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Short Communication

High-performance liquid chromatography with UV detection and diode-array UV confirmation of isonicotinic acid hydrazide in cattle milk☆

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Abstract

A method for the determination of isonicotinic acid hydrazide (isoniazid) in milk was developed. Milk was deproteinized with trichloroacetic acid. Isoniazid was condensed with cinnamaldehyde and assayed on a reversed-phase HPLC system, with good sensitivity and accuracy (10 μg/l) with UV detection at 330 nm. Use of solid-phase extraction with a C_{18} cartridge allows the detection limit to be lowered to 0.1 μg/l with UV detection and confirmation of isoniazid hydrazone from the diode-array UV spectrum.

1. Introduction

The hydrazide of isonicotinic acid (isoniazid) is still the primary drug for the treatment of human tuberculosis, but its fraudulent use in cattle-breeding, severely prosecuted under Italian law, represents a serious and widespread threat to public health. Administration of this drug to infected cattle can conceal a positive tuberculin test, thereby making its results unreliable and all attempts to eradicate tuberculosis from cattle farms useless. On the other hand, as isoniazid diffuses readily into all body fluids and cells, it is detectable in significant concentrations in commercially bound milk, thus exposing the consumers to a low, but prolonged, dose of this drug.

Phenomena of acute intoxication [1] are very unlikely to occur, as the levels of isoniazid found in milk are well below the therapeutic doses. Nevertheless, this does not exclude the development of either bacterial resistance, sensitivity to the drug or interaction phenomena with other hepatically metabolized drugs [2,3].

In the dairy industry, the presence of a bacteriostatic agent such as isoniazid in milk may inhibit or at least lengthen the dairy processes with considerable economic consequences. Therefore, it is very important to develop a technique for detecting the presence of isoniazid in milk. In addition, the method should allow the univocal and unambiguous identification of the drug.

The only method for detecting isoniazid in

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cattle milk dates back to 1965, when Ruffo [4] examined spectrophotometric and biological techniques. A number of other experimental methods, including spectrophotometric [5], spectrofluorimetric [6], polarographic [7] and chromatographic [8–13] methods, for detecting isoniazid and its metabolites in biological fluids (serum, urine) of patients receiving antitubercular therapy have been described. Among all the proposed methods, we turned our interest to the work of Lacroix *et al.* [8] owing to its possible application to milk analysis. The use of a suitable concentration method and the recording of the UV spectrum with a diode-array detector allows the detection of the drug in milk produced by illegally treated cattle (through its isoniazid hydrazone).

2. Experimental

2.1. Reagents

Isoniazid standard was obtained from Sigma (St. Louis, MO, USA) and cinnamaldehyde, trichloroacetic acid, acetic acid, sodium acetate trihydrate, *n*-hexane (analytical-reagent grade) and methanol (HPLC grade) from Carlo Erba (Milan, Italy). Deionized water was ultrapurified with an Elgastat UHQ system (Elga, High Wycombe, UK).

A 3-ml SPE C_{18} cartridge containing 500 mg of bonded silica gel (40 μm average particle diameter, 60 Å pore size) was obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Apparatus

All the experiments were performed on a Varian (Walnut Creek, CA, USA) LC Star liquid chromatograph equipped with a Model 9001 pump and a Rheodyne (Cotati, CA, USA) injection valve fitted with a 100-μl sample loop. A 5-μm LiChrospher 100-CN column (250 mm × 4 mm I.D.) supplied by Merck (Darmstadt, Germany) was used. The analytical column was usually protected by a 5-μm LiChrospher 100-CN guard column (4 mm × 4 mm I.D.) (Merck).

The detector was a Varian UV-200 operating at 330 nm and UV spectra were recorded on a Polychrom Model 9065 diode-array detector.

Instrument control, data storage and analysis were performed on a PC Grid 325sc (Fremont, CA, USA) Deskpro 386/25m IBM-compatible personal computer with math coprocessor, using Polyview 2.0 software (Varian).

2.3. Procedure

All experiments were carried out isocratically at a flow-rate of 1.0 ml/min. The mobile phase was methanol–water (40:60, v/v) containing 0.41 g of sodium acetate trihydrate and 10 ml of glacial acetic acid per litre of solution; prior to use, it was filtered through a 0.45-μm Millipore filter (Waters, Milford, MA, USA).

