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High-performance liquid chromatographic determination of 4-methylimidazole in sheep plasma and in ammoniated tall fescue hay[a]

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ABSTRACT

A method for 4-methylimidazole (4MI) extraction and quantitation in body fluids and forage samples was developed. The procedures involve ion-pair extraction of the compound with the quantitation done by ion-pair liquid chromatography. The results indicate that this high-performance liquid chromatographic method is sensitive, reproducible and more rapid than others that have been previously used. The mean recovery of 4MI from plasma and tall fescue (*Festuca arundinacea*) hay samples were above 95 and 85%, respectively The versatility of the procedure makes it suitable for the determination of 4MI in body fluids and in forage samples.

INTRODUCTION

Many imidazole and pyrazine derivatives have been isolated in model systems where simple sugars react with ammonia [1–5]. One of the compounds, 4-methylimidazole (4MI), has been shown to be a very potent convulsant agent that elicits neurological signs in mice [6,7], chicks and rabbits [6,8] and cattle [9]. Similar hyperexcitability was produced in cattle fed ammoniated invert molasses [10,12] or ammoniated forages [9,13–18].

Acceptable methods for monitoring 4MI in biological samples do not appear to be available. However, a few procedures for 4MI extraction, identification and quantitation in caramel color have been reported: solvent extraction and quantitative determination by gas chromatographic (GC) analysis without derivatization [19,20] or after N-acetyl derivatization [21], solvent extraction and reversed-phase chromatography for quantitation [21] and ion-pair extraction and reversed-phase chromatography for quantitation [22]. Only one method for the

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determination of 4MI in ammoniated forage has been published [13]. All the reported methods for the determination of 4MI in caramel color, except that of Thomsen and Willumsen [22], are very long (up to 8 h per duplicate extractions) and require large volumes of extracting solvents to be evaporated in later stages, thus increasing inaccuracies and loss of precision. Ray *et al.* [13] published a method that converts 4MI contained in ammoniated forage into the acetyl derivative before gas chromatographic–mass spectrometric (GC–MS) analysis. In addition to the large amounts of solvents used for extraction and the time required per duplicate extraction, the recovery rate that they reported was low (44.4 ± 12.8%) and inconsistent, and they recommended that their method to be used for screening for 4MI but not for kinetic studies.

Thomsen and Willumsen [22] have described a reversed-phase ion-pair liquid chromatographic procedure for the extraction and determination of 4MI in caramel color. The method is a substantial improvement over existing methods in that it is faster and has a very good recovery rate (100–103.8%). However, its detection limit (4 $\mu g\ g^{-1}$) may be a problem when very dilute samples are analyzed

Despite the possible involvement of 4MI in the etiology of bovine hysteria, an accurate and fast method for the extraction and quantitation of 4MI has been lacking. Therefore, it was necessary to develop a reliable microanalytical method for determining concentrations of 4MI in plasma, urine and ammoniated forage samples. The extraction part of this procedure is adapted from the method published by Thomsen and Willumsen [22].

EXPERIMENTAL

Ion-pair extraction

Plasma and urine samples from sheep infused with or orally fed 4MI as well as ammoniated forage samples were analyzed for 4MI contents. Sampling times as determined from other pharmacokinetic studies indicated that blood samples taken 2 h after 4MI administration and urine samples taken between 2 and 4 h post-infusion or post-feeding were suitable. Forage samples were tall fescue hay ammoniated with 3% anhydrous ammonia (dry weight basis) at 84°C and 35% moisture for two days.

