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Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 237–241

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Short communication

Optimization of HPLC analysis for the determination of propylthiouracil levels in plasma and serum

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Received 23 July 1999; received in revised form 10 September 1999; accepted 19 September 1999

Keywords: Propylthiouracil analysis; Serum PTU; HPLC; Hyperthyroidism

1. Introduction

The term thyrotoxicosis refers to the syndrome that results when a tissue is presented with overproduction of thyroid hormones, a condition that represents a serious public health problem in several countries.

The major agents employed in therapy for thyrotoxicosis are drugs of the thionamide class, such as propylthiouracil (PTU), methimazole (MMI) and carbimazole (CA); these antithyroid agents exert their action by inhibiting the oxidation and organic binding of thyroid iodide. In addition, this effect, PTU also impairs the conversion of T4 to T3 in peripheral tissues, being generally used in preference to other thionamides [1] for this additional action.

Various techniques have been applied to the determination of PTU in serum or plasma. The colorimetric procedures [2] were the first described, but are relatively insensitive or lack the necessary selectivity to distinguish between the parent drug and metabolites of PTU. Gas–liquid chromatography (GLC) [3], despite its sensitivity, shows disadvantages such as the necessity of elevating the column temperature after every two or three injections to remove slowly eluting compounds. High-pressure ion exchange chromatography [4] presents a lower detection limit when compared to GLC. On the other hand, high-performance liquid chromatography [5–8] and radioimmunoassay [9] are laborious and expensive procedures. Potentiometric and coulometric titration of PTU has also been proposed [10]. The choices among all of these methods are dictated by adequacy and cost.

We present here an optimisation of high-performance liquid chromatography for the determination of PTU levels in serum of rats treated with this drug: the proportion of the methanol in the mobile phase was modified leading to a reduction in the PTU retention time when compared to some data from the literature [6,11]. This condition, associated to the simplification of the extraction procedure to one step, allowed the determination of PTU in a large number of sam-

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PII: S0731-7085(00)00275-2

ples with the quickness, selectivity, sensitivity and resolution required to this purpose. The data presented are also important as background for additional studies requering determination of this drug and of other thionamides in serum or plasma [12].

2. Materials and methods

2.1. Standard solutions and chemicals

A stock solution containing 5 mg/ml 6-*n*-propyl-2-thyouracil (PTU, Sigma Chemical, St. Louis, MO) was prepared in methanol. The solution was stable for at least 3 months when stored at 4°C, and was used to prepare the working solutions (0.312–40.0 μg/ml) at the time of use.

The solvent used as mobile phase was of HPLC grade (Merck) and all other reagents were of analytical grade. Water was purified in a Millie-Q® Water System (Millipore).

2.2. Treatment of animals and serum preparation

Adult male Wistar rats (180 ± 10 g) were treated daily by gavage with variable doses of PTU (1, 5 and 50 mg/200 g body weight per day) for time intervals from 4 to 22 days. Animals were killed by decapitation, blood was left to clot at room temperature for 40 min, and serum separated by centrifugation at 4°C and kept at -70°C until analysis.

2.3. Equipment and chromatographic conditions

All analysis were performed on a Bil-Sil C_{18} Hl 90-5S column (250×4.5 mm^2) (BIO-RAD) the mobile phase consisting of methanol:water (1:1, v/v), with a flow-rate of 1 ml/min. and room temperature of 25°C. Samples were applied using a Rheodyne injector model 7125 with a 20 μl loop, connected to an HPLC Pump (Waters Model 510). An ultraviolet absorption detector model 460 operating at 214 nm and a electronic integrator were used to monitor the chromatographic process.

2.4. Sample preparation

The extraction procedure, modified from the literature [7,11], consisted of addition of 6 ml of dicloromethane to 1 ml of serum (pH 6.0 adjusted with 10% HCl). Mixtures were then submitted to vortex-mixing for 10 min. After centrifugation at $1800 \times g$ for 5 min the organic phase was carefully transferred to conical tubes and evaporated under a stream of nitrogen. The residue was suspended in methanol (HPLC grade) or in the mobile phase (50% methanol:50% water). After mixing for 30 s, 20 μl aliquots of these solutions were submitted to chromatographic analysis.

