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Simple, Sensitive and Rapid LC/MS/MS Method for Quantification of Residual Phenylbutazone in Milk

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A simple, sensitive and rapid liquid chromatography mass spectrometry method was developed and validation for estimation of residual phenylbutazone in milk. An analyte was extracted by liquid-liquid extraction with dichloromethane. A chromatographic separation was performed on reversed phase chromatography C-18 column with mobile phase 0.3 % formic acid in water and 0.3 % formic acid in methanol. The analyte was quantitated in positive ionization by mass spectrometer. The mass transitions m/z 309.3 > 162.1, 309.3 > 160.1, 309.3 > 146 and 309.3 > 104.0 were used. The lower limit of quantification was 1.0 ng/g with relative standard deviation of less then 15 %. The average recovery of phenylbutazone in milk fortified at the level of 1.0 ng/g was 77.38 % with relative standard deviation of 5.76 %.

Key Words: Liquid chromatography, LC/MS/MS, Phenylbutazone, Milk.

INTRODUCTION

From the aspect of food hygiene drug monitoring in products from food producing animals is very important, as residue levels might be a potential hazard to the consumer. In Sweden, the non-steroidal antiinflammatory agent phenylbutazone was used for treating cows suffering from arthritis and laminates, although knowledge of phenylbutazone residue levels in milk is lacking[1].

Non-steroidal antiinflammatory drugs (non-steroidal antiinflammatory drugs are widely used in human and veterinary medicine for their ability to either suppress or reduce the inflammatory process and the clinical signs associated with it, such as heat, pain, swelling, hyperaemia and loss of function[2]. Non-steroidal antiinflammatory drugs (NSAIDs) represent a heterogeneous group of compounds. They are often chemically unrelated, although most of them are organic acids. The prototype is aspirin and hence these compounds are often referred to as aspirin-like drugs. The follows of classes of compounds can be distinguished[2].

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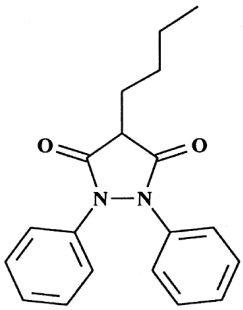

Structure of phenylbutazone

All aspirin type drugs are antipyretic, analgesic and antiinflammatory, but there important difference in their activity. Some are not suitable for either routine or prolonged use because of their toxicity, *e.g.* phenylbutazone. The most common side-effect is a propensity to reduce gastric or intestinal ulceration that can sometimes be accompanied by anaemia from the resultant blood loss. Other side-effects include disturbances in platelet function; the prolongation of gestation or spontaneous labour and changes in renal function[3]. Long term exposure to phenylbutazone includes kidney tumours in rats and liver tumours in mice. Each pharmacologically active substance administered to food-producing animals must be assigned a maximum residue limit (MRL)[2].

According to Export Inspection Agency (EIA), phenylbutazone is not permitted at any concentration in milk for human consumption. A method for determination of phenylbutazone in milk was proposed by HPLC with UV detection. The quantification limit of this method was very high (60 ng/mL)[2]. So we proposed a method for determination of the phenylbutazone in milk by LC/MS/MS with a quantification limit of 1 ng/mL. This method is more sensitive and accurate in a comparison of the HPLC method. In this paper we present simple and reliable method for the determination of phenylbutazone in milk, with relative good coefficient of variation of the results.

EXPERIMENTAL

Methanol, (HPLC grade purity) was purchased from J.T. Baker. Ethanol, dichloromethane and petroleum ether were purchased from MERCK. Analytical reagent grade formic acids, ammonia (25 %) and hydrochloric acid were obtained from Merck. During the process purified-water (Millipore) was used. Certified reference standard of phenylbutazone was purchased from Sigma-Aldrich.

The LC/MS/MS system consists of a series 200 pump and auto sampler (Perkin-Elmer) coupled to an API 3000 Triple Quadruple Mass Spectrometer (Applied Biosystems/MDS SCIEX).

Chromatographic condition: HPLC elution was performed on a C-18 column (LiChrocart® STAR RP-18 (3 µm), 55 mm) held at a temperature of 45 ºC using a

gradient solvent system. Mobile phase A was 0.3 % formic acid in water methanol and mobile phase B was 0.3 % formic acid in methanol. The flow rate was 0.2 mL/min. The gradient conditions are listed below:

Step	Time (min)	Flow rate (mL/min)	Gradient profile	Mobile phase A (%)	Mobile phase B (%)
0	0.0	200.00	0.0	50.0	50.0
1	0.2	200.00	0.0	50.0	50.0
2	0.5	200.00	1.0	10.0	90.0
3	4.0	200.00	0.0	10.0	90.0
4	4.2	200.00	1.0	50.0	50.0
5	6.0	200.00	0.0	50.0	50.0

Mass spectrometer condition: MS/MS determination was performed by operating the mass spectrometer in positive mode with a Turbo Ion Spray source, heated with 400 ºC. Capillary voltage was set at 2.5 KV. The Collision energy was separately optimized for the two selective ion transitions. Data were acquired according to the multiple reaction monitoring (MRM) approach. The optimum determined conditions by operating the mass spectrometer in positive (ESI+) mode of the interface were as below.

