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Milk as a Drug Analysis Medium: HPLC Determination of Isoniazid

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Taking into consideration the increasing importance of biomedical techniques in chemistry, the analysis of drugs in biological fluids has been introduced into an analytical course at our facility. The use of biological fluids presented us with safety concerns, so we devised a basic experiment, suitable for pharmaceutical chemistry students, which permits them to experiment with and safely handle biological fluids. This experiment could also be successfully adapted to different chemical laboratory analysis curricula.

The present laboratory experiment was designed with the following goals in mind:

1. Students should solve a real and interesting drug analysis problem.
2. The experiment should only require basic instrumental equipment.
3. Students should deal with a few simple chemical reactions, such as a derivatization, occurring in sample preparation.
4. An inexpensive biological fluid, in compliance with safety criteria, should be chosen as an analysis medium.

The last requirement led us to cow's milk as an effective biological fluid, which can model other proteinaceous samples such as blood plasma. Milk is cheap and safe, and allows for the development of some other interesting experiments or discussions with the students. Out of the various papers on the determination of drugs in milk, we chose to adapt the quantification of isonicotinic acid hydrazide (isoniazid) in milk *(1)* to our teaching purposes.

Rationale and Background

The hydrazide of isonicotinic acid (isoniazid; 4-pyridinecarbocylic hydrazide), **1**, is widely used today in the prevention and treatment of human tuberculosis and for bovine tuberculosis prophylaxis. Nevertheless, in several countries the addition of prophylactic isoniazid to cattle-feed is not approved by law because of its possible danger to public health. Traces of this drug can, in fact, transfer into milk, exposing consumers to long-term side effects and other undesired interaction phenomena. Furthermore, isoniazid can give a false positive tuberculin test in cattle, hiding the actual health conditions of the animals *(2, 3)*.

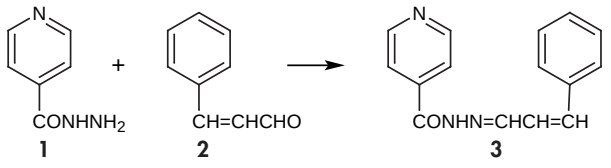
For these reasons, several experimental procedures, including spectrophotometric *(4, 5)* and chromatographic methods *(1, 6)*, have been developed to detect the presence of isoniazid in biological fluids and, particularly, in cow's milk. Some of these methods are based on the reaction of isoniazid, **1**, with cinnamaldehyde reagent, **2**, to form a derivative hydrazone, **3**, as shown in Scheme I. The isoniazid hydrazone derivative, **3**, is more strongly retained in the reversed stationary phase than isoniazid and exhibits a strong absorbance with a peak maximum at a wavelength of 330 nm. These peculiarities have been successfully applied for isoniazid determination both in plasma and in milk by means of an HPLC system with a UV detection *(1)*.

Biological matrix analysis is becoming increasingly important in pharmaceutical excretion–substance studies applied to nutrition, pharmacokinetics, quality control, environmental monitoring, and others. An interesting teaching subject is the treatment of complex matrixes with a high protein content. For this purpose the most suitable matrixes are blood and milk, but the latter is safer. Milk was chosen also for its relevance in popular topics (lactational transfer of drugs in breast milk and health control of dairy animals) and because its analytical treatment can raise some interesting problems for discussion in the classroom, especially concerning drug analysis *(7)*.

A complementary lecture on protein precipitation in a biological matrix could introduce the students to the experiment and inform them of the differences between the use of acid reagents and organic solvents as deproteinizing agents. In particular, the benefits of chemical-induced deproteinization (which avoids sample dilution) could be highlighted *(8)*.

Liquid chromatography, LC, is one of the more common techniques in chemical analysis. This experiment introduces students to LC practice and also allows them to apply theoretical concepts previously learned. In this case the understanding of separation processes can be emphasized by the instructor focusing on the resolution of the mixture of the biological matrix, analytes, and derivatization agents.

This experiment requires a simple isocratic HPLC system equipped with a UV detector. Stationary phases such as octadecylsilane and cyanopropylsilane allow for good retention times of compound **3**. A cyanopropylsilane column, for example, can elute compound **3** in about six minutes using a 40% methanol–acetic buffer mobile phase. Such equipment can resolve the above mixture without additional treatment after deproteinization.



Scheme I. Reaction of isoniazid, **1**, with cinnamaldehyde, **2**, to form a isoniazid hydrazone derivative, **3**.