2.5. Method validation

Calibration curves were prepared by the addition of 25 μl of a standard solution of PTU in methanol at concentrations of 0.312, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml in the mobile phase. In addition fixed volumes of standard solutions were mixed with 1.0 ml of normal rat serum (NRS), submitted to the extraction procedure, and the residue was resuspended in 1.0 ml of methanol and diluted in the mobile phase to the above concentrations. Volumes of 20 μl were then chromatographed as described.

The linearity of the method was evaluated for PTU concentrations in the range of 0.312–40.0 μg/ml. Recovery was assessed by extraction of PTU in the concentration range of 0.625–40.0 μg/ml added to normal rat serum and to normal rat plasma. The precision, accuracy and reproducibility of the method were evaluated by measuring ten samples of each PTU solution and interassay precision was evaluated by analysing samples in duplicate over 5 consecutive days. The quantitation limit, a parameter used to assess the sensitivity of the method, was obtained by the analysis of serum samples enriched with PTU at concentrations of 0.156–0.625 μg/ml. Selectivity was evaluated by analysing several drugs that could be used eventually during treatment with PTU. Drugs were firstly chromatographed at concentrations of 10 μg/ml under the conditions established previously. The same drugs were then added to NRS (1 mg/ml), submitted to the extrac-

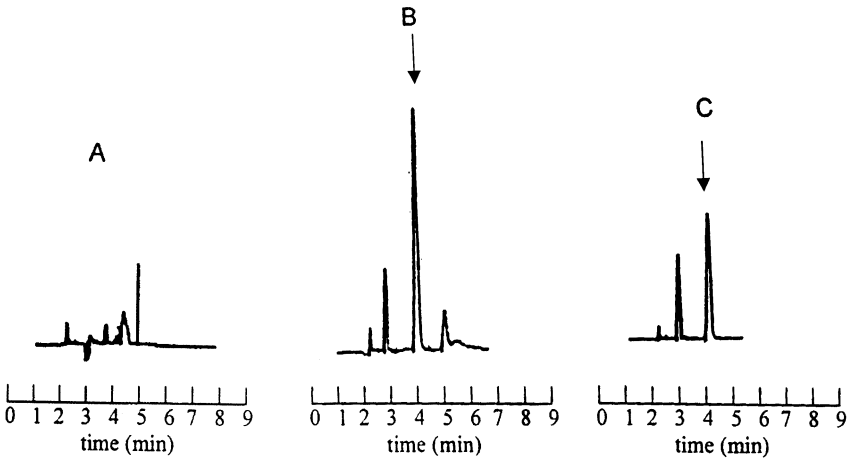

Fig. 1. Representative profiles obtained from rat serum analysed by HPLC as described. (A) Normal rat serum. (B) Serum spiked with PTU. (C) Serum from rats treated with 1 mg of PTU/200 g body weight per day for 16 days. Arrows indicate PTU elution (retention time at 4.2 min).

tion procedure, and chromatographed under the same conditions.

3. Results

The equation ($y = 31562 \pm 173x + 1.0 \pm 32.77$) was obtained from typical calibration curve through the least-squares linear regression. The method was linear in the concentration range of 0.312–40 μg/ml, and the detection limit was ~ 0.05 μg/ml.

Fig. 1 shows typical chromatograms obtained from analysis of NRS, NRS enriched with PTU, and serum from rats submitted to treatment with PTU (1 mg/200 g body weight per day for 16 days). PTU was eluted in sharp and well-defined peaks with a retention time of 4.2 min, allowing drug quantification by the determination of the integrated area.

Serum samples enriched with PTU in the concentration range of 2.5–20.0 μg/ml were evaluated to determine the absolute recovery of the method. The concentration of this drug after sample chromatography was determined through the calibration curves. The mean values obtained for each concentration (Table 1) indicate the efficiency of the extraction procedure (~ 100% recovery).

Standard solutions of PTU in the concentration range of 0.625–40 μg/ml were evaluated to determine the intra-assay and interassay coefficients of variation (C.V.) and the results are presented in Table 2.

