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Development and validation of a rapid, sensitive liquid chromatography–tandem mass spectrometry method using electrospray ionization for quantitation of centchroman in rat plasma and its application to preclinical pharmacokinetic study☆

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ABSTRACT

A highly sensitive and specific HPLC–ESI-MS/MS method has been developed and validated for the estimation of centchroman with 100 μL rat plasma using tamoxifen as an internal standard (IS). The assay procedure involved a single-step, liquid–liquid extraction of centchroman and IS from plasma with 2.5% (v/v) isopropanol in *n*-hexane, which yielded consistent recoveries of 109.5 and 107.8% for centchroman and IS in rat plasma, respectively. The total chromatographic run time was 3.8 min. Peaks were resolved using 0.01 M ammonium acetate (pH 4.5):acetonitrile (10:90, v/v) mobile phase on a Supelco Discovery C_{18} column. Specificity and matrix effect on ionization was determined and found that method was specific and there was no significant matrix effect. Linearity range was found to be 0.5–100.0 ng/mL with a correlation coefficient (r) of 0.9959 or better. The intra- and inter-day assay precision ranged from 3.3 to 9.0% and 5.5 to 6.8%, respectively, and intra- and inter-day assay accuracy was between 93.4–107.1% and 96.2–104.2%, respectively. Stability of centchroman in rat plasma was >89.0% in the battery of stability studies viz., bench-top, auto-sampler, freeze/thaw cycles and 30 days storage in a freezer at −80 °C. The assay was successfully applied to determine the pharmacokinetic parameters in *Sprague–Dawley* rats after an oral administration of centchroman at 20 mg/kg. As a result, the plasma half-life was 29.4 ± 2.3 h and the $AUC_{(0-\infty)}$ was 7345.1 ± 21.9 ng h/mL. The maximum plasma concentration (C_{max}) 117.5 ± 15.7 ng/mL was achieved at 9.0 ± 8.6 h (t_{max}).

1. Introduction

Centchroman (CDRI code: 67/20) {trans-2-2-dimethyl 1-3 phenyl-4-p(beta-pyrrolidinoithoxy phenyl) 7-methoxy chroman hydrochloride} is a synthetic nonsteroidal selective estrogen receptor modulator, used as post-coital contraceptive agent [1]. Antiferitlity effect of this is due to its inherent estrogenic and antiestrogenic properties and found to decrease monoamine oxidase in pituitary and median eminence without decreasing levels in uterus [2–4]. It also has antiosteoporotic and anticancer activity [5]. Pharmacokinetic parameters of centchroman in female volunteers and female rats are well characterized and reported. The absorption and disposition were found to follow first order kinetics in dose dependent fashion. Its major metabolite is 7-desmethyl centchroman (DMC). Its plasma protein binding is almost 100% which justifies its unusually long half-life but inter-individual variability has been found to exist [6]. Its pharmacokinetic interactions with several co-administered drugs have been studied in *Sprague–Dawley* rats and drug has been found safe for long term uses [2,7,8].

Preclinical evaluation of compounds in drug discovery and analytical method development and validation is inevitable. Few methods using HPLC with fluorescence detection in human plasma, human serum, milk, rat plasma and tissues have been reported so far [9,10]. All these methods have longer run time, cumbersome extraction procedures and require larger sample volume. To best of our knowledge, there are no bioanalytical methods using LC–MS/MS reported for the estimation of centchroman in rat plasma. Therefore, in our present work, we have developed and validated a highly sensitive and very rapid method for estimation of centchroman in rat plasma using LC–ESI-MS/MS to derive pharmacokinetic parameters in preclinical species. This method is more sensitive, selective with good accuracy, precision, and require lesser sample volume and useful for high throughput pharmacokinetic studies than previously reported methods.

