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Short communication

Simultaneous liquid chromatographic determination of centchroman and its 7-demethylated metabolite in serum and milk☆

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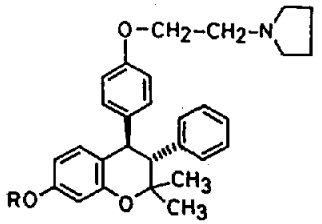
Abstract

A precise and sensitive high-performance liquid chromatographic assay was developed and validated for determination of centchroman (I) and its 7-demethylated metabolite (II) in human serum and milk. The serum, at alkaline pH, was extracted with diethyl ether. In the case of milk, after precipitation of the milk protein with acetonitrile, the supernatant was evaporated to dryness and then extracted with diethyl ether at alkaline pH. After solvent evaporation the residue was reconstituted in mobile phase. Separations were accomplished by reversed-phase liquid chromatography using a Spheri-5 cyano column. Recoveries of I and II were always >95%. Excellent linear relationships ($r > 0.999$) were obtained between the measured and added concentration ratios of the corresponding serum and milk concentrations over a range of 1 to 1000 ng/ml and 2.5 to 1000 ng/ml for I and II, respectively.

1. Introduction

Centchroman, 3,4-*trans*-2,2-dimethyl-3-phenyl-4-(*p*-pyrrolidinoethoxy) phenyl-7-methoxychroman (I, Fig. 1), is being marketed in India as a non-steroidal oral contraceptive [1–3] and is in phase III clinical trials for the treatment of advanced breast cancer [2]. Its putative metabolite [3,4-*trans*-2,2-dimethyl-3-phenyl-4-(*p*-pyrrolidinoethoxy) phenyl-7-hydroxychroman] (II, Fig. 1), is reportedly 20-fold more active than I [4].

A high-performance liquid chromatographic (HPLC) assay for the determination of I in serum has been reported [5]. It required a relatively large sample size (1–2 ml) for each analysis and had a sensitivity of 2 ng/ml. There is no report of the determination of either I or II



I: R=CH_3
II: R=H

Fig. 1. Structure of centchroman (I) and 7-desmethyl centchroman (II).

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in milk, which would be essential to assess the dose ingested by neonates via breast milk, if I is recommended as contraceptive agent in lactating women.

In this report, we describe an HPLC method which ensures sensitive and simultaneous determination of I and II using 0.5 ml of human serum or milk.

2. Experimental

2.1. Chemicals and reagents

Pure reference standards of I and II as free bases were prepared [6] in-house. Analytical grade potassium hydrogen orthophosphate, potassium hydroxide, and orthophosphoric acid were purchased from Glaxo (Bombay, India), while di-potassium hydrogen orthophosphate, HPLC grade acetonitrile and methanol were procured from S.D. Fine Chem. (Bombay, India). Diethyl ether (anaesthetic grade, IP; Ether India, Bombay, India) was distilled after treatment with 1% aqueous potassium hydroxide solution (250 ml/l) followed by washing with triply-distilled water to neutral pH. Triply-distilled water from an all-quartz apparatus was used in this study. Control serum was purchased from a local blood bank and cow milk was supplied by a milkman for use as control.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a solvent delivery pump (Model PU 4015, Pye Unicam, Cambridge, UK), a variable-wavelength fluorescence detector (Model RF-535, Shimadzu, Kyoto, Japan) set at an excitation wavelength of 280 nm and an emission wavelength of 310 nm, and a chart recorder/integrator (Model C-R5A Chromatopac, Shimadzu). The samples were injected onto a Spheri-5 cyano cartridge column (100 × 4.6 mm I.D., 5 μm) preceded by a precolumn packed with the same material (30 × 4.6 mm I.D., 5 μm; Pierce Chemical Co., Rockford, IL, USA) using a syringe loading injector (Model 7125, Rheodyne, Berkeley, CA, USA) with a fixed 50-μl loop. A Speedvac concentrator (Model SVC-200H, Savant Instruments, New York, NY, USA) was used to evaporate the organic solvent after extraction.

The mobile phase acetonitrile–20 m*M* potassium dihydrogen orthophosphate (adjusted to pH 3 with 20% orthophosphoric acid) (58:42, v/v) was filtered and deaerated before use. Chromatography was performed at ambient temperature. The flow-rate was 1.5 ml/min.