Preparation of standard stock solutions: Weigh accurately about 10 mg of certified reference standard of phenylbutazone in 10 mL volumetric flask and made a volume up to mark with methanol. Consider the purity, molecular weight, loss on drying or any other relevant information, after weighing the reference/working standard to obtain the actual concentration of the stock solution.

Sample preparation: Weigh 1 g sample of milk in a 15 mL centrifuge tube. Add 1.0 mL of ethanol, 100 µL of 25 % ammonia and 7 mL of petroleum ether. Vortex for 2 min and centrifuge at 3500 rpm for 10 min. Allow to stand for 5 min and discard the organic layer. Add 0.4 mL of 3 M HCl, 6 mL dichloromethane, shake for 10 min and centrifuge for 10 min at 3500 rpm. Withdrawal the organic layer into a dry test tube and evaporate to dryness under nitrogen. Dissolve the residue with 1.0 mL of mobile phase A and B (50:50), filter and inject on LCMS-MS.

RESULTS AND DISCUSSION

Mass spectrometer: In order to develop a method with desired sensitivity (1.0 ng/mL), it was necessary to use MS-MS detection, as the compound did not posses the UV absorbance or fluorescence properties needed to achieve this limit. To optimize the MS-MS condition, when precursor ion of phenylbutazone was set at $[M+H]^-$ (m/z 309.3) was observe as parent ion under ESI-MS-MS condition. The four product ions (309.3 > 162.1, 309.3 > 160.1, 309.3 > 146.0 and 309.3 > 104.0) under four different collision energy offset (28, 29, 30 and 45) were observed. The most sensitive mass transition was from m/z 309.3 > 160.1. The parameters presented in Table-1, are the results of mass spectrometry optimization. The positive ion Turboionspray mass spectrum of phenylbutazone is shown in Fig. 1.

TABLE-1

Scan type	MRM (MRM)
Polarity	Positive
Q1 Mass (amu)	309.3
Q3 Mass (amu)	162.1, 160.1,146,104.0
Dwell time	100
Ion source gas (gas 1) (psi)	8
Ion source gas (gas 2) (L/min)	7
Curtain gas (psi)	6
Collision gas (psi)	4
Ion spray voltage (V)	2500
Source temperature (ºC)	400
Declustering potential (V)	50
Focusing potential (V)	190
Entrance potential (V)	10
Cell Exit potential (V)	12
Collision energy (V)	28, 29, 30, 45

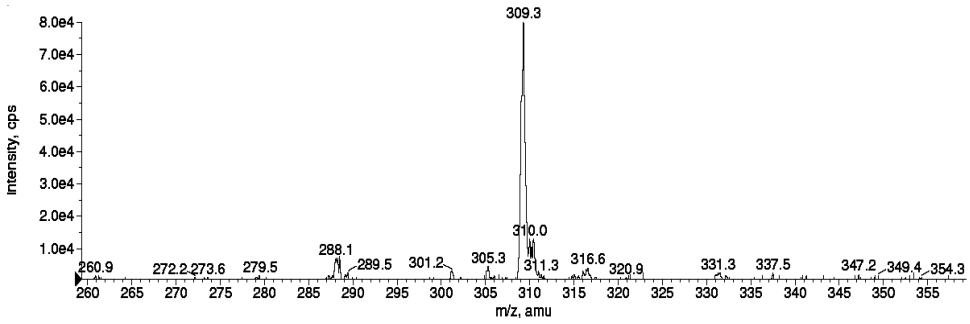

Fig. 1. Mass spectrum

Specificity: The specificity of the method was investigated by analyte blank milk, spiked milk and standard phenylbutazone (Fig. 2). Blank milk showed no significant interfering peak at the retention time of phenylbutazone.

Linearity and calibration curve: The seven points calibration curve was constructed by plotting peak area (Y) of the phenylbutazone *vs.* concentration (x). Calibration curve was linear over the concentration range 0.5 to 30 ng/mL for the phenylbutazone. The regression coefficient was calculated by (1/x) linear regression in analyst 1.4.1 software used in AB API 3000. The regression coefficient of the weighted calibration curve was 0.9995 for phenylbutazone. The calibration curves were shown in Fig. 3.