Plasma samples. A 500-μl volume of plasma was pipetted into a screw-capped glass tube and 3.5 ml of a 0.2 *M* potassium phosphate buffer (pH 6.0) were added. The tube was capped and vortex-mixed for 30 s. Then 4 ml of 0.1 *M* bis(2-ethylhexyl) hydrogenphosphate (BEHPA) in chloroform were added, and again the tube was vortex-mixed for 30 s to extract 4MI. After centrifugation at 3500 *g* for 10 min at ambient temperature, the aqueous phase (top layer) was removed by aspiration and 3 ml of the chloroform phase (bottom layer) were transferred to a new centrifuge tube containing 3 ml of 0.1 *M* phosphoric acid. The tube was vortex-mixed again for 30 s and 4MI re-extracted into the aqueous phase. After centrifugation at 3500 *g* for 10 min at ambient temperature, the aqueous phase

(top layer) was ready for final determination by high-performance liquid chromatography (HPLC).

Urine samples. A 100-μl volume of urine from 4MI-infused sheep (IV) and 50 μl of urine from orally administered sheep were pipetted into screw-capped glass tubes, and 20 and 5 ml of a 0.2 *M* phosphate buffer (pH 6.0) were added, respectively. Subsequent procedures were the same as for plasma samples.

Forage samples. Approximately 0.5 g of tall fescue (*Festuca arundinacea*) hay was put into a screw-capped glass test tube, and 10 ml of a 0.2 *M* potassium phosphate buffer (pH 7.0) were added. The tube was vortex-mixed for 30 s, mechanically shaken for 30 min and then centrifuged at 3500 *g* for 10 min. After filtration through a Whatman No. 1 paper, a 500-μl aliquot was pipetted into a screw-capped glass tube; 3.5 ml of the phosphate buffer were added, and the extraction was continued as previously described for plasma samples.

Preparation of standards. Standards were prepared by dissolving the appropriate amount of 4MI (Aldrich, Milwaukee, WI, U.S.A.) in the appropriate volume of 0.2 *M* potassium phosphate buffer to yield a 500 $\mu g\ ml^{-1}$ stock solution. Further dilutions were made using the same buffer.

Ion-pair chromatography

Apparatus. The HPLC system consisted of a Waters M-45 solvent delivery system coupled to a 7125 injection valve (Rheodyne) fitted with a 20-μl sample loop. Separation of 4MI was achieved using a 15 cm × 3.9 mm stainless-steel analytical column packed with 10-μm μBondapak C_{18} material (Waters, Milford, MA, U.S.A.). The analytical column was fitted with a disposable Guard-Pak precolumn insert packed with 10-μm particles of μBondapak C_{18} material (Waters). Detection of 4MI was achieved by a Waters Lambda-Max Model 481 LC spectrophotometer at 215 nm. Chromatograms were recorded on a Shimadzu Chromatopac C-R6A (Shimadzu, Columbia, MD, U.S.A.) plotting integrator that measures peak heights and areas. It was programmed to use areas for automatic calculation of concentrations of unknown samples. Calibration was done by the two-point calibration method. A Waters Model 720 system controller was used to monitor the conditions of the pump.

Mobile phase. The isocratic mobile phase was composed of water and methanol (20:80) plus 0.005 *M* PIC B_7 low-UV reagent (Waters) [23], and then filtered under vacuum using a 0.22-μm Durapore filter (Waters [23]). The eluent was degassed under vacuum before use.

RESULTS AND DISCUSSION

Imidazole derivatives have low volatilities and high polarities and this makes them less extractible with most organic solvents [22,24]. An ionized compound such as 4MI cannot be extracted efficiently into water-immiscible solvents, and an ion-pair extraction enhances solubility of the ionized solute (phosphate buffer)