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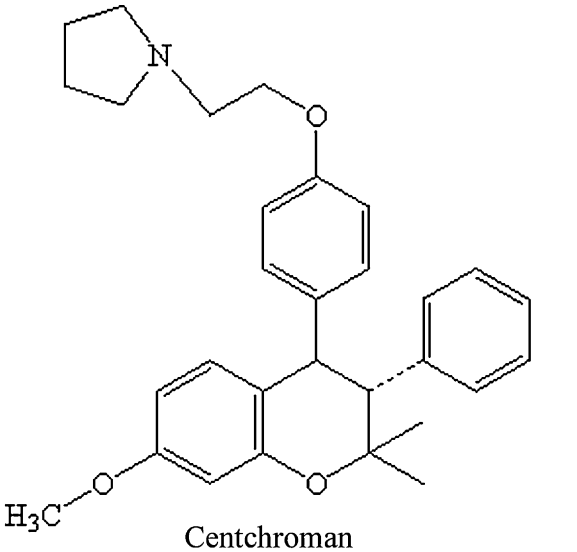
2. Experimental

2.1. Chemicals and reagents

Centchroman was synthesized at the Medicinal Chemistry Division of Central Drug Research Institute Lucknow, India, whereas tamoxifen (IS) was purchased from Sigma–Aldrich (St. Louis, USA). Chemical structure of centchroman and tamoxifen is shown in Fig. 1. HPLC grade acetonitrile and isoproapanol were purchased from Sisco Research Laboratories (SRL) Pvt. Limited (Mumbai, India). HPLC grade *n*-hexane was obtained from E Merck Limited (Mumbai, India). Ammonium acetate and glacial acetic acid AR were purchased from E Merck Limited (Mumbai, India). Ultra pure water was obtained from a Sartorious Arium 611 system. Heparin sodium injection I.P. (1000 IU/mL, Biologicals E. Limited, Hyderabad, India) was purchased from Pharmacy store (Lucknow, India). Blank, drug free plasma samples were collected from adult, healthy female *Sprague–Dawley* rats at Department of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood (25 IU/mL) at $2000 \times g$ for 10 min. Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance and experimental studies with animals. All experiments, euthanasia and disposal of carcass were performed in accordance with the guidelines laid by IAEC for animal experimentation.



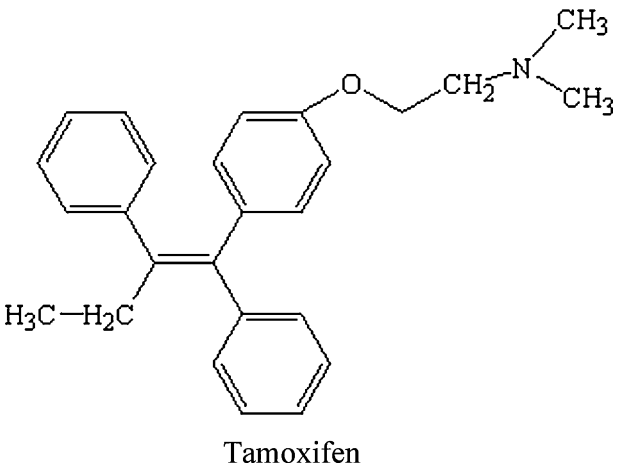


Fig. 1. Structural representation of centchroman and tamoxifen.

2.2. Instrumentation and chromatographic conditions

HPLC system consists of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin–Elmer instruments, Norwalk, USA) was used to inject 10 μL aliquots of the processed samples on a Supelco Discovery C_{18} column (4.6 mm × 50 mm, 5.0 μm). The system was run in isocratic mode with mobile phase consisting of 0.01 M ammonium acetate (pH 4.5) and acetonitrile in ratio of 10:90 (v/v). Mobile phase was duly filtered through 0.22 μm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min and delivered at a flow rate of 0.8 mL/min for chromatographic separation.

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The ion spray voltage was set at 5500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 10, 10, 20 and 10, respectively. Compounds parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were 100, 43, 10, 8 V and 95, 35, 10, 8 V for centchroman and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of *m*/*z* 458.5 precursor ion $[M+H]^+$ to the *m*/*z* 98.1 product ion for centchroman and *m*/*z* 372.5 precursor ion $[M+H]^+$ to the *m*/*z* 72.0 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada).