†In memory of Enzo Sottofattori who died unexpectedly February, 2003.

Experimental Procedure

Column and Mobile Phase

A 200 × 4.6 mm i.d. stainless-steel Hypersil BDS cyanopropyl column (Hypersil, Runcorn, United Kingdom) was used; the column was protected with a LiChroCart 4-4 guard cartridge system (Merck, Germany). The mobile phase consisted of a methanol–water mixture (40:60, v/v) containing 0.41 g of sodium acetate trihydrate and 10 mL of glacial acetic acid per litre of solution. The flow rate was 1.0 mL/min. The detection wavelength was 330 nm, at room temperature.

Preparation of Calibration Curves

In order to obtain standards for the calibration curve, a stock solution of isoniazid with a concentration of 100.0 mg/L was given to the students, who were asked to dilute it with distilled water to yield final concentrations of 1.00, 5.00, 10.00, and 50.00 mg/L (labelled respectively as A, B, C, and D).

Students working in pairs injected 20-mL aliquots into the chromatograph, in triplicate. Each group plotted the obtained peak area of isoniazid versus the concentration and calculated the calibration curves by using linear regression analysis.

Sample Preparation

A 50.00-mL aliquot of the stock solution containing isoniazid was diluted by the instructors with milk in a 200.0-mL volumetric flask. Samples of the diluted solutions, 5.00, 10.00, 20.00, 25.00, and 50.00 mL, were dispensed randomly by means of a buret into a series of numbered 100-mL flasks, which upon dilution would result in 1.25, 2.50, 5.00, 6.25, and 12.50 mg/L isoniazid concentrations. Each pair of students was asked to choose one of the above numbered volumetric flasks with an unknown volume of solution and to dilute the sample to a volume of 100.00 mL by adding milk.

This experiment can be successfully carried out with most commercially available canned or bottled cow's milk (e.g., homogenized, pasteurized, UHT, lowfat, whole, etc.). In fact, the milk butterfat concentration does not affect the reproducibility of the experiment. We used homogenized, fresh, whole cow's milk with 2% fat content.

Sample Treatment

The deproteinization procedure consisted of adding 0.50 mL of 20% (w/w) aqueous trichloroacetic acid to 2.00 mL of sample solution, which had been previously transferred into a test tube by means of a graduated pipette. The solution was mixed in a vortex for 30 s, left at room temperature for 10 min, and centrifuged at 4000 rpm for 10 min.

The students were then instructed to remove the coagulated proteins by carefully pipetting 500.0 μL of supernatant and transferring it into a centrifuge tube containing 10.00 μL of a 1% (w/v) cinnamaldehyde–methanolic solution to activate the derivatization process. Aliquots of 20 mL of this solution, previously mixed in a vortex for a few seconds and left at room temperature for 5 min, were subsequently injected into the chromatograph.

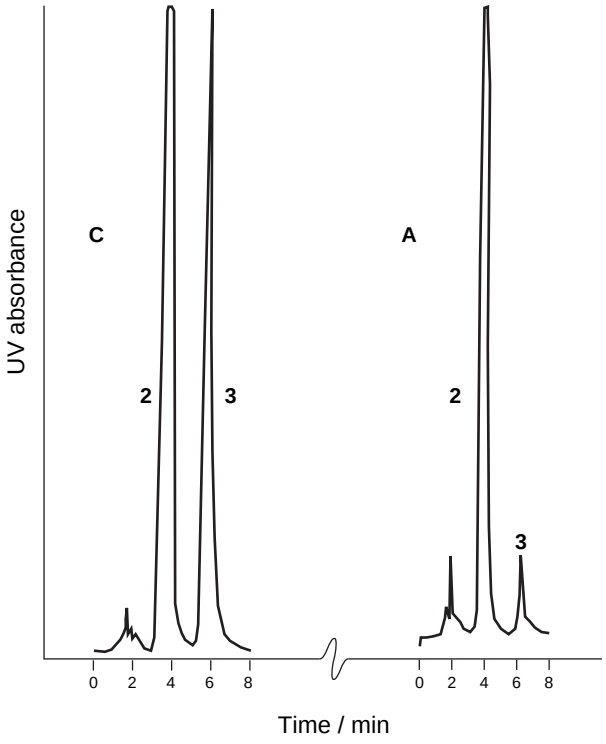


Figure 1. Chromatograms at the calibration concentrations of (A) 1.00 mg/L and (C) 10.00 mg/L of isoniazid. Numbers refer to the structures in Scheme I.

