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Determination of Menbutone in Bovine Milk and Meat Using Micellar Liquid Chromatography: Application to Injectable Dosage Forms

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Abstract A simple, sensitive, and rapid method was developed for the routine identification and quantification of menbutone in different matrices by micellar liquid chromatography. Separation was performed in less than 4 min using a C_{18} column with UV detection at 234 nm. A micellar solution composed of 0.12 M sodium dodecyl sulfate, 8 % *n*-butanol, and 0.3 % triethylamine in 0.02 M phosphoric acid at pH 6.0 was used as the mobile phase. The method was fully validated in accordance with International Conference on Harmonization (ICH) guidelines. The limits of detection and quantitation were 0.95 and 2.86 ng mL^{-1}, respectively. The method showed good repeatability, linearity, and sensitivity according to the evaluation of the validation parameters. The micellar method was successfully applied for the analysis of menbutone in its commercial injections with a mean % recovery value of 99.73± 1.634 % and in spiked bovine milk and meat samples with a mean % recovery values in the range of 98.00–100.60 %. High extraction efficiency was obtained without matrix interference in the extraction process and in the subsequent chromatographic determination. No organic solvent was used during the pretreatment step. Hence, this method can be considered as an interesting example for *green* chemistry.

Keywords Micellar liquid chromatography · Menbutone · Dosage forms · Bovine milk · Meat

Introduction

Menbutone (4-methoxy-γ-oxo-1-naphthalene butanoic acid) (Fig. 1) (O'Neil 2006) is a specific stimulant of exocrine glands of the digestive tract: digestive tonic and choloretic in bovine, ovine, porcine, and equine species (Symonds 1982). It helps in any course affecting the digestive system as diarrhea, anorexia, and gastroenteritis (Ackerman 2007). After injection into the body, it increases biliary, pancreatic, and peptic secretion by two to five times compared with the normal levels of these secretions.

In modern farming practice, drugs are used in a large scale and are applied in animal husbandry for different reasons. They are used to prevent diseases, cure animals, or as feed additive to promote growth. In the veterinary clinic, animals digestive diseases account for about 30 % of the total number of clinical diseases and loss of appetite, indigestion, constipation, and other gastrointestinal dysfunctions are almost concomitant diseases or secondary in most animals. Animal indigestion makes animal growth stunting, production performance degradation, and even death, often resulting in large economic losses, hindering the development of animal husbandry (Ackerman 2007). All drugs administered to milk and meat-producing animals may lead to residues in the milk and meat. There is no current legislation which establishes limits of menbutone residues in meat and milk (Codex Alimentarius Commission 2006). As a result of this lack of regulations, a zero-tolerance policy is applied for menbutone residues in baby food and formulae which means that the presence of this compound is illegal at any level (Rodriguez et al. 2008). The use of menbutone may cause accumulation of its residues into the

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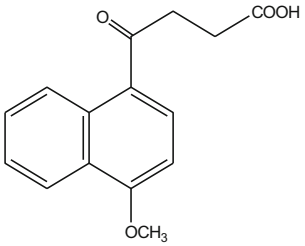

Fig. 1 Chemical structure of menbutone

animal tissues and milk which ultimately find their ways into food products derived from animal origin (Lund and Lassen 1969). Assays are needed to test the level of menbutone in animal products before they are brought to the market since the safety factors of it are not known.

Micellar liquid chromatography (MLC) allows complex matrices to be analyzed without the need of extraction and with direct injection of the samples (Berthod and Garcia-Alvarez-Coque 2000). Micelles tend to bind proteins competitively, thereby releasing protein-bound drugs and proteins, rather than precipitating into the column. Proteins are solubilized and washed harmlessly away, eluting with the solvent front. This means that costs and analysis times are cut considerably (Kalyankar et al. 2014). Micellar mobile phases usually need less quantity of organic modifier and generate less amount of toxic waste in comparison to aqueous-organic solvents, so that they are less toxic, non-inflammable, biodegradable, and relatively inexpensive (Rambla-Alegre 2012). MLC has been proven to be a useful technique in the determination of diverse groups of compounds in several matrices (Belal et al. 2013; 2009; Soltani and Jouyban 2012; Mourya et al. 2011; Malinowska and Stepnik 2012; Rizk et al. 2014), including food samples (Szymanski 2008; Nasr et al. 2013a, b; Beltran-Martinavarro et al. 2012).