2.3. Preparation of stock and standard solutions

Primary stock solutions of centchroman for preparation of calibration curve (CC) and quality control (QC) samples were prepared from separate weighings. The primary stock solutions of the analyte and IS were prepared in methanol (1.0 mg/mL) and stored at 4 °C. Appropriate dilutions were made in methanol for centchroman to produce working stock solution of 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005 μg/mL and on the day of analysis this set of stocks was used to prepare standards for the calibration curve. Another set of working stock solutions of centchroman was made in methanol (from second primary stock) at 0.8, 0.4, 0.015 and 0.005 μg/mL for preparation of QC samples. Working stock solutions were stored at approximately 4 °C and found stable for 1 month (data not shown). Individually, QC and CC working stock solutions of centchroman were spiked into blank plasma for QC and CC samples. A working stock solution of IS (0.4 μg/mL) was prepared in methanol from primary stock solution of 1.0 mg/mL in methanol. Calibration standards were prepared by spiking 90 μL of control pooled rat plasma with the appropriate working solution of centchroman (10 μL) and IS (10 μL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control rat plasma in bulk at four concentration levels [0.5 ng/mL (lower limit of quantitation, LLOQ), 1.5 ng/mL (QC low), 40.0 ng/mL (QC medium) and 80.0 ng/mL (QC high)] and 100 μL volumes were aliquoted into different tubes and stored at −80 ± 10 °C until analysis.

2.4. Recovery

The recovery of centchroman and IS, through liquid–liquid extraction procedure, was determined by comparing the responses

of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from post-extracted plasma standard sample at equivalent concentrations [11]. Recoveries of centchroman were determined at LLOQ, QC low and QC high concentrations viz., 0.5, 1.5, and 80.0 ng/mL, whereas the recovery of the IS was determined at a single concentration of 40.0 ng/mL.

2.5. Sample preparation

A simple liquid–liquid extraction method was followed for extraction of centchroman from rat plasma. To 100 μL of plasma aliquot, IS solution (10 μL of working stock) equivalent to 4.0 ng was added and mixed for 15 s on a cyclomixer (Spinix Tarsons, Kolkata, India), followed by extraction with 2.0 mL of 2.5% (v/v) isopropanol in *n*-hexane. The mixture was vortexed for 3 min, followed by centrifugation for 5 min at 2000 × *g* on Sigma 3-16K (Frankfurt, Germany). An aliquot of 1.8 mL of organic layer was separated and evaporated to dryness under vaccum in speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 μL of the mobile phase and 10 μL was injected onto analytical column.

2.6. Validation procedures

A full validation according to the FDA guidelines was performed for the assay in rat plasma [12].

2.6.1. Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples collected from six different rats to investigate the potential interferences at the LC peak region for analyte and IS.

2.6.2. Matrix effect

The effect of rat plasma constituents over the ionization of centchroman and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 6$) with the response of analytes from neat standard samples (10 μL of required working stock sample spiked into 90 μL of methanol instead of blank plasma) at equivalent concentrations [11,13]. The matrix effect for centchroman was determined at LLOQ, QC low and QC high concentrations, viz., 0.5, 1.5 and 80.0 ng/mL whereas the matrix effect over the IS was determined at a single concentration of 40.0 ng/mL.

2.6.3. Calibration curve

The calibration curve was acquired by plotting the ratio of sum of peak area of centchroman to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.995 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [12].

2.6.4. Precision and accuracy

The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e., 0.5, 1.5, 40.0 and 80.0 ng/mL. The inter-assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% standard deviation (S.D.) from the nominal values and a precision of within ±15% relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision [12].

2.6.5. Stability experiments

All stability studies were conducted at two concentration levels, i.e. QC low and QC high, using six replicates at each concentration levels. Replicate injections of processed samples were analyzed up to 18 h to establish autosampler stability of analyte and IS at 4 °C. The peak areas of analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. The stability of centchroman in the biomatrix during 6 h exposure at room temperature in rat plasma (bench top) was determined at ambient temperature (25 ± 2 °C). Freeze/thaw stability was evaluated up to three cycles. In each cycle samples were frozen for at least 12 h at −80 ± 10 °C. Freezer stability of centchroman in rat plasma was assessed by analyzing the QC samples stored at −80 ± 10 °C for at least 30 days. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., ±15% R.S.D.).

