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Short communication

Sensitive high-performance liquid chromatographic assay method for the determination of guggulsterone in serum☆

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Abstract

Guggulsterone (I) is a new hypolipidemic agent, being developed at CDRI (Lucknow, India). A sensitive high-performance liquid chromatographic assay in serum has been developed and validated for the determination of guggulsterone in serum for pharmacokinetic studies. This assay method consists of extraction of the drug with hexane from spiked human serum samples. Separation was achieved using C_{18} reversed-phase column coupled with photodiode array detector, and an acetonitrile–water mixture as mobile phase. The method described herein is simple and has limit of quantitation of 10 ng/ml as compared to 200 ng/ml by the previous reported method. The standard curve was linear over the range of 10–1000 ng/ml in mobile phase as well as in normal human serum. Analytical recovery of I added to serum was >90%. The reproducibility was determined by the inter- and intra-assay variations which were <10%.

1. Introduction

Hypercholesterolemia is a heterogenous group of disorders in lipid metabolism characterised by substantially elevated levels of total plasma cholesterol and low-density lipoprotein–cholesterol complex [1]. Hypercholesterolemia with or without triglyceridemia is one of the independent risk factors linked to increased mortality due to myocardial infarction [2]. Recently an Ayurvedic drug consisting of plant sterols and steroids derived from *Commiphora mukul* collectively known as "Guggulipid" has been marketed as hypolipidemic drug [3] in India. In the guggulipid, 4,17(20)-pregnadiene-3,16-dione (Guggulsterone, I, Fig. 1) is the main ingredient among several other components such as diterpenes, steroids, esters and higher alcohols [4]. Guggulsterone possesses marked cholesterol and lipid lowering activity [5–7] and is being developed in this institute as a new hypolipidemic agent.

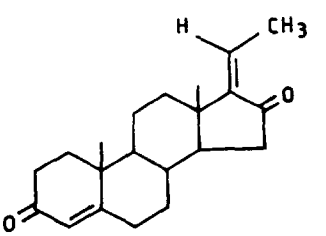

Fig. 1. Structure of guggulsterone (I).

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A high-performance liquid chromatographic (HPLC) method for the determination of I in blood plasma has been reported [8]. Limit of quantitation was 200 ng/ml with UV detection. Lack of sensitivity and associated problems of extraction with chloroform necessitated the development of a simple and sensitive assay method for the determination of I in serum for pharmacokinetic studies. The assay was used for quantitation in rat serum after oral administration of I.

2. Experimental

2.1. Reagents and standards

Analytical standard (>99%) of I was supplied by Cipla, Bombay, India. HPLC-grade acetonitrile was obtained from S.D. Fine Chem. (Bombay, India). Hexane [E. Merck (India) Limited, Bombay, India] was purified before use. Triply distilled water from an all-quartz apparatus was prepared and used as solvent.

2.2. Apparatus and chromatographic conditions

The HPLC system was equipped with a Model Pye Unicam PU 4003 controller and pump (Pye Unicam, Cambridge, UK), a Model 7125 injector with a 100-μl loop (Rheodyne, Berkeley, CA, USA) and a Waters Model 991 Photodiode array detector (Millipore, Milford, MA, USA). Separation was accomplished on a 100×4.6 mm I.D. (10 μm) C_{18} cartridge analytical column coupled with 30×4.6 mm I.D. guard column (Pierce Chemical, Rockford, IL, USA) of the same material. Chromatograms were recorded and integrated by Waters 991 software (version 5.11, Millipore) on a NEC personal computer and plotted by a Waters 5200 printer/plotter (Nihon Millipore, Tokyo, Japan). A Model SVC-200 H Savant Speed-Vac concentrator (Savant Instruments, New York, USA) was used to evaporate the organic solvent after extraction.

The mobile phase consisted of acetonitrile and water in the ratio 65:35. It was filtered and degassed before use. Chromatography was performed at ambient temperature at a flow-rate of 1 ml/min. Effluents were monitored at 248 nm.

2.3. Stock and standard solution preparation

Stock solution of I (100 μg/ml) was prepared by dissolving 10 mg of I in 100 ml of methanol. Working standards in mobile phase (10–50 μg/ml) were prepared by sequential dilution of stock solution of I. The standards in mobile phase were made from working standards in the range of 10–1000 ng/ml. Serum calibration standards were prepared by adding I to drug-free serum to obtain concentrations ranging from 10 to 1000 ng/ml. The serum standards were vortex-mixed and stored at −80°C until analysis.

2.4. Extraction

Drug-free or spiked serum (0.5 ml) was extracted in a 10-ml (75 mm × 5 mm) glass test tube with 2×3 ml hexane by vortex-mixing for 1 min and centrifuging for 10 min at 1000 *g*. Organic layer was separated by snap-freezing the aqueous layer in liquid nitrogen and evaporated to dryness under reduced pressure in Savant Speed-Vac concentrator. The residue was reconstituted in 250 μl of mobile phase and analysed by HPLC using a 100-μl injection loop.