Hazards

Isoniazid standard solutions used in the experiment do not present a serious hazard, but could be dangerous if ingested. The methanolic solution of the derivatizing acid agent is toxic with its major risk being inhalation; however, in this experiment the methanol solution is minimized (10 μL), so risks are reduced. Trichloroacetic acid solution can be harmful if swallowed and skin contact may cause burns. Although the volume required is very small, students must wear protective gloves and safety goggles. Only pasteurized milk should be handled to avoid exposure to contaminants in cow's milk.

Result and Discussion

Two examples of chromatograms obtained at isoniazid concentrations of 1.00 and 10.00 mg/L are shown in Figure 1. Peaks of cinnamaldehyde, **2**, and hydrazone, **3**, are well resolved and eluted at 4 and 6 min, respectively. In preliminary analysis, applying the method proposed by Defilippi, et al. *(1)* using a 250 × 46 mm cyanopropyl column of another brand (Merck, Germany), the retention times were about 7 and 12 min, respectively. The shorter column used in the final analysis decreased the retentivity and reduced the back-pressure, improving the efficiency of the analysis.

The samples given to the student were in the calibration range of 1.00 to 10.00 mg/L, although linearity was satisfied in the 1.00 to 50.00 mg/L range. It was noted that the analytical error increased with the sample concentration. It is relevant to note that in the 1.00 to 10.00 mg/L inter-

Table 1. Selected Examples of Calibration Data Obtained by the Students and the Instructors

Operators[a]	Precision (%RSD)	Recovery (%)	Correlation coefficient
Instructors	0.85–1.38	98.1–100.0	.999
Students, group #4	0.77–2.08	98.0–101.4	.995
Students, group #7	0.90–1.85	97.3–101.1	.998
Students, group #15	0.80–1.65	98.2–101.1	.999

[a]All data collected in triplicate.

Table 2. Results Obtained by the Students

Concentration /mg L^{-1}	Av exp value (10 samples)	Av absolute error	Av relative error	Set of data RSD	Coefficient of variation %
1.25	1.33	0.08	6.58[a]	0.088	7.09
2.50	2.50	0.01	0.01	0.136	5.42
5.00	5.05	0.05	1.03	0.170	3.40
6.25	6.15	0.10	1.57	0.255	1.09
12.50	10.77	1.73	13.81[b]	0.472	3.77

[a]Large error is attributed to high dispersion of the data in the most dilute samples.

[b]Large error is attributed to systematic analyte loss.

val, the peak area of the unreacted cinnamaldehyde is quite constant, so it can give an immediate feedback on possible rough errors in the sample preparation. Students are required to replicate their analysis runs, so the use of an internal standard in this experiment is not strictly necessary.

This method was validated by the authors. Among the validation parameters, students were required to focus only on the calibration curve validation parameters. Examples of calibration curves data are reported in Table 1. In most cases RSD and recovery values were acceptable. Table 2 reports the average values calculated on the basis of ten experimental values obtained by the students. For each set of data, corresponding to the five concentrations of isoniazid, some basic statistical parameters were calculated.

Experimental data in Table 2 show a relevant grade of dispersion in the most diluted samples and a lack of accuracy in the most concentrated samples. A possible loss of isoniazid probably caused by a coprecipitation of analytes from the mother matrix during deproteinization could account for the error at high concentrations *(9)*. This systematic loss provided an interesting starting point for discussion with the students concerning the analysis of procedural errors and possible ways of improving the accuracy (e.g., use of automatic micropipettes, faster centrifugation, etc.).

Conclusions

The analysis of isoniazid in milk allows students to approach basic subjects such as the derivatization of drugs and their determination in a treated biological matrix using a simple, informative, and safe experiment, which is of great value in analytical instruction and critical data evaluation. This easy and fast (four hours over two days) experiment permits each student to operate the chromatograph, thus giving the opportunity to become familiar with some basic analytical parameters such as purity index (estimable by means of a diode array detector response), et cetera.

Students were asked to complete a laboratory report describing the experimental procedures and the processing of data. The feedback was positive and matched the goals for which the experiment was designed.

Considering the interest and the safe manipulation of milk as a drug analysis medium, we plan to apply the outlines of this project to other courses and adapt this simple experiment to the analysis of other drugs, such as antibiotics or antipsychotics.

[w]Supplemental Material

Instructions for the students and notes for the instructor are available in this issue of *JCE Online*.

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