2.4. Standards

Isoniazid standard solution was obtained diluting the pure drug with milk produced by safely non-treated cattle to the required concentration. The results were identical with those obtained from a standard solution prepared using water.

2.5. Sample treatment

Without concentration

A 2-ml volume was added to 0.5 ml of 20% (w/w) aqueous trichloroacetic acid, mixed for 30 s, left at room temperature for 10 min, then centrifuged at 5500 *g* for 15 min. A 600-μl volume of the clear solution was added to 5 μl of a 1% (w/v) methanolic solution of cinnamaldehyde, vortex mixed for a few seconds, left at room temperature for 5 min and injected in the HPLC system.

Concentration of isoniazid hydrazone

An 80-ml volume of milk was added to 20 ml of 20% (w/w) aqueous trichloroacetic acid, left at room temperature for 5 min and then centrifuged at 5500 *g* for 15 min. A 75-ml volume of this solution was filtered on a Millipore cellulose acetate filter (0.45 μm), then added to 1 ml of a 1% (w/v) methanolic solution of cinnamal-

dehyde, mixed for a few seconds, left at room temperature for 15 min and then passed through an SPE C_{18} cartridge (preconditioned first with 6 ml of methanol and then with 6 ml of water) at 5 ml/min. The cartridge was dried for 10 min, washed with 3 ml HPLC mobile phase under a weak vacuum (0.2 ml/min), then with 5 ml of *n*-hexane (0.3 ml/min) and dried for 10 min. Finally, the isoniazid hydrazone was eluted with 6 ml of methanol. The methanolic solution was evaporated under vacuum (bath temperature 35–40°C) and the residue was dissolved in 0.200 ml of HPLC mobile phase and injected into the HPLC system.

3. Results and discussion

HPLC of the analytical sample obtained according to the two methods in Section 2.5 allows the identification of the different components as a function of their retention times (t_R = 5.6 and 7.4 min for cinnamaldehyde and isoniazid hydrazone, respectively) and from the diode-array UV spectra of single chromatographic peaks (Fig. 1).

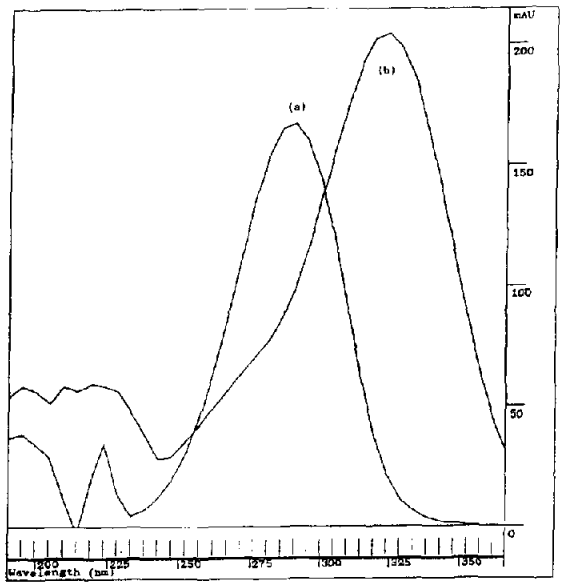

Fig. 1. Diode-array UV spectra of (a) cinnamaldehyde and (b) isoniazid hydrazone.

Fig. 2 shows chromatograms of milk serum samples containing (a) 1, (b) 10 and (c) 100 μg/l of isoniazid obtained according to the sample treatment procedure without concentration reported in Section 2.5. Isoniazid hydrazone is strongly retained by a C_{18} cartridge together with many other components and with cinnamaldehyde. Selective elution with 3 ml of the HPLC mobile phase at a flow-rate of 0.2 ml/min and 5 ml of *n*-hexane at a flow-rate 0.3 ml/min allows the isolation and recovery of isoniazid hydrazone.

The two chromatograms in Fig. 3 refer to (a) an isoniazid-free milk serum sample and (b) an isoniazid-containing sample concentrated 375-fold from the initial 0.1 μg/l according to the second sample treatment procedure in Section 2.5. At the lower concentration it is not possible to obtain a UV spectrum of isoniazid hydrazone.

Fig. 4a shows the chromatogram of a milk serum sample containing initially 1 μg/l isoniazid, concentrated 300-fold. This is the threshold concentration for recording the diode-array UV spectrum of the peak corresponding to isoniazid hydrazone (Fig. 4b).

