**), including differences in their pharmacological actions and pharmacokinetic behaviour.

Two methods have been reported for the determination of isomeric mixture of **1** [8,9], and one for the determination of individual isomers, **1a** and **1b**, in pharmaceutical preparations [10]. There is no published assay technique for simultaneous estimation of the individual isomers of **1** in biological fluids. To support the development of **1** as a candidate hypolipidaemic agent, a rapid, accurate and precise high-performance liquid chromatographic assay for the simultaneous estimation of **1a** and **1b** in rat serum has been developed, validated and used for estimation of **1a** and **1b** in the serum of rats.

2. Experimental

2.1. Reagents and solvents

Isomeric mixture **1** was supplied by Cipla Ltd., Bombay, India. Pure standards (>99%) of **1a** and **1b** isomers were obtained by column chromatography of **1** followed by recrystallization. HPLC-grade hexane, methanol and acetonitrile were obtained from Merck Bombay, India, and dipotassium hydrogen phosphate salt was supplied by S.D. Fine Chemical, Bombay, India. Triply distilled water from an all-quartz apparatus was used for preparing buffer.

2.2. Apparatus and chromatographic conditions

The HPLC instrument consisted of a Model PU 4003 controller and pump (Pye Unicam Ltd., Cambridge, UK), a Model 7125 injector with a 20-μl loop (Rheodyne, Burkeley, CA, USA). Separation from endogenous serum components was achieved on C_{18} (10 μm) cartridge analytical column (100× 4.6 mm I.D.) coupled with a guard column (30×4.6 mm I.D., Pierce Chemicals Co., Rockford, IL, USA) containing the same material. Column eluant was monitored at 248 nm on a Waters 991 photodiode array detector, and recorded and integrated using Waters 991 software version 5.11 (Millipore Corporation, Milford, MA, USA) on a NEC personal computer. A Model SVC-200 Savant Speed-Vac concentrator (Savant instruments, New York, USA) was used to evaporate the organic solvent after extraction.

The mobile phase, acetonitrile–methanol–50 m*M* dipotassium hydrogen orthophosphate (adjusted to pH 7.2 with 20% orthophosphoric acid)–tetrahydrofuran (40:25:34.5:0.5), was filtered and degassed before use. The chromatography was performed at ambient temperature with a flow-rate of 1 ml/min.

2.3. Preparation of stock and standard solution

Stock solutions of **1a** and **1b** (1 mg/ml) were separately prepared by dissolving 5 mg of each in 5 ml of methanol and stored at 4°C. Combined (**1a**+ **1b**) standard solutions of strength 1, 10 and 100 μg/ml were prepared from the stock solutions of **1a** and **1b** in acetonitrile. The mobile calibration standards in the range of 25–2500 ng/ml were prepared by diluting specific volumes of standard solution of **1a** and **1b** in a ratio of 1:1 in 10 ml mobile phase and stored at 4°C. These standard solutions were found to be stable for a period of 6 months at 4°C. Serum calibration standards were prepared by transferring appropriate volumes of specific stock solutions of **1a** and **1b**, in a ratio of 1:1, into dry tubes and evaporating the solvent under a stream of nitrogen before adding the required volume of serum to get a calibration range of 25–2500 ng/ml, vortex-mixed, stored at −20°C and thawed before use.

2.4. Sample preparation

Drug-free or spiked serum (0.25 ml) was extracted in a 10-ml (75×5 mm) glass tube with 2× 2 ml hexane by vortex-mixing for 1 min and centrifuging for 10 min at 1000 *g*. The organic layer was separated by snap-freezing the aqueous layer in liquid nitrogen, and the organic phase was evaporated to dryness under reduced pressure in a Savant-Vac concentrator. The residue was reconstituted in 50 μl of mobile phase and analysed on HPLC.

2.5. Validation

2.5.1. Accuracy and precision

Aliquots (0.25 ml) of spiked serum samples ($n=5$) at four concentration levels of 25, 50, 250, 2500 ng/ml were processed as mentioned above, and the concentrations were determined by interpolation on the corresponding calibration graphs. The accuracy of the method was calculated on the basis of the differences in the mean calculated and added concentrations, while the precision was obtained by calculating the within-day and inter-day coefficients of variation (C.V.). Acceptance limits of ±20% at the lower limit of quantitation (LLOQ) and ±15% at other concentrations in the calibration range were used for validation [11].

2.6. Effect of freeze–thaw cycles on the stability of *1a* and *1b* in serum

Replicates ($3\times5=15$) of serum calibration standards at low (50 ng/ml), medium (250 ng/ml) and high (2500 ng/ml) concentrations were prepared. One set of three concentrations in triplicate was analysed on day 1 (no freeze–thaw cycle) before storing the remaining sets at −20°C in sealed tubes. The other similar sets were analysed after one, two, four and seven freeze–thaw cycles. Thawing was achieved by keeping the tubes at room temperature for 30 min. Results of day 1 were taken as standards (100%) and the rest were compared with the first day result as percent deviation.