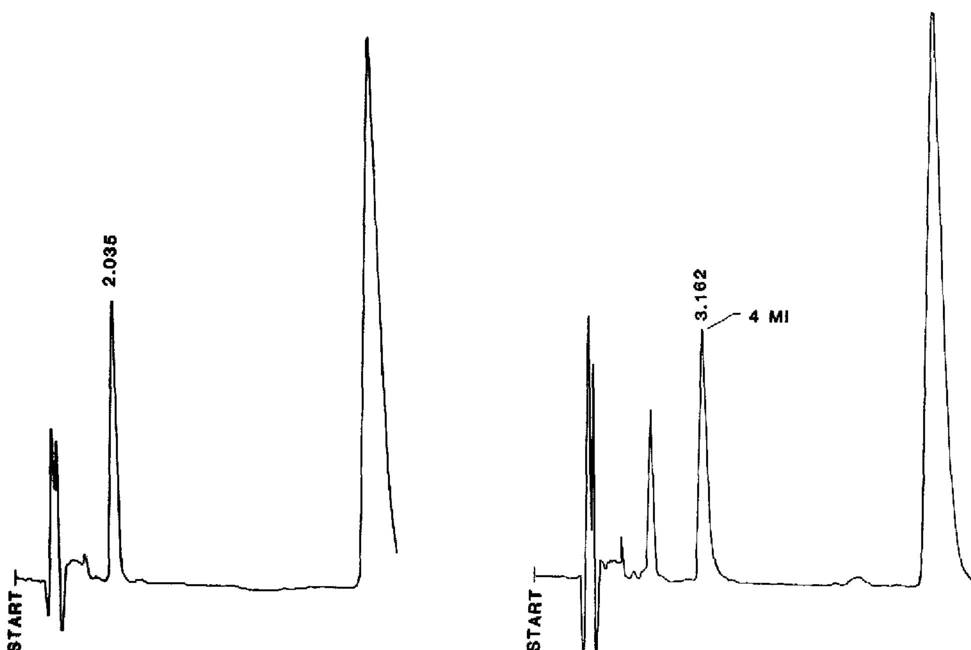

Fig. 1. HPLC profile of extracted plasma blank showing no peak at the 4MI retention time.

Fig. 2. HPLC profile of plasma from sheep infused with 4MI showing 12.12 $\mu g\ ml^{-1}$ 4MI at retention time 3.162 min. Actual amount of 4MI on column was 30.3 ng

into the organic phase (chloroform) by formation of an ion pair whose behavior in the organic phase is that of a non-ionic or neutral species [23]. The ion-pair extraction used in this method was based on that of Thomsen and Willumsen [22] which used BEHPA as an ion-pair agent to help obtain a more hydrophobic product easily extracted by chloroform.

The pH of solvents is important because extreme values may result in reduced efficiency of extraction. Thomsen and Willumsen [22], working with caramel color, determined that pH 6.0 was optimum for full recovery of 4MI. Since we were working with a different material (biological samples and ammoniated tall fescue), it was necessary to determine the pH that would give the highest extraction efficiency. pH values, ranging from 5 to 11 were tested keeping other analytical parameters the same. Criteria used to evaluate which pH value was the best were qualitative (shape and resolution of the curve and retention time) and quantitative (peak height). The highest extraction efficiency was obtained with a potassium phosphate buffer of pH 6.00 for plasma and urine samples and pH 7.00 for tall fescue hay samples.

A reversed-phase system was selected because 4MI is water-soluble, and since it is also ionic or ionizable, ion-pair chromatography was chosen. The retention time could be regulated by changing the methanol proportion, but when it ex-

ceeded 20%, a loss in peak height or area and in resolution between 4MI and interfering peaks occurred. The mobile phase composition was changed from water–methanol (80:20) through various intermediate proportions to water–methanol (20:80) and the latter was shown to be the best in terms of detection response, resolution and shape of peaks and baseline. Davis and Hartford [21] used a heptasulfonate (regular PIC B_7 reagent) as the ion-pairing reagent, but in this study a low-UV PIC B_7 reagent was used to avoid interference caused by regular PIC B_7 that absorbs around the wavelength used for 4MI determination (215 nm). At that wavelength, regular PIC B_7 could not be zeroed and ran off scale even at the lowest sensitivity (5 a.u.f.s.).