Recovery: The extraction recovery is calculated by comparing the peak area of phenylbutazone in aqueous standard with the peak area of phenylbutazone added to the blank milk. The extraction recoveries of phenylbutazone at the level 1.0, 2.5, 10.0 and 30.0 ng/mL are listed in Table-2 and show an overall mean per cent recovery of 80.99 % for phenylbutazone.

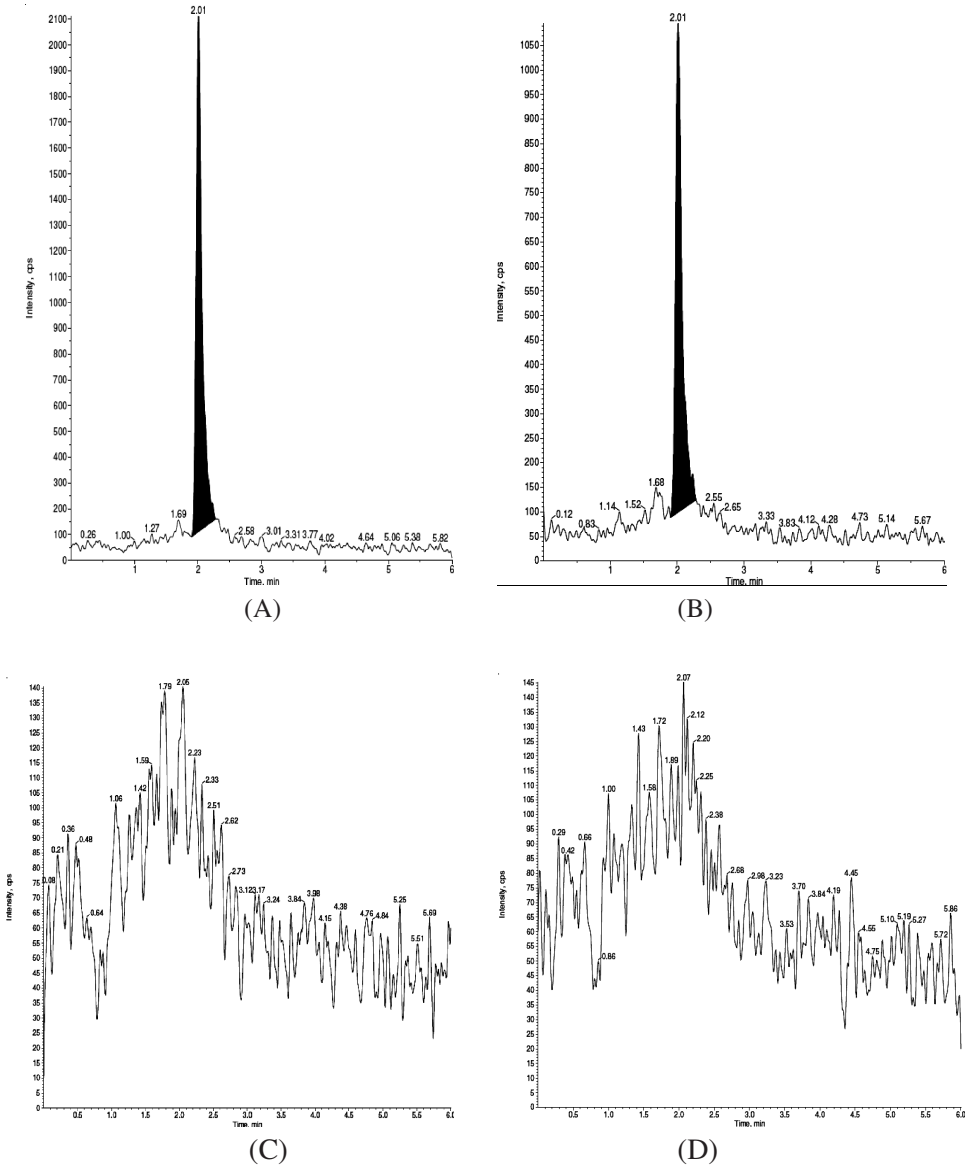

Fig. 2. (A) Standard phenylbutazone 1.0 ppb, (B) Standard phenylbutazone 0.5 ppb, (C) Blank milk, (D) Mobile phase

Precision and accuracy: Table-2 shown a summery of intra and inter day precision and accuracy for phenylbutazone in milk. The intra-day and inter-day accuracy of phenylbutazone in milk sample with the precision (% RSD) are tabulated in Table-2. So it is expected that the purposed method will be applicable to estimation of phenylbutazone in milk.