2.6.6. Dilution integrity

Dilution of biological matrix is required if some study sample concentrations are expected to be higher than the upper limit of quantitation (100.0 ng/mL). Dilution integrity experiments were carried out by four times dilution of plasma samples containing 320.0 ng/mL of centchroman with blank plasma to obtain samples containing 80.0 ng/mL (HQC) of centchroman.

2.7. Pharmacokinetic study in rats

Rat *in vivo* oral pharmacokinetic study was performed to show the applicability of newly developed and validated bioanalytical method. Study was performed in female *Sprague–Dawley* rats ($n = 5$, weight range 200–220 g). Centchroman was administered orally at a dose of 20 mg/kg in 0.25% sodium carboxy methyl cellulose (CMC) suspension. Blood samples were collected from the retro-orbital plexus of rats under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.5, 1.0, 2.0, 3.0, 5.0, 9.0, 24.0, 48.0, and 74.0 h post-dosing. Plasma was harvested by centrifuging the blood at 2000 × *g* for 5 min and stored frozen at −80 ± 10 °C until analysis.

Rat plasma (100 μL) samples were spiked with IS and processed as described above and data was accepted based on the performance of QCs prepared using rat blank plasma (four QCs each at three concentration levels). The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration; (ii) not less than 50% at each QC concentration level must meet the acceptance criteria. Plasma concentration–time data of centchroman was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, USA).

3. Results

3.1. Liquid chromatography

Method development began with the optimization of chromatographic conditions including mobile phase composition and column type. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 4.0–7.0, along with altered flow rates (in the range of 0.4–1.0 mL/min) were tested for complete chromatographic resolution of centchroman and IS (data not shown). Mobile phase

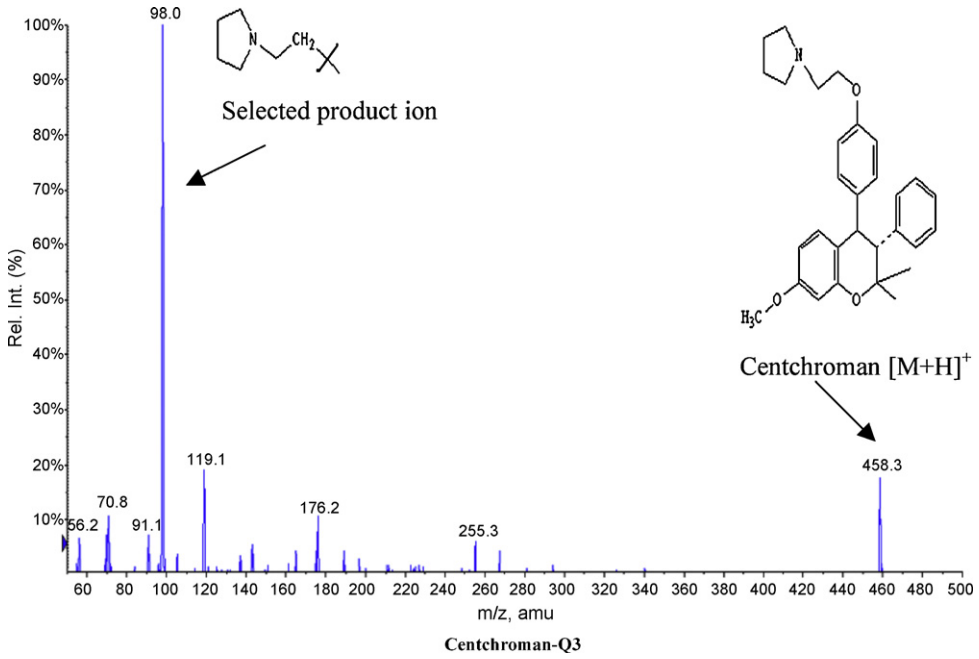


Fig. 2. MS/MS spectra of centchroman and tamoxifen showing prominent precursor to product ion transitions.