In reviewing the literature, it was found that few methods have been reported for the estimation of menbutone. These methods include spectrophotometry (Fouad et al. 2013), HPLC-UV detection (Luo et al. 2013a, b), and HPLC-MS (Shoichiro and Hisaya 2008; Hirosh et al. 2010). The reported spectrophotometric and HPLC-UV methods for the estimation of menbutone are not sensitive enough to determine trace amounts of the drug substance. The HPLC-MS method, although reliable and sensitive enough, is tedious and cannot be used for routine analysis of the drug due to the associated costs and the non-availability of MS instrumentation in every laboratory as well as the requisite expertise in operating MS systems, especially in developing and under-developed countries.

Menbutone is very potent in its digestive activity, and the regular effective doses are very small; hence, its detection in milk and meat requires a highly sensitive and selective method. Analytical methods for the determination of menbutone in animal tissues and milk are, however, scarce. Therefore, it was desirable to develop a simple and fast chromatographic method that can be applied in quality control laboratories for the determination of menbutone. The results obtained were promising.

Experimental

Materials

Pure drug sample was kindly provided by the Egyptian Company for Chemicals and Pharmaceuticals, Cairo, Egypt. Its purity was 100.62 % according to the manufacturer's method. Each milliliter of menbutone 10 % injections (batch number 081133) is labeled to contain 100.0 mg of menbutone, a product of the Egyptian Company for Chemicals and Pharmaceuticals, Cairo, Egypt, which was purchased from a local pharmacy. Bovine milk and meat were purchased from the local market.

Reagents and Chemicals

All reagents and solvents used were of HPLC grade. High purity water was used throughout the study. Orthophosphoric acid (85 %, *w*/*v*), 1-propanol, and *n*-butanol were obtained from Sigma-Aldrich (Germany). Methanol and acetonitrile were obtained from Fisher Scientific (UK). Sodium dodecyl sulfate (SDS) was obtained from Oxford Laboratory, Mumbai (India). Triethylamine was obtained from S D Fine-Chem Limited (India). Nylon filters and syringe filters were obtained from Sartorius Stedim (Göttingen, Germany).

Instrumentations

Chromatographic analyses were carried out using a Shimadzu Prominence HPLC System (Shimadzu, Japan) with a LC-20 AD pump, DGU-20 A5 degasser, CBM-20A interface, and SPD-20A UV-Vis detector with 20 μL injection loop. Centrifugation was carried out using a TDL-60 B Centrifuge (Anke, Taiwan). Ultrasonic bath used was BHA-180 T (Abbott, USA). Tissue homogenization was made using Tissue Master 125 with a 7-mm stainless steel generator probe (Omni International, GA, USA). The pH was measured with a Jenway pH meter, 4510 (Essex, UK). The mobile phase was filtered through a Charles Austen Pumps Ltd. Filter, model B100 SE (England, UK) using 0.45-μm milli-pore filters (Gelman, Germany).

Chromatographic Conditions

MLC was performed on a Shim-Pack VP-ODS column (150 mm×4.6 mm i.d., 5 μm particle size; Shimadzu, Japan) using micellar mobile phase consisting of 0.12 M sodium dodecyl sulfate, 8 % butanol, and 0.3 % triethylamine in

0.02 M orthophosphoric acid buffered at pH 6.0. The mobile phase was filtered and sonicated for 30 min before use. The flow rate was 1.0 mL min^{-1}, and sample injection volumes were 20 μL at room temperature (25 °C). The UV detector was set at 234 nm.

Standard Solutions

Stock solution of 0.2 mg mL^{-1} of menbutone was prepared by dissolving 10.0 mg menbutone in 50 mL of methanol, and then the solution was sonicated in an ultrasonic bath for 5 min. Working solutions were prepared by diluting the stock solution with the mobile phase. Stock solution was found to be stable for 1 week if stored in a refrigerator.

Construction of Calibration Curves

Working solutions containing 0.05–2.0 and 2.0–20 μg mL^{-1} of menbutone were prepared by serial dilution of aliquots of the stock solution. Then, 20-μL aliquots were injected (triplicate) and eluted with the mobile phase under the reported chromatographic conditions. The average peak areas were plotted versus the concentrations of the drug in micrograms per milliliter. Alternatively, the corresponding regression equations were derived.

