Journal of Chromatography, 616 (1993) 291–296
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6857

Determination of thyreostatic residues in cattle plasma by high-performance liquid chromatography with ultraviolet detection

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(First received December 9th, 1992; revised manuscript received March 20th, 1993)

ABSTRACT

A procedure is described for the concurrent assay of thiouracil, methylthiouracil, propylthiouracil, phenylthiouracil and methimazole in bovine plasma. In this procedure, reversed-phase high-performance liquid chromatography is performed after liquid–liquid extraction of plasma with ethyl acetate. Compounds are quantified by ultraviolet detection using a wavelength of 276 nm for thiouracil, methylthiouracil, propylthiouracil and phenylthiouracil and 258 nm for methimazole. The linearity range, precision, recovery and detection limits were determined and the method was shown to be applicable to samples of plasma from young bulls experimentally treated with methylthiouracil.

INTRODUCTION

Thyreostatics, also known as antihormones, belong to category of drugs that are capable of inhibiting the production of thyroid hormones. In addition to their application in human therapy in treating hyperthyroidism, thyreostatics are also fraudulently utilized to fatten animals for slaughter because of their effect of enhancing water retention in tissues.

The presence of these substances in the edible parts of the animals represents a potential risk to the consumer and, for this reason, specific legislation has been promulgated in our country [1,2] as well as in the EC [3] prohibiting their use in animal breeding.

Various techniques have been proposed for analysing residues of thyreostatics in meat, urine or plasma samples of cattle; these include thin-layer chromatography [4,5], high-performance liquid chromatography with UV and/or electrochemical detection (ED) [6–9], gas chromatography with electron-capture nitrogen–phosphorus, or mass spectrometric detection [10,11]. Concerning cattle plasma, methods have been described for determining only one thyreostatic, namely methylthiouracil [4,5].

We now present a method for the determination of thiouracil (TU), methylthiouracil (MTU), propylthiouracil (PTU), phenylthiouracil (PhTU) and methimazole or tapazole (TAP) at levels of a minimum of 200 μg/l in plasma sam-

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0378-4347/93/$06.00

ples of cattle by reversed-phase HPLC with UV detection. For the identification of thyreostatics, the UV spectrum analysis is proposed.

EXPERIMENTAL

Chemicals and chromatographic standards

Pure standard TU, MTU, PTU, and PhTU were obtained from Carlo Erba (Milan, Italy) and TAP was obtained from Aldrich (Milan, Italy). The methanol used was of HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical reagent grade. High-purity water was produced with a Milli-Q system (Millipore, Bedford, MA, USA).

Chromatographic equipment

The HPLC analyses were performed on a Perkin-Elmer system (Norwalk, CT, USA) equipped with a Series 3 liquid chromatograph connected to an LC-75 spectrophotometric detector with autocontrol. The samples were introduced with a Rheodyne 7125 injection valve (Berkeley, CA, USA) equipped with a 100-μl loop. Chromatograms were recorded on a Perkin-Elmer Sigma 10 B chromatography data station. The column used was LiChrosorb RP-18 (250 mm × 4 mm I.D.; particle size 10 μm, Merck). The analytical column was usually protected by a precolumn (25 mm × 3 mm I.D.) packed with LiChroprep RP-18 (Merck).

Separation of TU, MTU, PTU and PhTU was achieved by using a linear gradient of methanol (solvent A) and 0.025 *M* phosphate buffer at pH 3 (solvent B), increasing from 10% solvent A at time $t = 0$ to 70% solvent A at $t = 30$ min, with a constant flow-rate of 1 ml/min at room temperature. The determination of TAP was performed with the same gradient, but the time of elution was interrupted at 10 min. The eluent was monitored at 276 nm when the analysis was performed for TU, MTU, PTU and PhTU and at 258 nm when only TAP was assayed. The two different wavelengths correspond to the zones of maxima of the UV spectra of compounds tested.

Preparation and handling of samples and standards

Blank blood samples were taken from young and adult cattle. The samples were collected in heparinized tubes and immediately centrifuged (1500 *g*, 10 min, 4°C). The plasma was stored at −20°C in glass vials.

Plasma was handled as follows. In a test tube, 0.5 ml of plasma were added to 100 mg of EDTA disodium salt and 3 ml of ethyl acetate. After vortex-mixing for 3 min, the suspension was centrifuged at 5000 *g* for 10 min. The organic phase was transferred into another test tube and evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 200 μl of methanol. An aliquot of the solution (10 μl) was injected into the HPLC system with a Hamilton microsyringe (Bonaduz, Switzerland).