comprising of 0.01 M ammonium acetate (pH 4.5):acetonitrile (10:90, v/v) was delivered at a flow rate of 0.8 mL/min was found to be suitable during LC optimization and enabled the determination of electrospray response for centchroman and IS. Versatility, suitability, and robustness of the method was checked with several C_{18} columns from various manufacturers was evaluated (data not shown) and found that chromatographic resolution, selectivity and sensitivity were good with Supelco Discovery C_{18} column (4.6 mm × 50 mm, 5.0 μm).

3.2. Mass spectrometry

In order to optimize ESI conditions for centchroman and IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for centchroman and IS revealed peaks at *m/z* 458.5 and 372.5, respectively as protonated molecular ions $[M+H]^+$. The product ion mass spectrum for centchroman shows the formation of characteristic product ions at *m/z* 267.4, 255.2, 176.0, 143.0, 119.0, 98.1 and 71.0 (Fig. 2). Following detailed optimization of mass spectrometry conditions (provided in instrumentation and chromatographic conditions section), the *m/z* 458.5 precursor ion to the *m/z* 98.1 was used for quantification for centchroman. Similarly, for IS *m/z* 372.5 precursor ion to the *m/z* 72.0 was used for quantification purpose.

3.3. Recovery

A simple liquid–liquid extraction with 2.5% isopropanol in *n*-hexane proved to be robust and provided cleanest samples. The results of the comparison of pre-extracted standards versus post-extracted plasma standards were estimated for centchroman at 0.5, 1.5 and 80.0 ng/mL and the absolute mean recovery was 109.5%. The absolute recovery of IS at 40.0 ng/mL was 107.8%.

3.4. Validation procedures

3.4.1. Matrix effect, specificity and selectivity

In this study, the matrix effect was evaluated by analyzing LLOQ (0.5 ng/mL), QC low (1.5 ng/mL) and QC high samples (80.0 ng/mL). Average matrix effect values obtained were 8.1, 10.2 and 9.5% at LLOQ, QC low and QC high, respectively. Matrix effect on IS was found to be 3.3% at tested concentration of 40.0 ng/mL.

In the present study, the specificity and selectivity has been studied by using independent plasma samples from six different rats. The blank samples showed neither significant differences between them nor area values higher than the 20% of the LLOQ's area at the analyte retention time or higher than 5% of the internal standard area at its corresponding retention time.

Fig. 3 shows a typical overlaid chromatogram for the control rat plasma (free of analyte and IS), rat plasma spiked with centchroman at LLOQ and IS and an *in vivo* rat plasma sample obtained at 8 h after oral administration of centchroman. The retention time of centchroman and IS were 2.4 and 2.7 min, respectively. The total chromatographic run time was 3.8 min.

3.4.2. Calibration curve

The plasma calibration curve was constructed using eight calibration standards (viz., 0.5–100.0 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor $(1/X^2)$. The average regression ($n = 3$) was found to be ≥0.995. The lowest concentration with R.S.D. < 20% was taken as LLOQ and was found to be 0.5 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 89.4–108.8; while the % precision values ranged from −11.9 to 8.1 (Table 1).

3.4.3. Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples are presented in Tables 2 and 3. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

3.4.4. Stability

The predicted concentrations for centchroman at 1.5 and 80.0 ng/mL samples deviated within the nominal concentrations