3.1. Linearity

Linearity was tested by injecting a series of standard solutions containing isoniazid in the range 10–4000 μg/l of cattle milk (this range covers samples with or without concentration). The correlation coefficient (r) for the equation $y = 4.19 \cdot 10^{-3}x + 2.18 \cdot 10^{-3}$ is 0.9998 (x = peak-area counts; y = concentration in μg/l).

3.2. Detection limit

The detection limit without diode-array UV spectral confirmation is about 10 μg/l in non-preconcentrated samples and about 0.1 μg/l in samples that have been preconcentrated on an SPE cartridge. The detection limit with diode-array UV spectral confirmation is about 200 μg/l without preconcentration on an SPE cartridge

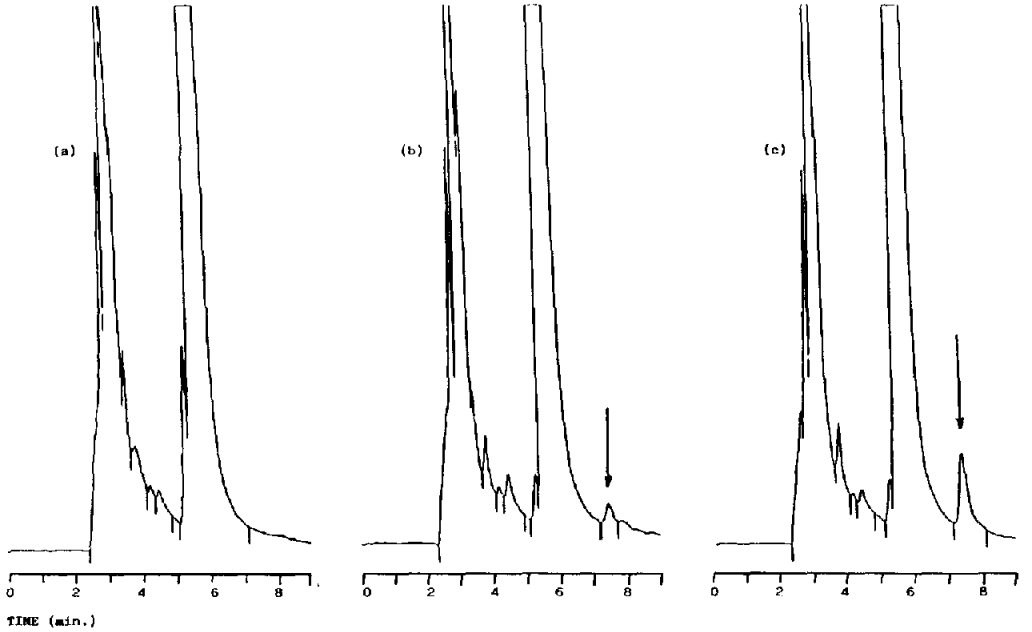

Fig. 2. Chromatograms of isoniazid hydrazone in cattle milk serum samples, obtained using UV detector without concentration. Concentration: (a) 1; (b) 10; (c) 100 μg/l. Chromatographic conditions are described in detail in the text.

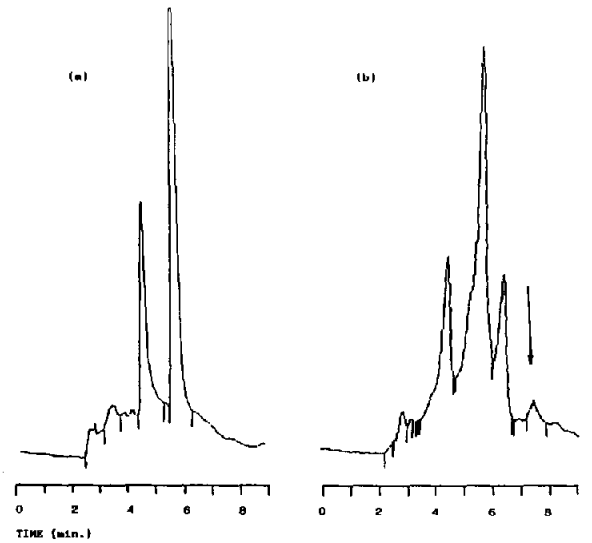

Fig. 3. Chromatograms obtained with UV detection of C_{18} SPE concentrated milk serum samples: (a) isoniazid free; (b) containing 0.1 μg/l, concentrated 375-fold.

and about 1 μg/l with 300-fold preconcentrated samples.