2.5. Accuracy and precision

Compound I was added to serum ($n = 5$) at three concentration levels. Aliquots (0.5 ml) of serum spiked with 10, 100 and 500 ng/ml of I were processed as described above and concentrations were determined against the standard curve in serum. The accuracy of the method was calculated based on the difference between the mean calculated and added concentrations, while precision was determined by calculating the within and inter-day coefficients of variation (C.V.).

Table 1
Extraction efficiency of I from spiked serum samples ($n = 5$)

Concentration (ng/ml)	Extraction efficiency (%)	Coefficient of variation[a] (%)
10	93.21	3.97
100	91.66	6.70
500	101.02	5.33

[a] Coefficient of variation = (S.D./mean) · 100.

2.6. Extraction efficiency

Serum spiked with 10, 100 and 500 ng/ml of I was analysed according to the described method without any added internal standard (Table 1). Carefully measured aliquots of the reconstituted extract were injected and peak heights of I were measured. Absolute recovery of I was calculated by comparing these peak heights with those obtained by direct injection of drug standards in mobile phase.

3. Results and discussion

3.1. Chromatography

The HPLC method described herein overcomes the problems associated with the previous method like lack of sensitivity, extraction with chloroform, introduction of large amounts of endogenous impurities into the column. Fig. 2 illustrates chromatograms of a standard containing 100 ng/ml of I in mobile phase (A), drug-free serum from a normal rat (B), serum standard containing 100 ng/ml of I (C), and a serum sample from a rat treated with a single 50 mg/kg oral dose of I (D). Under the chromatographic conditions the retention time of I was 6.2 ± 0.2 min. Extracted serum impurities did not interfere since they eluted either at the solvent front or after the peak of interest.

The quantitation limit of I in serum was 10 ng/ml after 2-fold concentration using 0.5 ml of serum. This method provides adequate sensitivity and specificity for monitoring serum levels of I.

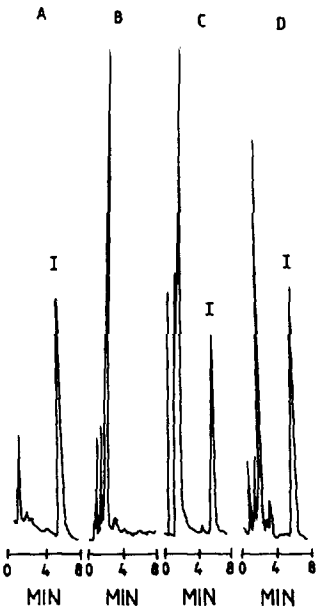

Fig. 2. Chromatograms of (A) standard containing 100 ng/ml of I, (B) drug-free serum, (C) serum containing 100 ng/ml of I, and (D) rat serum at 4 h after a 50 mg/kg oral dose of I.

3.2. Selectivity and peak purity

The complementary use of the photodiode-array detection for identifying the eluted compound by its retention time/wavelength absorption is more reliable than the UV detection alone, which gives only the retention time [9,10]. The photodiode-array detection allowed the observation of the full UV spectrum of each peak as it eluted from the chromatographic column. Hence the detection of other components could be observed. Peak purity and UV absorption at 248 nm of eluted peaks in treated rat serum samples were checked by spectral analysis in photodiode-array detector and compared with the standard spectral data. The data were found to be identical in both the cases and purity of the peaks were more than 99% indicating the absence of interference from endogenous substances of serum.

Table 2
Precision and accuracy of the method

Spiked concentration (ng/ml)	Concentration found (mean ±S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy (% of mean deviation)
Within-day (n = 5)			
10	11.16 ± 0.54	4.02	+11.60
100	93.63 ± 0.59	6.82	−6.37
500	488.24 ± 2.16	4.70	−2.35
Day-to-day (n = 5)			
10	11.06 ± 0.53	4.01	+10.60
100	106.15 ± 0.84	8.03	+6.15
500	479.95 ± 3.03	7.62	−4.01

3.3. Linearity and reproducibility

Linear least square regression analysis of the calibration graph demonstrated linearity in the range 10–500 ng/ml. A typical standard curve could be described by the equation $y = 0.092x - 0.027$ ($r = 0.999$). The reproducibility and accuracy of the method were determined by processing spiked serum samples at 10, 100 and 500 ng/ml with respect to the calibration curve run each day. Five samples were analysed at each concentration level. The within-day coefficients of variation ($n = 5$) were less than 7% and day-to-day coefficients of variation ($n = 5$) of samples analysed on five different days were less than 8% (Table 2).

3.4. Application of the method in clinical pharmacokinetics

The assay method described here was applied to determine the concentration–time profile of I in normal healthy Sprague–Dawley rats after a single oral dose (50 mg/kg). Chromatograms of blank and spiked rat serum are shown in Fig. 2B and 2C. No interfering peaks were present at the elution region of I. Further studies are in progress to evaluate the pharmacokinetic parameters of I.

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