Application to Injections

Five menbutone 10 %® injections were mixed, and an aliquot of the mixed solution equivalent to 20 mg was transferred to a 100-mL volumetric flask and completed to volume with methanol to obtain a solution claimed to contain 0.2 mg mL^{-1} of menbutone. Solutions were analyzed following the details under the "Construction of calibration curves" section. The nominal content of the ampoules was determined using the calibration curve or the regression equation.

Bovine Milk and Meat Sample Preparation

2.5 g of the bovine meat was accurately weighed, and 5 mL of the milk sample was spiked each with aliquots of menbutone solution. The spiked meat samples were homogenized and completed to 25 mL of 0.12 M SDS solution of pH 6.0. The samples were homogenized at 5000 rpm for 5 min; then, the homogenate was sonicated for 15 min and then centrifuged at 3000 rpm for 5 min. Milk samples were only sonicated for 2 min without centrifugation. The supernatant of all the samples was filtered through 0.45-μm membrane filters using a vacuum pump. The filtrates were diluted with the mobile phase and filtered through a syringe filter. Twenty-microliter aliquots were injected (triplicate) and eluted with the mobile phase under the above chromatographic conditions. The average peak area was plotted versus the concentration of menbutone in micrograms per milliliter to get the calibration curve. The nominal contents of the drug in these preparations were determined using the corresponding regression equations.

Results and Discussion

The proposed method permits the quantitation of menbutone in pharmaceutical dosage forms, bovine milk, and meat. Figure 2a shows a typical chromatogram of the peak of the drug (t_R=3.1 min) under the described chromatographic

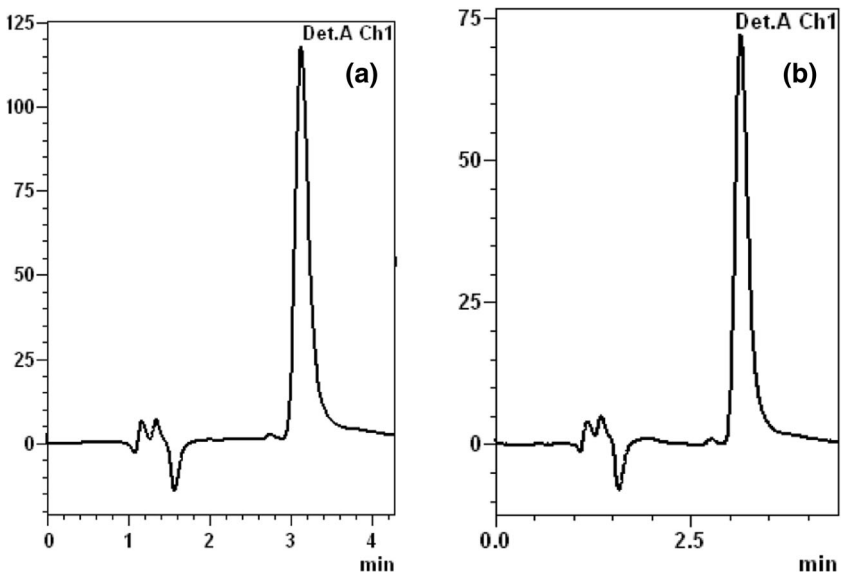

Fig. 2 Chromatograms showing **a** menbutone standard (10.0 μg mL^{-1}) and **b** menbutone (6.0 μg mL^{-1}) in its dosage form

Table 1 Assay results for the determination of menbutone in pure form using the proposed and comparison methods

Ranges	Proposed method			Comparison method		
	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	% recovery	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	% recovery
0.05–2.0 μg mL^{-1}	0.05	0.0494	98.80	2.5	2.452	98.08
	0.1	0.0996	99.60	10.0	10.074	100.74
	0.25	0.251	100.40	25.0	24.979	99.92
	0.5	0.500	100.00			
	0.75	0.749	99.87			
	1.0	0.999	99.90			
	2.0	1.999	99.95			
Mean % ± SD			99.79±0.496			99.58±1.362
t test	0.376			(2.306)		
F test	7.54			(19.3)		
2.0–20 μg mL^{-1}	2.0	1.973	98.65	2.5	2.452	98.08
	6.0	6.042	100.70	10.0	10.074	100.74
	10.0	9.984	99.84	25.0	24.979	99.92
	15.0	14.999	99.99			
	20.0	19.991	99.96			
Mean % ± SD			99.83±0.740			99.58±1.362
t test	0.344			(2.447)		
F test	3.388			(19.2)		

Each result is the average of three separate determinations. The values between parentheses are the tabulated *t* and *F* values at $P=0.05$

conditions. The proposed method offers high sensitivity as low as 0.945 ng mL^{-1} of menbutone could be detected accurately.