2.7. In-process stability of *1a* and *1b* in the dry extract at −20°C

To study the stability in the processed samples (dry serum extracts), serum calibration standards prepared at low, medium and high concentrations were extracted as mentioned above. One set in triplicate was reconstituted with mobile phase and analysed on the same day (day 1). The remaining tubes containing dry residue were sealed and stored at −20°C. Other similar sets were reconstituted and analysed on days 2, 3, 4 and 8. Results of day 1 were taken as standard (100%) and the rest were compared with the first day result as percent deviation.

2.8. Extraction efficiency

Spiked serum samples ($n=5$) were analysed according to the method described above. Aliquots of the reconstituted extract were injected on HPLC and the peak heights of **1a** and **1b** were measured. Absolute recovery of **1a** and **1b** was calculated by comparing the observed peak heights with those obtained by direct injection of mobile calibration standard.

3. Results and discussion

3.1. Selectivity and specificity

The representative chromatograms of mobile standard **1a** (A), **1b** (B), an extract of drug-free rat serum (C), spiked serum (D), serum sample from dosed rats (50 mg/kg, p.o.) at 4 h (E) and serum sample of rat at 24 h (F) are shown in Fig. 2. Interfering peaks were not present in chromatograms at the elution position of **1a** (7.2±0.2 min) or **1b** (5.5±0.2 min).

3.2. Linearity and reproducibility

Linear least-square regression analysis of the calibration graph demonstrated linearity in the range of 25–2500 ng/ml for both **1a** and **1b**. A typical standard curve could be described by the equations $y=0.341x-2.36$ (**1a**) and $y=0.33x-2.34$ (**1b**) with a correlation coefficient (r) of >0.999 for both isomers. The reproducibility and accuracy of the method were determined by processing spiked serum samples at 25, 50, 250, 2500 ng/ml of **1a** and **1b** with respect to the calibration curves run each day. Five samples were analysed at each concentration level. The within-day coefficients of variation ($n=5$) were less than 4 and 3%, and day-to-day coefficients of variation ($n=5$) of samples analysed on five different days were less than 3% for **1b** and **1a** (Table 1).

3.3. Recovery

Recovery of **1a** and **1b** from the serum was calculated by comparing the peak heights obtained from serum calibration standards with the calibration

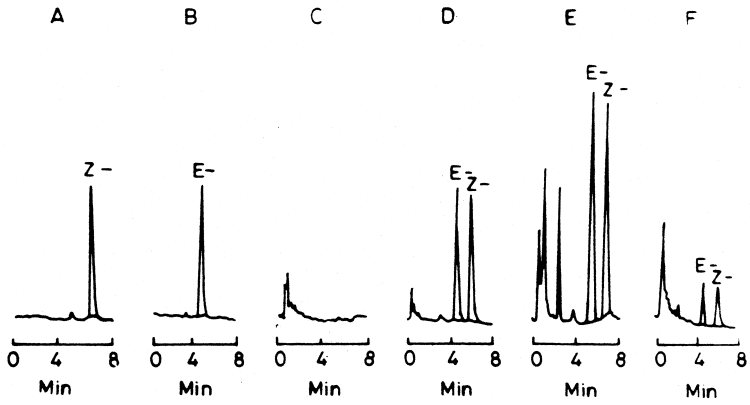

Fig. 2. Chromatogram of (A) standard containing 200 ng/ml of **1a**, (B) standard containing 200 ng/ml of **1b**, (C) drug-free rat serum, (D) serum containing 100 ng/ml of **1a** and 100 ng/ml of **1b** after two-fold concentration, (E) serum sample of rat at 4 h (**1a**, 144.88 and **1b**, 152.65 ng/ml), (F) serum sample of rat at 24 h (**1a**, 24.61 and **1b**, 28.44 ng/ml) post-oral dose of **1a** (50 mg/kg).

curve in mobile phase. Recovery of both isomers from serum at 25, 50, 250 and 2500 ng/ml was more than 90% (Table 2).

3.4. Stability in freeze–thaw cycle

The concentrations after one, two, four, and seven freeze–thaw cycles were compared with concentrations obtained from spiked serum samples not subjected to freeze–thaw cycles: **1a** and **1b** were stable up to four freeze–thaw cycles at all three concentration levels [12]. The observed variations in accuracy and precision during the freeze–thaw cycles were within ±10% (Fig. 3) and similar to the inter/intra-day variations. This indicated that errors were inherent with the analysis. There was no trend in calculated concentrations after different freeze–thaw cycles, indicating that **1a** and **1b** were stable in serum matrix under these conditions. Also, no extra peaks of degradation products were observed. Later analysis of QC samples, in triplicate, at low (50 ng/ml), medium (250 ng/ml) and high (2500 ng/ml) levels of *E*- and *Z*-isomers, upon storing at −20°C for 1, 13 and 30 days, showed that the two