Methanolic stock standard solutions were prepared at concentrations of 0.2 mg/ml for TU, MTU, PTU, TAP and 0.1 mg/ml for PhTU. For each thyreostatic, six working standard solutions were prepared in methanol by diluting the stock solutions so as to obtain concentrations of 20, 10, 5, 2.5, 1 and 0.5 μg/ml. By using these working standard solutions a calibration curve was created for each substance in order to verify the linearity of the response in the range of the concentrations studied. Plasma standards at the concentrations of 0.2, 0.4, 1, 2, 4 and 8 mg/l were prepared by adding 0.5 ml of blank bovine plasma to known amounts of the methanolic working solutions after evaporation of the solvent. Absolute recovery was determined by comparing the peak areas of these known amounts of the five thyreostatics added to the bovine plasma that underwent the complete assay procedure (final volume 0.2 ml of methanol) with those from the analysis of the same amounts of standard working standard working solutions (final volume 0.2 ml of methanol).

RESULTS AND DISCUSSION

Extraction

In order to improve the phase separation during sample extraction with ethyl acetate, a mod-

ification of the method of Pochard *et al.* [5] for MTU determination in bovine plasma is proposed. Using 3 ml of ethyl acetate greatly improved the recovery reproducibility.

High-performance liquid chromatography

In order to establish the optimum mobile phase conditions required for the baseline resolution of TU, MTU, PTU, PhTU and TAP on the C_{18} column, a systematic study was conducted on the influence of pH, phosphate buffer concentration and methanol percentage on the retention times (t_R) of the substances examined. Regarding the phosphate buffer, we also tried 0.05–0.02 and 0.01 *M* concentrations and pH values of 4 and 5; however, in all these cases the peaks were either enlarged or the TAP–MTU separation was incomplete or the two peaks overlapped. The mobile phase system described in the Experimental section appears to obtain the best separation available. For a good quantitation and for obtaining the same sensitivity of all tested compounds, we were obliged to use two different wavelengths and precisely 276 nm for the simultaneous determination of the four thiouracil derivatives and 258 nm for the TAP assay. Fig. 1 shows the spectra of the five compounds. At the two wavelengths used, the detection limits of HPLC system were the same for all substances. Figs. 2A and B and 3A and B show chromatographic recordings from bovine plasma without

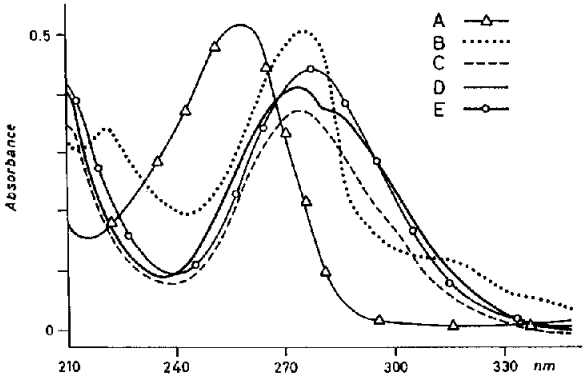

Fig. 1. UV spectra of tested compounds in methanol. A = TAP; B = PhTU; C = PTU; D = TU; E = MTU. Concentration of each compound: 4 mg/l.

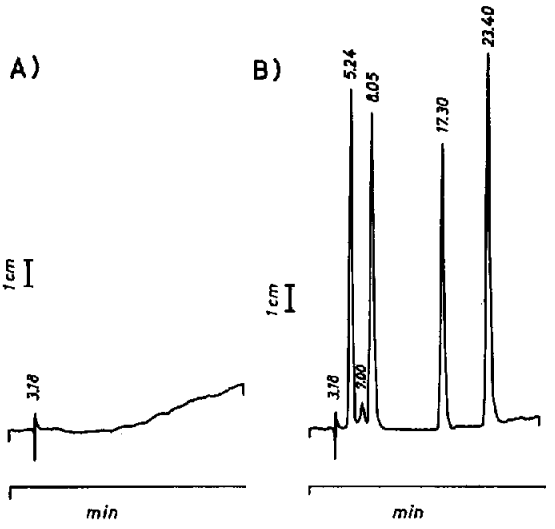

Fig. 2. (A) Chromatogram of a bovine blank plasma. (B) Chromatogram of the same bovine blank plasma spiked with TU, MTU, PTU, PhTU and TAP standard (each 4 mg/l). Sample pretreatment and HPLC conditions as described in the text. UV absorption at 276 nm; detector sensitivity = 0.040 a.u.f.s. Peaks: t_R 5.24 = TU; t_R 7.00 = TAP; t_R 8.05 = MTU; t_R 17.30 = PTU; t_R 23.40 = PhTU.

and with added thyreostatic agents, respectively. No influence from other substances naturally present in blood was observed at the two wavelengths used.