Method Development and Optimization

Two different columns were used for performance investigations, including Shim-Pack VP-ODS C_{18} (250 mm×4.6 mm i.d., 5 μm particle size) and Shim-Pack VP-ODS C_{18} (150 mm×4.6 mm i.d., 5 μm particle size). The experimental studies revealed that the second column was more suitable, since it produced good peak shape in a reasonable time.

Different organic modifiers were tried including acetonitrile, methanol, ethanol, propanol, and butanol. The retention time decreased through the addition of propanol or butanol, which have greater elution strength. Butanol was selected as it yielded better efficiencies and lower retentions than propanol. The influence of the concentration of SDS on the retention time and peak shape of menbutone was studied using a concentration of 0.05–0.150 M. It was found that an increase in

Table 2 Accuracy and precision data for the determination of menbutone using the proposed method

Range	Amount taken (μg mL^{-1})	Intra-day[a]			Inter-day[b]		
		Amount found ± SD (μg mL^{-1})	Accuracy (R%)	Precision (% RSD)	Amount found ± SD (μg mL^{-1})	Accuracy (R%)	Precision (% RSD)
0.05–2.0 (μg mL^{-1})	0.1	0.098±0.001	98.00	1.02	0.0986±0.0013	98.60	1.318
	0.5	0.502±0.007	100.40	1.39	0.513±0.012	102.60	2.339
	1.0	1.003±0.012	100.30	1.196	1.018±0.017	101.80	1.67
2.0–20 (μg mL^{-1})	2.0	1.999±0.028	99.95	1.401	2.011±0.039	100.55	1.939
	10.0	9.947±0.122	99.47	1.126	9.972±0.219	99.72	2.253
	20.0	20.056±0.0826	100.28	0.412	20.103±0.115	100.52	0.572