Table 1
Accuracy and precision for **1a** and **1b**

Spiked Serum concentration (ng/ml)	Stereoisomers					
	Z-isomer			*E*-isomer		
	Obs.	C.V. (%)	Accuracy (%)	Obs.	C.V. (%)	Accuracy (%)
Within-day (n=5)						
25	24.97±0.50	2.00	−0.12	24.93±0.50	2.00	−0.28
50	48.71±1.80	3.69	−2.58	49.54±0.63	0.64	−0.92
250	249.76±1.90	0.76	−0.10	251.21±1.86	0.37	+0.48
2500	2505.16±5.8	0.23	+0.21	2500.39±2.41	0.05	+0.02
Day-to-day (n=5)						
25	25.07±0.41	1.63	+0.28	24.88±0.58	2.33	−0.48
50	50.75±1.23	2.42	+1.5	48.78±2.85	2.92	−2.45
250	249.01±1.30	0.52	−0.40	265.05±6.16	1.16	+6.02
2500	2500.39±7.37	0.29	+0.02	2500.29±1.70	0.03	+0.01

Obs., observed concentration (mean±S.D.).

Table 2
Absolute recoveries of **1a** and **1b** from spiked serum ($n=5$)

Concentration (ng/ml)	Stereoisomers			
	Z-isomer		*E*-isomer	
	Extraction efficiency (%)	C.V.[a] (%)	Extraction efficiency (%)	C.V.[a] (%)
25	92	6.29	91	4.8
50	94	4.9	92	7.3
250	95	6.5	92	6.2
2500	95	6.3	93	5.9

[a]C.V., (S.D./mean)×100.

isomers are stable under the storage conditions for 30 days. The deviations in the calculated concentrations in the QC samples after 13 and 30 days of storage were less than 10% and no trend was observed in the recovery of the isomers.

3.5. In-process stability

Considering the results of day 1 as standards (100%) the results of days 2, 3, 4 and 8 were compared as percent deviation. The result showed that both **1a** and **1b** are stable up to 8 days at all three concentrations (Fig. 4). No trends were observed, and the observed variations were due to inter/intra-batch variations and were within acceptance limits.

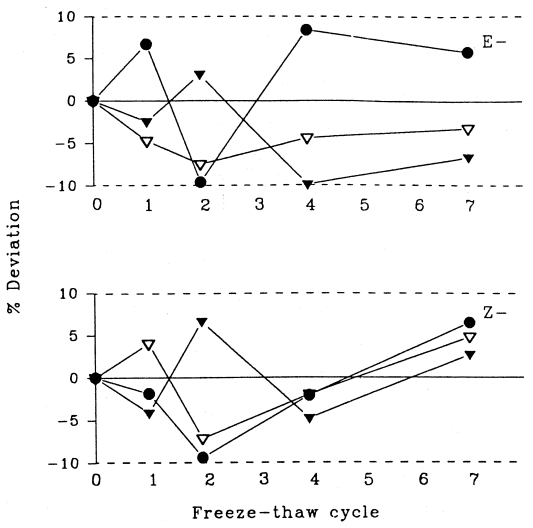

Fig. 3. Effect of freeze–thaw cycles on the stability of **1a** and **1b** in spiked serum samples.

3.6. Applicability of the assay method in pharmacokinetic studies

The developed assay method was found to be sensitive and specific. This method was applied for the estimation of **1a** and **1b** in rat serum after a single oral dose (50 mg/kg) of **1a**. It was of interest to note that the chromatograms of serum samples from dosed rat showed an additional major peak at a retention time of 5.50 min (Fig. 2). This peak was identified on the basis of its UV spectrum and retention time as **1b** (*E*-isomer). Repeated analysis of analytical standards and spiked serum samples during the assay validation showed that the $Z \rightarrow E$ conversion does not take place in the spiked serum

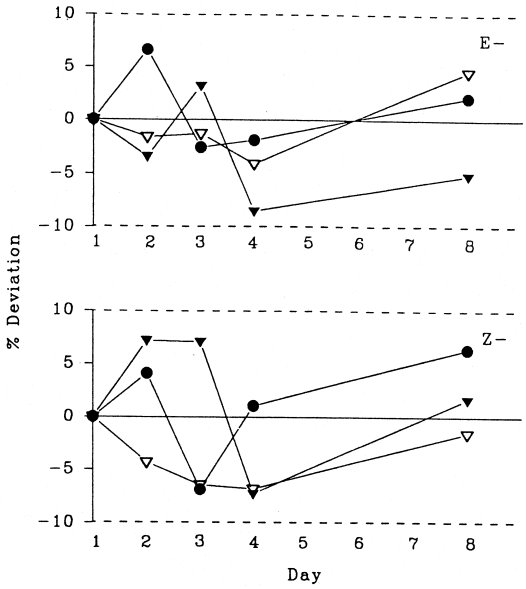

Fig. 4. Stability of **1a** and **1b** in dry extract of serum at −20°C.

samples, and hence the formation of the *E*-isomer can be attributed solely due to the in vivo process. Detailed studies to characterize the mechanism and site of this conversion, and their disposition are in progress.

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