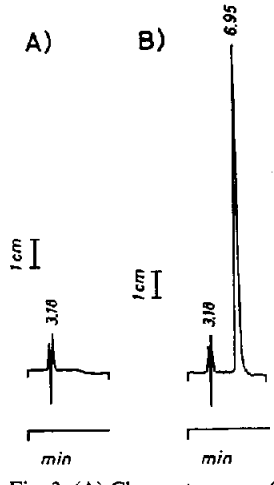

Fig. 3. (A) Chromatogram of a bovine blank plasma. (B) Chromatogram of the same bovine blank plasma spiked with TAP standard (4 mg/l). Sample pretreatment and HPLC conditions as described in the text. UV absorption at 258 nm; detector sensitivity = 0.040 a.u.f.s. Peak at t_R 6.95 = TAP.

TABLE I

REGRESSION LINES OF TU, MTU, PTU, PhTU AND TAP

Range 0.2–8 mg per l of plasma.

Compound	Slope	Intercept	Correlation coefficient (r^2)
TU	0.4030	−0.0277	0.9996
MTU	0.4432	0.0430	1.0000
PTU	0.3445	−0.0143	0.9966
PhTU	0.4505	−0.0031	0.9998
TAP	0.4335	−0.0115	1.0000

TABLE II

RECOVERY AND PRECISION RESULTS FOR BOVINE PLASMA SAMPLES SPIKED WITH TU, MTU, PTU, PhTU AND TAP (n = 5)

Compound	Concentration (mean ± S.D.) (mg/ml)		Recovery[a] (%)	Precision[b] (%)
	Added	Found		
TU	0.2	0.12 ± 0.01	59.5	5.9
	0.4	0.26 ± 0.01	63.9	3.9
	1	0.63 ± 0.03	63.0	4.3
	2	1.31 ± 0.08	65.3	6.3
	4	2.69 ± 0.15	67.2	5.4
	8	5.82 ± 0.25	72.8	4.3
MTU	0.2	0.11 ± 0.01	57.4	5.1
	0.4	0.24 ± 0.01	60.0	4.7
	1	0.65 ± 0.03	64.8	4.0
	2	1.23 ± 0.07	61.7	6.3
	4	2.56 ± 0.19	64.0	5.0
	8	5.38 ± 0.26	67.2	4.9
PTU	0.2	0.12 ± 0.01	62.0	6.1
	0.4	0.25 ± 0.01	63.4	3.6
	1	0.67 ± 0.02	66.6	3.5
	2	1.46 ± 0.05	73.0	3.4
	4	2.26 ± 0.19	74.1	6.6
	8	5.98 ± 0.25	74.7	4.1
PhTU	0.2	0.12 ± 0.01	61.7	5.5
	0.4	0.25 ± 0.01	63.2	3.8
	1	0.69 ± 0.03	68.5	2.8
	2	1.58 ± 0.04	78.8	2.8
	4	3.17 ± 0.09	79.4	2.9
	8	6.92 ± 0.25	86.5	2.4
TAP	0.2	0.12 ± 0.01	59.7	5.2
	0.4	0.24 ± 0.01	60.3	4.8
	1	0.68 ± 0.02	68.0	3.2
	2	1.48 ± 0.08	73.8	5.4
	4	2.95 ± 0.10	73.8	3.4
	8	6.15 ± 0.24	76.9	3.9

[a] (Found/added) × 100.

[b] Coefficient of variation.

Linearity

Linearity was tested by injecting a series of standard solutions containing concentrations of the five thyreostatics in the range 0.2–8 mg/l of plasma for each substance. These levels were suitable for the determination of thyreostatics in plasma of unlawfully or experimentally treated cattle [4,8]. Linear regression analysis data are shown in Table I.