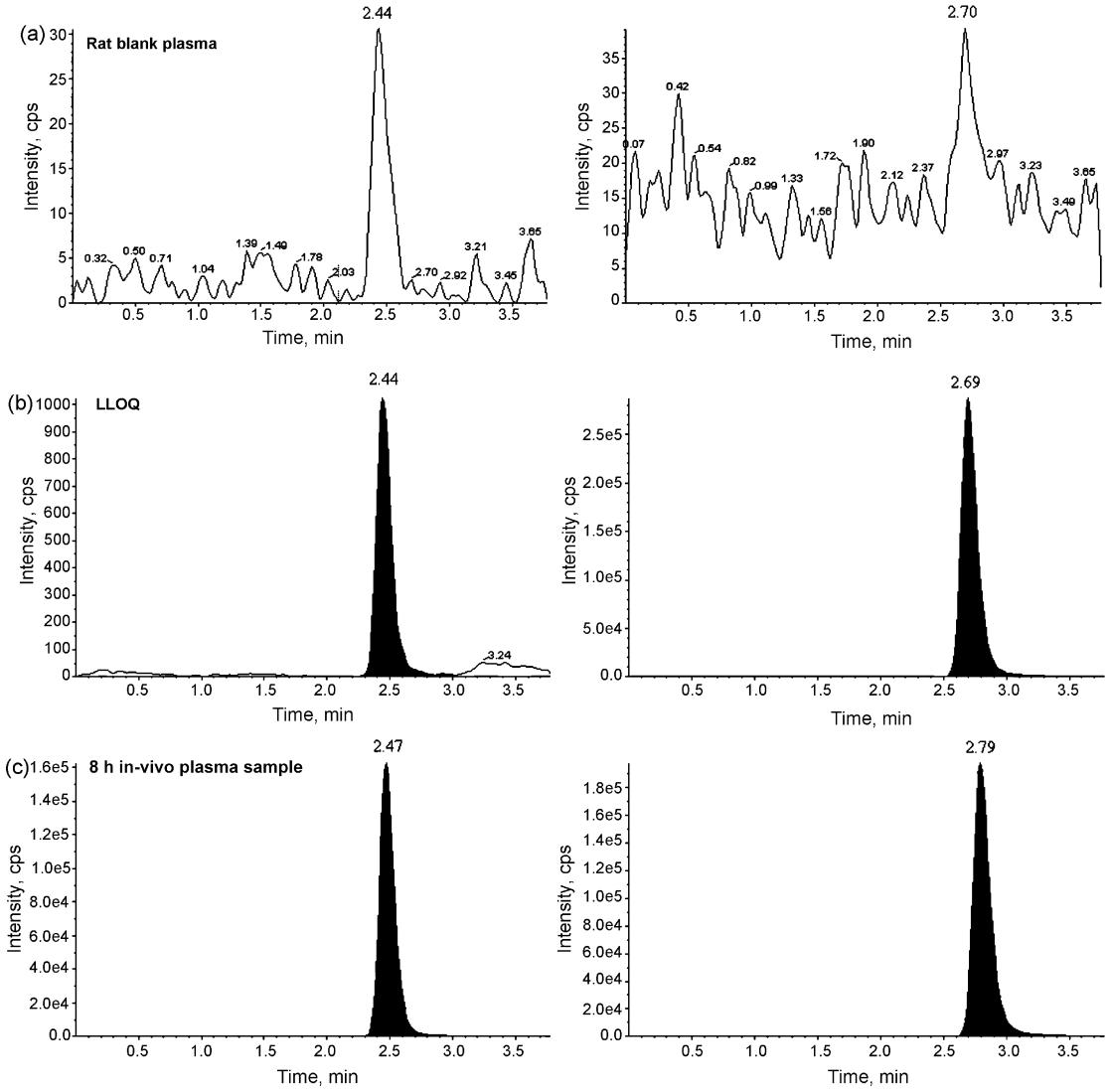


Fig. 3. Typical MRM chromatograms of centchroman (left panel) and IS (right panel) in (a) rat blank plasma, (b) rat plasma spiked with centchroman at LLOQ (0.5 ng/mL) and IS (c) a 8 h *in vivo* plasma sample showing centchroman peak obtained following oral dose of centchroman.

in a battery of stability tests, viz., in-injector (18 h), bench-top (6 h), repeated three freeze/thaw cycles and at −80 ± 10 °C for at least for 30 days (Table 4). The results were found to be within the assay variability limits during the entire process.