Each result is the average of three separate determinations

[a] Within the day

[b] Three consecutive days

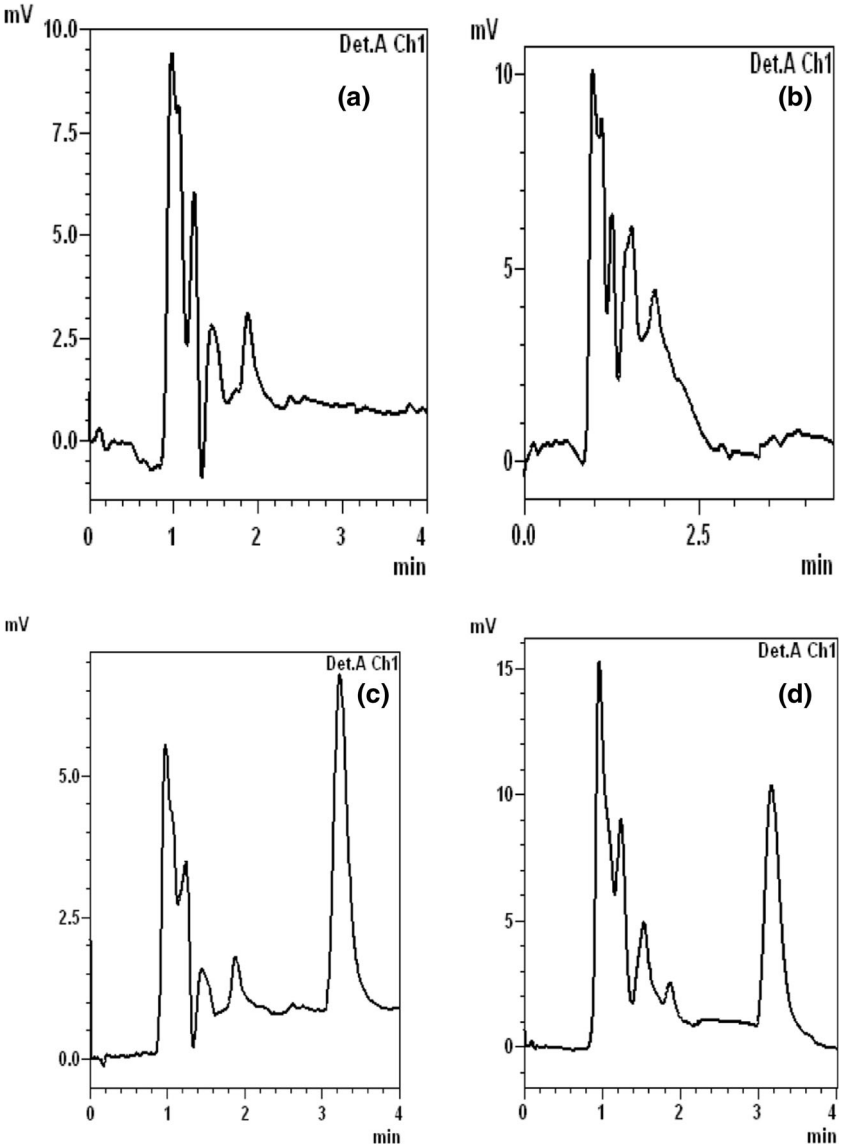

Fig. 3 Chromatograms showing **a** blank for milk sample, **b** blank for meat sample, **c** menbutone in milk sample (0.5 μg mL^{-1}), and **d** menbutone in meat sample (0.5 μg mL^{-1})

the concentration of SDS decreased the retention time of menbutone with a higher number of theoretical plates. Proportions of butanol and SDS in the mobile phase were altered to get good peak shape and desired retention times. 0.12 M SDS and 8 % *n*-butanol were the best, giving good peak shape and the highest number of theoretical plates. Retention times increased when the concentration of surfactant decreased. Micellar mobile phases of pH values ranging from 3.0 to 6.5 were tested. pH over the range 3.0–5.0 caused a delay in elution with peak broadening. There was a slight decrease in retention time over the range 5.5–6.5. Thus, pH 6.0 was most appropriate, where it offered a good combination of peak symmetry and analysis time. The temperature of the column was varied from 25 to 40 °C (with 5 °C interval). Similarly, the effect of flow rate was examined at 0.8, 1.0, and 1.2 mL min^{-1}. It was found that a column temperature of 25 °C and a flow rate of 1.0 mL min^{-1} were the optimum conditions for good separation within reasonable retention times. Proper choice of the detection wavelength is crucial for sensitivity of the method. Quantitation was achieved with UV detection at 234 nm based on highest peak area for the drug. After optimization of these variables, best peak shape and lowest peak tailing were achieved with good sensitivity within a reasonable analytical run time. Finally, 0.12 M sodium dodecyl sulfate/8 % *n*-butanol at pH 6.0 was selected as the optimum mobile phase in which the analyte eluted at approximately 3 min with an adequate shape.

Method Validation

The validity of the proposed method was assessed by studying the following parameters in accordance with the International Conference on Harmonization (ICH) Q2B recommendations (ICH and Q2 (R1) 2005): linearity, limit of detection (LOD),

Table 3 Assay results for the determination of menbutone in injection using the proposed and comparison methods

Parameters	Proposed method			Comparison method		
	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	% recovery	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	% recovery
Data	2.0	1.957	97.85	2.5	2.555	102.20
	6.0	6.095	101.58	10.0	9.918	99.18
	10.0	9.849	98.49	25.0	25.026	100.10
	15.0	15.179	101.19			
	20.0	19.909	99.55			
Mean % ± SD			99.73±1.634			100.49±1.547
t test	0.65			(2.447)		
F test	1.116			(19.2)		

Each result is the average of three separate determinations. The values between parentheses are the tabulated *t* and *F* values at $P=0.05$

limit of quantification (LOQ), accuracy, precision, selectivity, sample solution stability, mobile phase stability, and robustness.

Linearity

Under the above-described experimental conditions, linear relationships were established by plotting peak areas against the drug concentrations. The working concentration ranges were found to be linear over two ranges, 0.05–2.0 and 2.0–20 μg mL^{-1}. Linear regression analysis of the data gave the following equations:

$$P = 0.097 + 135C;\ r = 0.9999\ \left(0.05\text{–}2.0\ \ \mu g\ \ mL^{-1}\right)$$
$$P = 3.19 + 135.2C;\ r = 0.9999\ \left(2.0\text{–}20\ \ \mu g\ \ mL^{-1}\right)$$

where C is the concentration of the drug in micrograms per milliliter and P is the peak area. The high values of the correlation coefficients (r value 0.999) indicate good linearity of the calibration graphs in both cases.