The following figures show chromatograms of blank plasma (Fig. 1), plasma from a sheep intravenously infused with 4MI (Fig. 2), a sample of untreated tall fescue hay (Fig. 3) and a sample of treated tall fescue hay (Fig. 4). The retention times were 3.16 and 4.94 for plasma and forage, respectively. No interference from extracted blank plasma was observed. The resolution of 4MI in ammoniated forage was not complete but the quantitation was still satisfactory.

Linearity, recovery and low-level detection

For recovery tests, six different concentrations of the standard were used for plasma and three for ammoniated tall fescue (Table I). Addition of the standard

Fig. 3. HPLC profile of extracted non-ammoniated fescue hay showing no peak at the 4MI retention time

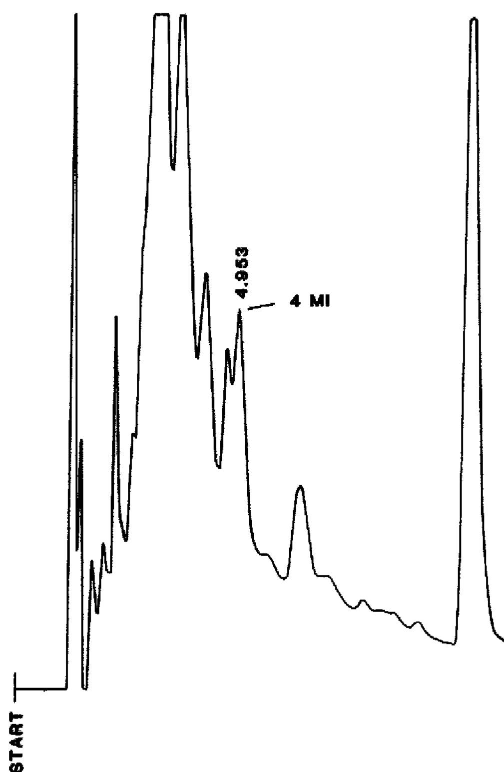

Fig. 4 HPLC profile of extracted ammoniated fescue hay showing 61.2 μg g^{-1} 4MI at retention time 4 93 min. Actual amount of 4MI on column was 5.1 ng.

TABLE I

RECOVERY OF 4MI FROM PLASMA AND TALL FESCUE HAY CONTAINING KNOWN AMOUNTS OF STANDARD

Sample size: 0.5 ml for plasma, 0.5 g for ammoniated fescue; n = 4.

Sample	Added (μg ml^{-1})	Found (mean ± S.D.) (μg ml^{-1})	C V. (%)	Recovery (%)
Plasma	1	1 0±0.08	8	100
	5	4.9±0.19	3.8	98 6
	10	9.5±0.23	2.4	94.6
	15	14.1±0.38	2.7	94.3
	20	18.3±0.57	3 1	91.3
	25	24.1±0.48	2 0	96.4
Fescue	25	23.1±1.34	5.8	92.56
	50	43.0±0.59	1.4	86.03
	100	83.6±1.49	1.8	83 57

to the sample was made before the extraction (before addition of the phosphate buffer), and the recovery rate was over 80%. Various other methods yielding recovery values over 90% have been reported by Wilks and co-workers [19,20], Fuchs and Sundel [26], Thomsen and Willumsen [22] using caramel color and Weiss *et al.* [17] using ammoniated forage.

The linearity was tested by extracting 50, 100, 200, 400 and 800 μl of the same plasma or forage sample. The results obtained (Table II) show determination coefficients better than 0.99 and small intercepts (0.12 and −0.33 for plasma and tall fescue samples, respectively). This led us to use standard solutions of two concentrations for calibration.