3.3. Recovery and precision

The recovery and precision of the proposed concentration method were determined by analyses of cattle milk samples containing different concentrations of isoniazid and are reported in Table 1. The accuracy, defined as (amount found/amount expected)·100, ranged from a minimum of 68% to a maximum of 98%.

4. Conclusions

The method described is of interest for its very good sensitivity in screening and in the detection of isoniazid illegally administered to cattle. The high sensitivity (0.1 μg/l) allows the detection and withdrawal of contaminated milk from the market, to the advantage of consumers' health.

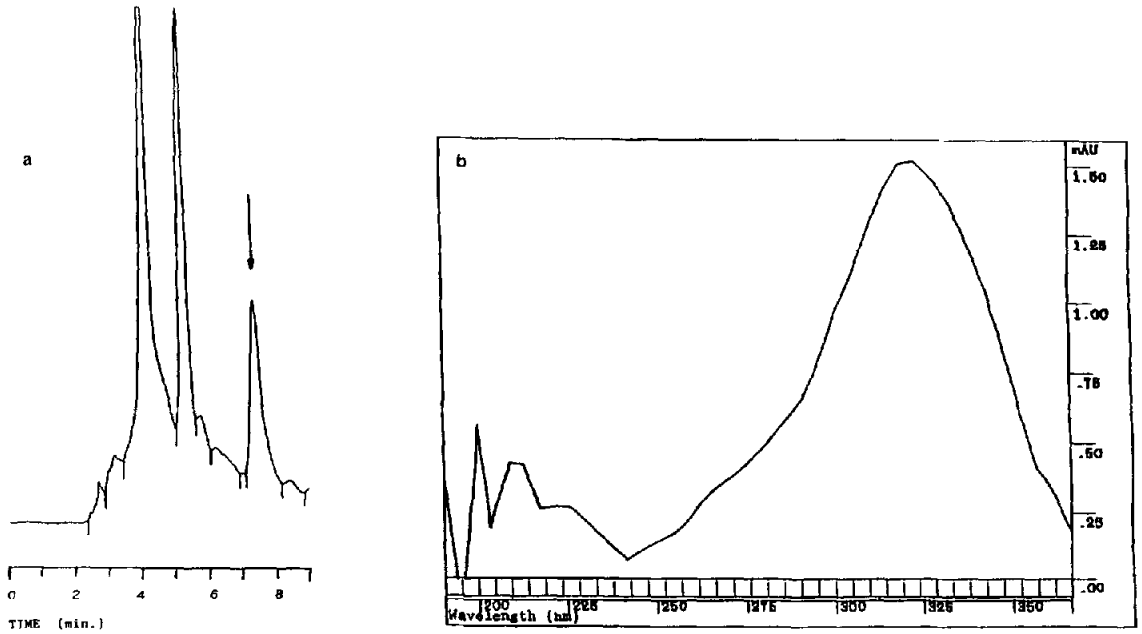

Fig. 4. (a) Chromatogram obtained with UV detection and (b) diode-array UV spectrum of isoniazid hydrazone in a cattle milk serum sample (1 μg/l) after 300-fold C_{18} SPE concentration.

Table 1
Accuracy and precision of isoniazid determination

Concentration of isoniazid in milk (μg/l)	Concentration factor	Isoniazid found		Accuracy (%)	No. of trials
		Mean (μg/l)	R.S.D. (%)		
0.10[a]	375	0.07	30	69	10
0.20[a]	300	0.15	22	75	10
0.50[a]	300	0.41	18	81	9
1.0[a]	300	0.85	12	85	10
4.0[a]	200	3.6	6	90	10
8.0[a]	200	7.4	6	92	10
13[b]	150	12	9	90	8
42[b]	75	40	5	95	6
56[b]	40	55	6	98	7
77[b]	40	75	4	97	8

Concentration factors of 375, 300, 200, 150 and 75 were obtained from 75 ml of milk serum recovered with 0.200, 0.250, 0.375, 0.500 and 1.000 ml, respectively, of HPLC mobile phase. The concentration factor of 40 was obtained from 40 ml of milk serum recovered with 1.000 ml of HPLC mobile-phase. Chromatographic conditions are detailed in the text.

[a] Spiked samples.

[b] Real samples.

5. References

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