Detection limit

The detection limit was better than 200 μg/l of plasma for the five compounds tested at a signal-to-noise ratio of 2 and with an injection volume of 10 μl.

Recovery and precision

The recovery and precision of the HPLC method described were determined by analysing bovine blank plasma samples spiked with thyreostatic standards at six different concentrations. The values reported in Table II are the mean of the data obtained in five replications. From the results one can see that the coefficients of variation (C.V.), ranging from 2.4 to 6.6%, were constant over the whole concentration range studied. The recovery for the five substances studied, defined as (amount found/amount added) × 100%, depended on the concentrations utilized and ranged from a minimum of 57.4% for MTU to a maximum of 86.5% for PhTU.

Identification of peaks in biological extracts

The chromatographic peaks were identified by a combination of methods. Peak identification was initially performed on the basis of the retention time and by co-injection of reference compounds. Secondly, identification was completed by means of the absorption spectra obtained with the stopped-flow mode. The spectra of the reference compounds obtained in the range 210–320 nm were compared with the spectra of the unknown peaks with the flow stopped at the point where the compounds of interest were eluting.

Application

Plasma samples from two young bulls who were orally administered MTU at a mean daily dosage of 5 g were analysed with the method described. Blood samples were collected eight days after the beginning the administration and the

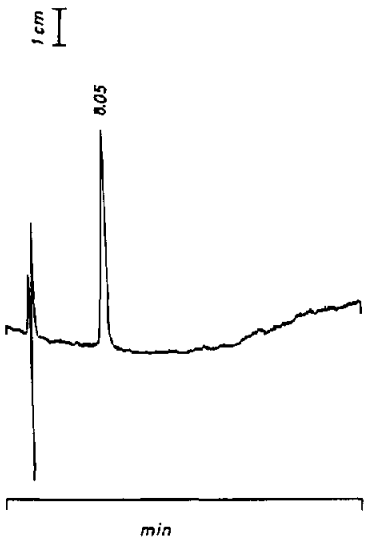

Fig. 4. Chromatogram of plasma of young bull treated orally with MTU. Chromatographic conditions same as in Fig. 2. Concentration found for MTU: 1.3 mg/l. Animal treatment and blood sample collection as described in the text.

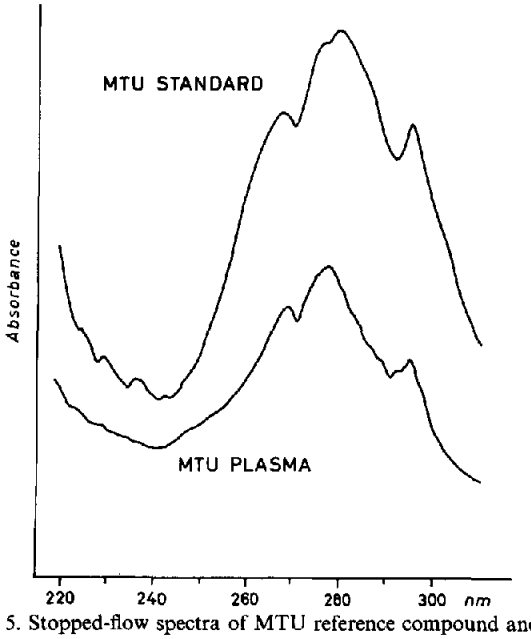

Fig. 5. Stopped-flow spectra of MTU reference compound and of chromatographic peak in plasma sample of a young bull treated orally with MTU.

analyses were performed within 48 h to prevent any possible degradation as observed for MTU by us and by Melander *et al.* [12] for TAP in human serum. The amounts of MTU in plasma were 1.5 and 2.8 mg/l, respectively, without correction for recovery.

The values are in good agreement with previously reported levels [4,8]. Fig. 4 shows a chromatogram from the plasma sample of a young bull treated with MTU and Fig. 5 the stopped-flow spectra of MTU.

CONCLUSIONS

The chromatographic system reported for the determination of some thyreostatic substances represents an advancement on current literature systems since it provides improved and complete resolution for the five compounds tested. The disadvantages of using two different wavelengths for obtaining the same sensitivity for the five compounds are believed to be more than compensated for by the completeness of the detection of all the thyreostatics tested. The technique described therefore seems ideally suited for the determination of TU, MTU, PTU, PhTU and TAP in plasma of animals treated unlawfully.

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