TABLE II

LINEARITY OF EXTRACTION OF DIFFERENT QUANTITIES OF THE SAME PLASMA OR FORAGE SAMPLE

Run No.	4MI extracted (μg)				
	50 μl	100 μl	200 μl	400 μl	800 μl
Plasma					
1	1.5	2.3	4.7	9 2	17.8
2	1.2	2.2	4.9	9 1	18.5
3	1.3	2.4	4.9	9 2	18 7
4	1.2	2.3	5 0	9.5	18.1
Mean	1.3	2.3	4 9	9.3	18.5
S.D	0.1	0.1	0.1	0.2	0.8
C.V. (%)	10.9	3.5	2.3	1.9	4.5
$r=0.999$					
Fescue					
1	1.4[a]	5.1	14.4	30 1	56 4
2	0.3	5.5	15.7	28.0	55 8
3	0.7	5.3	15.2	28 7	59.2
4	0.9	5.4	16.3	30 2	57.6
Mean	0.8	5.4	15.7	29 2	57 3
S.D.	0.5	0.2	0.5	1.1	1.5
C.V. (%)	56.1	3 0	3.2	3.6	2.6
$r=0.998$					

[a] 50 μl omitted from regression.

Linearity of extraction and detection of ammoniated tall fescue hay were very good in the range 100–800 μl. The values obtained when 50 μl of the sample filtrate were extracted and quantitated were not used in the regression analysis of the results. A sample size of 0.5 g appears to be adequate to start the extraction, but at least 100 μl of the filtrate should be used in the later steps of the extraction procedure.

The reproducibility of the method was evaluted by performing six replicate extractions of the same sample (Table III). Day-to-day precision was estimated for the same sample over a period of four days. The average content was 0.73 μg ml^{-1} with coefficient of variation (C.V.) of 4% within extractions and 0.73 μl ml^{-1} with a C.V. of 1% within days for plasma. Ammoniated tall fescue samples gave an average content of 64.36 μg g^{-1} with a C.V. of 3.86% within extractions and 64.36 μg g^{-1} with a C.V. of 1.55% within days. The C.V.s are below 5%, an indication of a good reproducibility.

TABLE III

REPRODUCIBILITY OF EXTRACTION OF PLASMA AND FORAGE SAMPLES DAY-TO-DAY PRECISION

Run No.	4MI extracted			
	Day 1	Day 2	Day 3	Day 4
Plasma (μg ml^{-1})				
1	0.77	0.77	0 70	0.68
2	0.71	0.78	0.70	0.76
3	0.72	0.68	0 76	0 72
4	0.76	0.70	0 68	0 74
5	0.70	0.73	0.78	0 69
6	0.69	0.70	0 72	0.70
Mean	0.73	0.73	0.72	0 72
S.D.	0.03	0.04	0.04	0.03
C.V. (%)	4.5	5.6	5.4	4 3
Fescue (μg g^{-1})				
1	67 5	62.7	65.7	61 2
2	68.7	64.1	62.7	67 6
3	61.7	64.9	66.6	65 4
4	62 0	62.9	65.5	66.2
5	63 2	64 1	59.2	65 2
6	66.9	64.2	60.0	66 7
Mean	65 0	63.8	63.3	65.4
S.D.	3 0	0 8	3.1	2 2
C.V. (%)	4.7	1.3	4.9	3 4

Tests for the absolute detection limit were not run. The smallest levels of 4MI obtained with plasma (0.11 μg ml^{-1}) and ammoniated tall fescue (0.54 μg g^{-1}) were far lower than the 4 μg g^{-1} obtained by Thomsen and Willumsen [22] using an ion-pair liquid chromatography procedure for the determination of 4MI in caramel colors. Total time for analysis of plasma and urine samples was 8.5 min which allowed the extraction and analysis of twenty samples in duplicate per day or twelve samples in duplicate of ammoniated tall fescue hay.

Once extracted and stored in a refrigerator, 4MI contained in plasma, urine or ammoniated forage was very stable for at least six months. The same extracts analyzed after six months of storage gave the same concentrations (± 2%). This method presents an advantage over the derivatization that yields derivatives that are stable for several days only [22]. An instrument problem in the course of an analysis would not lead to the loss of samples and possible repeat of the experiment because the extracts can be satisfactorily stored in a refrigerator.

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