2.3. Stock and standard solutions

Stock solutions of I and II (each 400 μg/ml) were separately prepared by dissolving 10 mg in 25 ml of methanol. Combined working standards of both I and II were prepared from the stock solutions in mobile phase in the range of 5 to 2500 ng/ml. The solutions were vortex-mixed and stored at 4°C.

Subsequent dilutions (500 ng/ml–200 μg/ml) were prepared from a 1:1 mixture (v/v) of the stock solutions of I and II with methanol so that the desired volumes (20–250 μl) could conveniently be delivered. The serum and milk standard samples were prepared by diluting appropriate quantities of the above combined mixture with 10 ml control serum or milk. The quantities added corresponded to concentrations of both I and II ranging from 1 to 1000 ng/ml. The standards were vortex-mixed, aliquots (0.5 ml) stored at −60°C and thawed before use.

2.4. Sample preparation

Serum

To 0.5 ml drug-free or spiked serum in a 15-ml glass tube was added 50 μl potassium hydroxide (1%, w/v) and the sample was vortex-mixed for 15 s. The alkaline sample was extracted with 2 × 3 ml diethyl ether by vortex-mixing for 1 min and centrifugation for 10 min at 1000 *g* followed by freezing of the aqueous portion in liquid nitrogen and decanting the organic phase into a 10-ml conical glass tube. The combined extract was evaporated to dryness under reduced pressure. The residue was dissolved in 0.1 ml mobile

phase and 85–95 μl of the reconstituted solutions were injected onto the HPLC system.

Milk

To 0.5 ml drug-free or spiked milk was added 2 ml acetonitrile in a 5-ml glass tube, followed by vortex-mixing for 1 min, standing at 4°C for 30 min and centrifugation for 10 min at 1000 *g*. The supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 0.2 ml di-potassium hydrogen orthophosphate (0.1 *M*, pH 8.7), and then extracted with 3 ml diethyl ether and processed as described above for serum.

2.5. Method validation

Selectivity and linearity

Drug-free serum or milk samples were routinely processed and analysed as described above. The resultant chromatograms were examined for the presence of endogenous impurities which could possibly interfere with the measurement of I and/or II.

The slopes, intercepts and correlation coefficients of the calibration curves were calculated by linear regression analysis.

Extraction efficiencies

The extraction efficiencies were determined by processing and analysing 0.5-ml aliquots of serum or milk standards containing 5, 50 and 500 ng/ml of I and II each as described above. The recoveries of I and II were calculated by comparing their peak heights with those obtained from the respective buffer standards of I and II injected directly.

Accuracy and precision

Drug-free serum or milk samples ($n = 6$) spiked with 5, 50 and 500 ng/ml of I and II were processed as described and concentrations were determined from the standard curves corresponding to each compound. The accuracy of the method was calculated from the differences between the mean calculated and added concentrations, while precision was determined by calculating the within- and inter-day coefficients of variation (C.V.). Limit of determination was defined as the concentration in serum and milk that resulted in a peak of approximately 3 times the noise level.

3. Results and discussion

Representative chromatograms of a standard containing 100 ng/ml (A), an extract of drug-free human serum or milk (B) and serum containing 20 ng/ml or milk containing 25 ng/ml of I and II (C) are shown in Figs. 2 and 3, respectively. Interfering peaks were not present in either chromatograms at the elution position of I (5.6 min) or II (4.0 min). The chromatographic peak of II appeared 1 min prior to I with a peak height approximately 2.5 times smaller than that of I.

The required accuracy and precision were achieved by the external calibration method and extraction efficiency was higher than 95% (Table 1), thus inclusion of an internal standard was not

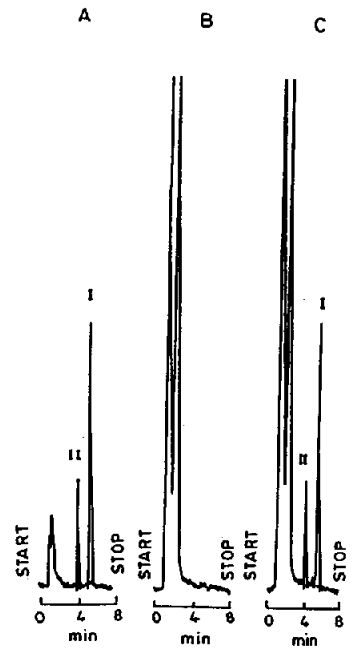

Fig. 2. Chromatograms of (A) standard containing 100 ng/ml of I and II, (B) drug-free serum, and (C) serum containing 20 ng/ml of I and II after 5-fold concentration.