Several drugs were analysed as possible interferents. The interference of these substances with the method was investigated by chromatography of solutions prepared in methanol (10 μg/ml) under the conditions established. The elution profile of these drugs was monitored for 0.9–30 min after injection, none of them being eluted with the retention time of PTU (Table 3). Similar results were obtained when the same drugs (1 mg/ml) were added to normal NRS and submitted to the extraction procedure.

Table 1
PTU recovery from normal serum spiked with this drug[a]

PTU concentration (μg/ml)[b]	PTU recovery	
	(μg/ml)[c]	(%)
5	4.7	93.66
10	10.3	103.21
20	20.1	100.50

[a] Values are from five determinations
[b] Expected concentration.
[c] Detected concentration.

Table 2
Intra and interassay C.V. of PTU determinations by the method described[a]

PTU concentration (μg/ml)	C.V. (%)	
	Intra-assay	Inter-assay
0.625	2.62	7.10
1.250	2.08	2.30
2.500	3.71	2.80
5.000	2.81	3.00
10.000	3.98	2.10
20.000	2.76	3.10

[a] Ten samples were determined for each concentration.

Table 3
Retention time of the drugs studied as interferents

Drug concentration (μg/ml)	Retention time (min)	
	Before extraction	After extraction
Atenolol	ND[a]	ND
Carbamazepine	8.55	ND
Clonazepam	16.32	16.32
Metoprolol	ND	ND
Phenobarbital	6.22	6.75
Hyoscine	1.57	ND
Paracetamol	3.05	ND
Progesterone	ND	ND
Propranolol	ND	3.10
Verapamil	1.87	ND

[a] ND, not detectable.

Table 4
PTU concentration in serum of treated rats as determined by the method described

Period of treatment (days)	PTU concentration in serum (μg/ml)[a]	
	5 mg	50 mg
4	0.3345 ± 0.0388	2.765 ± 0.5445
8	0.4200 ± 0.0424	4.550 ± 0.8344
12	0.4880 ± 0.0240	7.930 ± 0.0424
16	1.1292 ± 0.3636	23.865 ± 0.1768
22	2.0587 ± 0.4166	40.360 ± 0.9899

[a] Serum rats treated with PTU (5 and 50 mg/200 g body weight per day).

Table 4 shows the results of PTU determination according to the proposed method in serum of rats treated with variable doses (1, 5 and 50 mg/200 g body weight per day) for different time intervals. As the time of treatment increases, so does the serum PTU concentration, with maximum values being dose-dependent.

4. Discussion

The method proposed here was used for the determination of variable levels of PTU in large number of serum samples from rats treated with this drug. Conditions were investigated to allow simplification of the extraction procedures described in the literature and good resolution in HPLC separation using a C_{18} column (HI 90-5S 250×4.5 mm^2 BIO-RAD). The proportion of methanol in the mobile phase, associated with the choice of the wavelength of 214 nm to monitor of PTU elution from the column, provided the required resolution and sensitivity. Volumes of 1 ml serum were used for convenience; however, the resolution of the PTU peak and the low noise levels observed allow the analysis of smaller sample volumes in contrast to previously reported data [5].

Under our conditions, PTU was eluted from the HPLC column, with a similar or lower retention time (4.20 min) compared to literature data [5,6,11]. The detection limit was around 0.05 μg/ml, similar to other chromatographic methods [6,7] and a linear response occurred at concentrations of 0.312–40 μg/ml, therefore covering a wide range of PTU levels that may occur in serum of patients during variable stages of therapy and drug regimens, and in animals under variable experimental conditions as exemplified here. The intra and inter assay C.V. were of 3.7% ($n = 10$) and 2.8% ($n = 10$), respectively. The advantage of a single (one-step) extraction and procedure was associated with an almost complete ($\sim 100\%$) recovery.

5. Conclusion

This method is selective, with no interference

some drugs that are frequently administered in combination with PTU for the treatment of hyperthyroidism, sensitive and rapid (~ 7 min/sample chromatography), allowing the analysis of a large number of samples within a short period of time, and may be applied clinically to monitor serum PTU levels in patients treated with this drug.

Acknowledgements

The authors are grateful to FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) for financial support and Dr Antonio Cardozo dos Santos for supplying drugs for the interferents studies.

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