Limit of Quantitation and Limit of Detection

The LOQ was determined by establishing the lowest concentration of the analyte that can be measured according to ICH Q2B recommendations (ICH and Q2 (R1) 2005) and below which the calibration graph is nonlinear and was found to be 2.864 ng mL^{-1}. The LOD was determined by establishing the minimum level at which the analyte can be reliably detected; it was found to be 0.945 ng mL^{-1}.

Accuracy and Precision

To prove the accuracy of the proposed method, the results of the assay of menbutone in pure form were compared with those of the comparison method. The comparison method is the manufacturer's method, which depends on measuring the UV absorbance of the aqueous solution of the drug at 321 nm.

Statistical analysis of the results obtained using Student's t test and variance ratio F test (Miller and Miller 2005) revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively, as illustrated in Table 1.

Intra-day precision was achieved by determination of three different concentrations of menbutone on three successive times in the same day. Inter-day precision was performed as intra-day precision, but on three successive days. Small values of % relative standard deviation (RSD) revealed the precision of the proposed method. The results are illustrated in Table 2.

Selectivity

The selectivity of the proposed MLC method was established by its ability to determine menbutone in commercial injection solution without interference from any additives (Fig. 2b). Furthermore, to evaluate the specificity of the method to determine the cited drug in bovine milk and meat, a blank sample was prepared and injected under the recommended chromatographic conditions. No interfering peaks were observed at the retention time of the drug, which proved the homogeneity and purity of the peak (Fig. 3a–d).

Sample Solution Stability and Mobile Phase Stability

Evaluation of the stability of menbutone solution was achieved by quantification of the drug on eight successive days and comparison to freshly prepared solution. No significant changes were observed for up to 7 days. The stability of the mobile phase was also checked, and it was found to be stable for up to 3 days with no significant changes.

Table 4 Assay results for the determination of menbutone in bovine milk and meat using the proposed method

Method	Bovine milk			Bovine meat		
	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	% recovery	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	% recovery
Data	0.1	0.099	99.00	0.1	0.098	98.00
	0.5	0.501	100.20	0.5	0.503	100.60
	1.0	0.999	99.90	1.0	0.998	99.80
Mean			99.70			99.47
SD			0.624			1.332
% RSD			0.626			1.339

Each result is the average of three separate determinations

Robustness

To assess the robustness of the proposed MLC method, the chromatographic conditions were deliberately altered, viz flow rate (1±0.1), detection wavelength (234±2 nm), pH of the mobile phase (6.0±0.2 pH units), concentration of *n*-butanol (8± 0.5 %), and concentration of SDS (0.12±0.01 M). The efficiency of the separation of menbutone was not affected, indicating the reliability of the proposed method. Therefore, the method is robust enough to the small changes in the experimental conditions.

Applications

Application of the Proposed Method to the Determination of Menbutone in Its Injection Solution

The developed MLC method was applied successfully for the assay of the drug in its 10 %® injection solution as shown in Fig. 2b. The results obtained were statistically compared with those of the comparison method using the *t* test and *F* test (Miller and Miller 2005). The results show that there were no significant differences between the developed and comparison methods regarding accuracy and precision, respectively, as illustrated in Table 3.

Application of the Proposed Method to the Determination of Menbutone in Bovine Milk and Meat

The applicability of the procedure developed here to determine menbutone was tested by analyzing the drug in bovine milk and meat. All the samples were bought at a local supermarket. Table 4 shows the results of the analysis of menbutone determined in all the samples after homogenization with micellar solution, sonication, centrifugation, and filtration. The data obtained (Table 4) show satisfactory recoveries for menbutone in all samples, and the results fall in the range of 98.00–100.60 %. Figure 3c, d shows the chromatograms obtained from the spiked samples of menbutone analyzed with the optimum mobile phase.

Conclusion

The proposed method was found to be reliable, simple, quick, and accurate for the determination of menbutone in its ampoules. The proposed method is also useful for food quality testing and control as it was successfully applied to determine the content of menbutone in bovine milk and meat samples. One advantage of this procedure is the possibility of injecting the samples directly into the chromatographic system without previous treatment other than homogenization, dilution, and filtration, thus avoiding tedious extraction steps from matrices. Validation according to ICH regulations provides satisfactory results in terms of sensitivity, linearity, accuracy, and recoveries. It is noteworthy that the use of micellar mobile phase endows the procedure advantages such as non-toxicity, non-inflammability, biodegradability, and low cost.

Conflict of Interest The authors declare no conflict of interest. This article does not contain any studies with human or animal subjects.

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