3.4.5. Dilution integrity

Dilution integrity experiments carried out at six replicates by four times dilution with blank plasma and assay precision and accu-

Table 1
Precision and accuracy data of back-calculated concentrations of calibration samples for centchroman in rat plasma ($n = 3$).

Nominal concentration (ng/mL)	Observed concentration (ng/mL, mean ± S.D.)	Precision[a] (%)	Accuracy[b] (%)
0.5	0.5 ± 0.0	0.5	100.5
1.0	1.0 ± 0.1	1.1	101.1
2.0	2.2 ± 0.0	8.1	108.8
5.0	4.5 ± 0.6	−10.8	90.3
10.0	10.3 ± 0.7	3.3	103.4
20.0	20.6 ± 1.4	2.9	103.0
50.0	44.7 ± 0.9	−11.9	89.4
100.0	100.7 ± 3.9	0.7	100.7

[a] Expressed as % R.S.D. (S.D./mean) × 100.
[b] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 2
Intra-day assay precision and accuracy for centchroman in rat plasma ($n = 6$).

Nominal concentration (ng/mL)	Observed concentration (ng/mL, mean ± S.D.)	Precision[a] (%)	Accuracy[b] (%)
0.5			
(Day-1)	0.5 ± 0.0	4.4	102.2
(Day-2)	0.5 ± 0.0	6.7	97.2
(Day-3)	0.5 ± 0.0	6.8	101.2
1.5			
(Day-1)	1.4 ± 0.1	4.2	93.4
(Day-2)	1.5 ± 0.1	6.7	97.6
(Day-3)	1.5 ± 0.1	5.0	97.4
40.0			
(Day-1)	41.5 ± 2.3	5.5	103.7
(Day-2)	41.6 ± 2.2	5.3	104.0
(Day-3)	40.2 ± 3.6	9.0	100.5
80.0			
(Day-1)	85.7 ± 2.8	3.3	107.1
(Day-2)	85.0 ± 4.6	5.4	106.3
(Day-3)	79.7 ± 7.1	9.0	99.6

[a] Expressed as % R.S.D. (S.D./mean) × 100.
[b] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 3
Inter-day assay precision and accuracy for centchroman in rat plasma.

Nominal concentration (ng/mL)	Observed concentration[a] (ng/mL, mean ± S.D.)	Precision[b] (%)	Accuracy[c] (%)
0.5	0.5 ± 0.0	6.2	100.2
1.5	1.4 ± 0.1	5.5	96.2
40.0	41.1 ± 2.7	6.5	102.7
80.0	83.4 ± 5.6	6.8	104.2

[a] $n = 3$ days with six replicates per day.
[b] Expressed as % R.S.D. (S.D./mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

Table 4
Stability of centchroman in rat plasma.

Nominal concentration (ng/mL)	Stability	Mean ± S.D.,[a] $n = 6$ (ng/mL)	Precision[b] (%)	Accuracy[c] (%)
	0 h (for all)	1.5 ± 0.1	5.0	97.4
	18 h (auto-sampler)	1.4 ± 0.1	6.1	98.4
1.5	6 h (bench top)	1.5 ± 0.1	7.8	104.1
	3rd freeze–thaw	1.4 ± 0.1	5.4	93.3
	30 days at −80 °C	1.5 ± 0.2	9.6	103.0
	0 h (for all)	79.7 ± 6.4	8.1	99.6
	18 h (auto-sampler)	76.3 ± 5.3	6.9	89.7
80.0	6 h (bench top)	85.1 ± 4.9	5.8	106.8
	3rd freeze–thaw	82.9 ± 3.7	4.5	104.1
	30 days at −80 °C	84.2 ± 2.5	3.0	105.7

[a] Back calculated plasma concentrations.
[b] Expressed as % R.S.D. (S.D./mean) × 100.
[c] Calculated as (mean determined concentration/nominal concentration) × 100.

racy were determined in a similar manner as described in Section 2.6.4. The % accuracy observed for the mean of back-calculated concentrations for diluted QCs was 103.9. The precision (% R.S.D.) for diluted QCs was 3.2%. The results suggested that samples whose concentrations were greater than the upper limit of calibration curve could be re-analyzed by appropriate dilution.