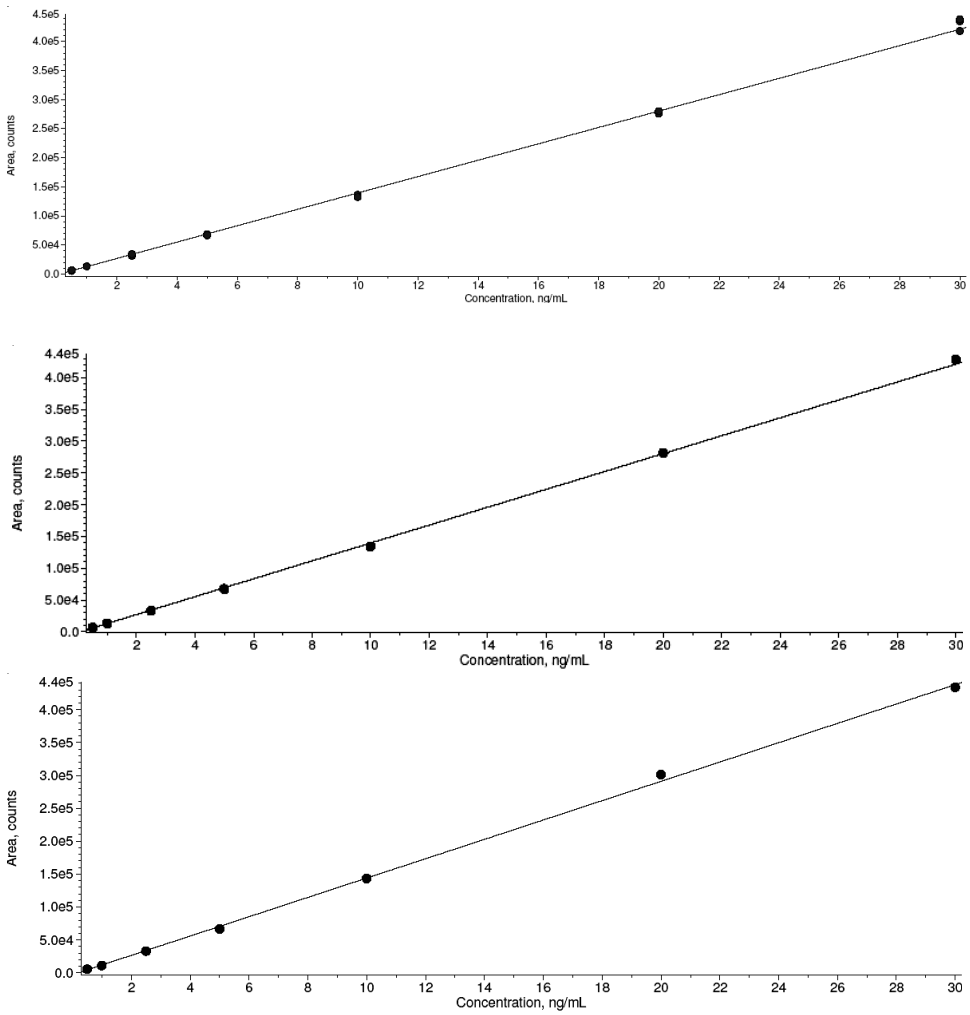

Fig. 3. Calibration curves

Limit of detection and limit of quantification: The limit of detection of phenylbutazone milk is 0.5 ng/mL (Signal to noise ratio was greater than 3:1) and lower limit of quantification of phenylbutazone milk is 1.0 ng/mL (signal to noise ratio was greater than 10:1). Over all results indicates that the present method is sensitive, accurate, precise and enough for monitoring of the phenylbutazone in milk.

Conclusion

The method has been described for the determination of phenylbutazone residue in milk. The method was validated over the concentration 1.0 to 30.0 ng/mL with over all recovery 80.99 and 4.47 % of RSD. The method is particularly well suited to routine regulatory analysis.

TABLE-2

	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	No. of samples	Precision (% RSD)	Accuracy	Over all % RSD	Average Recovery
Day 1	1.0158	0.7944	3	6.06	79.44	4.47	80.99
	0.9824	0.7044			70.44		
	0.9870	0.7629			76.29		
	2.5391	2.0381	3	2.17	81.52		
	2.5311	2.1236			84.94		
	2.4551	2.0569			82.28		
	10.0611	8.5378	3	4.19	85.38		
	10.5524	7.8845			78.84		
	10.4512	8.0374			80.37		
	30.5772	25.5092	3	2.35	85.03		
	29.9472	25.0441			83.48		
	31.4256	24.3432			81.14		
Day 2	1.0051	0.8377	3	5.20	83.77		
	1.0051	0.7880			78.80		
	0.9962	0.7558			75.58		
	2.4881	2.0498	3	1.14	81.99		
	2.4538	2.0275			81.10		
	2.6205	2.0034			80.14		
	10.4827	7.7938	3	3.37	77.94		
	9.8124	8.3364			83.36		
	10.0482	8.0412			80.41		
	29.9445	25.5473	3	3.65	85.16		
	29.7516	25.8137			86.05		
	30.7353	24.1091			80.36		

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