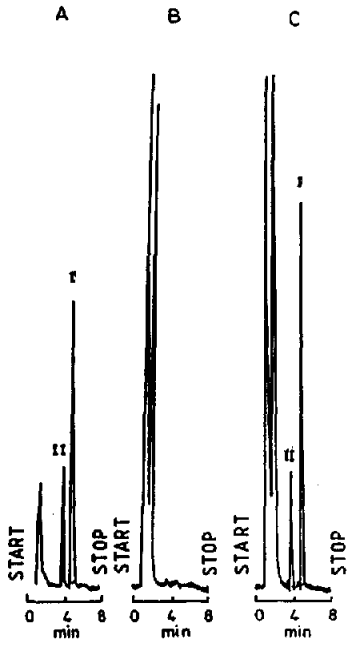

Fig. 3. Chromatograms of (A) standard containing 100 ng/ml of I and II, (B) drug-free milk, and (C) milk containing 25 ng/ml of I and II after 5-fold concentration.

deemed necessary. A linear relationship existed between peak heights and concentrations of I (1–1000 ng/ml) and II (2.5–1000 ng/ml). Linear regression analysis of standard curves yielded correlation coefficients all exceeding 0.999.

The reproducibility and accuracy of the method were determined by processing spiked human serum and milk at three concentration levels (low, medium and high) with respect to their calibration curves run each day. Six samples were analysed for each concentration. The suitability of the assay accuracy and precision is indicated in Table 2. The inter-day assay variation was also calculated by comparing the linear regression slopes of standard curves. Over a period of one month the slopes (mean ± S.D.) were 224.5 ± 16.3 and 68.2 ± 5.0 (serum), and 223.9 ± 17.0 and 65.4 ± 3.0 (milk) for I and II, respectively.

The lower limits of quantitation in human serum and milk were 1 ng/ml for I and 2.5 ng/ml for II after 5-fold concentration.

The level of accuracy, precision, selectivity and sensitivity of the method described here allows it to be used for pharmacokinetic studies in serum and milk, as is currently being done.

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Table 1
Absolute recoveries of I and II from spiked serum and milk ($n = 6$)

Concentration (ng/ml)	I		II	
	Recovery (%)	C.V.[a] (%)	Recovery (%)	C.V.[a] (%)
Serum				
5	96.9	4.7	96.0	5.0
50	95.3	7.8	99.6	8.5
500	97.8	6.5	98.7	4.2
Milk				
5	99.0	5.7	104.1	2.9
50	100.4	8.9	97.7	4.8
500	102.8	6.0	96.1	6.7

[a] C.V. = (S.D./mean) · 100.

Table 2
Accuracy and precision for I and II

Nominal serum or milk concentration (ng/ml)	Within-day			Day-to-day		
	Concentration found (mean ± S.D.) (ng/ml)	C.V. ($n = 6$) (%)	Accuracy (% of mean deviation)	Concentration found (mean ± S.D.) (ng/ml)	C.V. ($n = 7$) (%)	Accuracy (% of mean deviation)
I: Serum						
5	5.5 ± 0.2	3.9	+9.6	5.6 ± 0.3	4.6	+11.0
50	50.5 ± 3.9	7.7	+1.0	50.9 ± 3.4	6.6	+1.7
500	493.3 ± 9.8	2.0	−1.3	500.2 ± 15.5	3.1	+0.1
I: Milk						
5	4.9 ± 0.3	6.6	−2.0	4.9 ± 0.3	6.2	−2.6
50	51.6 ± 5.2	10.1	+3.1	52.8 ± 5.3	11.0	+5.6
500	522.2 ± 40.1	7.7	+4.4	523.0 ± 36.7	7.0	+4.6
II: Serum						
5	5.1 ± 0.2	4.4	+2.6	5.2 ± 0.2	4.6	+3.6
50	49.6 ± 4.0	8.0	−0.8	49.9 ± 3.5	7.0	−0.1
500	481.5 ± 7.7	1.6	−3.7	488.0 ± 20.5	2.2	−0.4
II: Milk						
5	5.3 ± 0.2	3.0	+5.8	5.3 ± 0.2	2.7	+6.0
50	51.6 ± 2.5	4.8	+3.2	52.1 ± 2.4	4.5	+4.2
500	509.5 ± 33.9	6.7	+1.9	512.3 ± 28.3	5.5	+2.5

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