3.5. Pharmacokinetic study in rats

The rat plasma samples generated following administration of centchroman were analyzed by the newly developed and validated method along with QC samples. During samples analysis it was found that some sample concentrations were falling above the calibration range, these samples were reanalyzed after dilution along with the diluted QCs. All the QCs met the acceptance criteria (data not shown). The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of centchroman in rats. Profiles of the mean plasma concentration versus time are shown in Fig. 4. Maximum concentration in plasma (C_{max} 117.5 ± 15.7 ng/mL) was achieved at 9.0 ± 8.6 h (t_{max}). The half-life ($t_{1/2}$) of centchroman was 29.4 ± 2.3 h, while the $AUC_{(0-\infty)}$ was 7345.1 ± 21.9 ng h/mL.

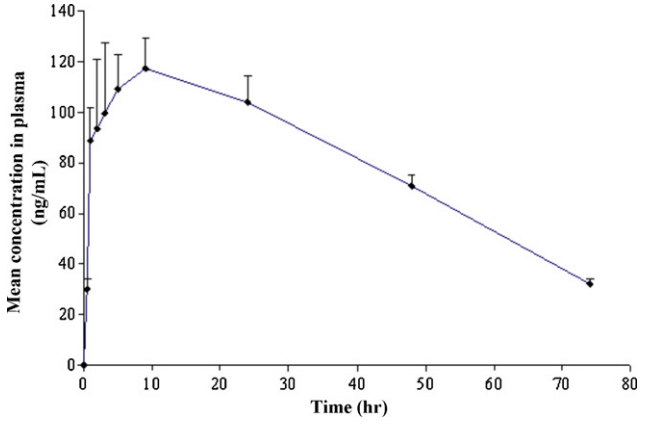


Fig. 4. Mean ± S.E.M. plasma concentration–time profile of centchroman in rat plasma following oral dose of centchroman ($n = 5$).

4. Discussion

In this study, we have developed and validated a LC–MS/MS method for quantitation of centchroman in rat plasma for the first time. Deuterated/isotopically labeled internal standard is ideal for LC–MS/MS analysis; however, these are not commercially/easily available due to cost/infrastructure constraint. Therefore, we explored the possibility for the best IS available commercially due to the non-availability of structurally similar analogue of centchroman in our lab. During LC optimization, we found that tamoxifen behave similarly as centchroman in terms of response (intensity) and peak shape and selected it for validation. Validation results show that there is no significant matrix effect on selected IS. Further, the ionization of IS was consistent and reproducible through out the validation and results were also found well with in the acceptable limits. Validated methods are essential for the determination of plasma concentrations in pre-clinical species plasma (for pre-clinical pharmacokinetics and toxicokinetic studies) and for clinical studies. The current validated method developed for centchroman offers significant advantages in terms of sensitivity and selectivity, sample preparation, faster run time and lower volume of sample requirements as compared to previously reported methods [9,10]. This method utilizes a short run time of 3.8 min for each sample analysis. The method utilized small volume of rat plasma, i.e., 100 μL. Sample preparation is very simple and it involves liquid–liquid extraction of plasma with 2.5% isopropanol in *n*-hexane. Due to good sensitivity (LLOQ – 0.5 ng/mL) of the assay, it offers a suitable platform for the determination of centchroman in pre-clinical studies. The applicability of the method in pre-clinical pharmacokinetic studies has been demonstrated in rats.

5. Conclusion

In conclusion, we have developed and validated a highly sensitive, specific, and reproducible and high-throughput LC–ESI-MS/MS assays to quantify centchroman using commercially available IS from small volumes of rat plasma for the first time. From the results of all the validation parameters and applicability of the assay, we can conclude that the present method can be useful for pre-clinical pharmacokinetic studies of centchroman with desired precision and accuracy along with high-throughput.

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