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Short communication

Simple, rapid and sensitive determination of protionamide in human serum by high-performance liquid chromatography

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Abstract

A simple high-performance liquid chromatography method has been developed that allows the sensitive determination of protionamide (2-*n*-propyl-pyridine-4-carboxylic acid thioamide, PTH) in human serum. After pretreatment of the serum with trichloroacetic acid (TCA) and centrifugation the supernatants were neutralized using $NaHCO_3$. PTH was separated on a Kromasil 100 C_4 column (acetonitrile–sodium tetraborate buffer pH 8–dibutylamine) and determined photometrically at 291 nm. The lower limit of quantification for 300 μl serum precipitated with 60 μl TCA and injection of 50 μl was 27 μg/l and linearity was observed up to 15 mg/l. © 1998 Elsevier Science B.V.

Keywords: Protionamide

1. Introduction

Protionamide (PTH) is a thioisonicotinamide derivative (Fig. 1 shows the chemical structure) with antibacterial activity against mycobacteria like *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The number of analytical methods for determination of PTH published in the literature is very low and the papers are at least 13 years old [1–4]. An extraction procedure combined with a normal-phase chromatography was described [3,4]. In consideration of the new techniques and materials used in analytical high-performance liquid chromatography (HPLC) during the last years the development of a simple, rapid, and sensitive method was performed.

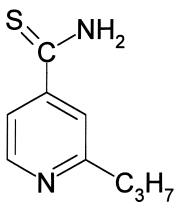

Fig. 1. Chemical structure of protionamide.

2. Experimental

2.1. Materials

The chemicals used were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany). Acetonitrile, HPLC grade, was from Baker (Groß Gerau, Germany). The analytical col-

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umn, Kromasil 100 C_4 (5 μm, 100×3 mm I.D.) and a precolumn (5×3 mm I.D.) with the same material were obtained from Knauer (Berlin, Germany). The HPLC equipment consisted of a WISP 712 autosampler from Waters (Eschborn, Germany), a gradient former Spectroflow 430 combined with a HPLC pump Spectroflow 400, a programmable absorbance detector Spectroflow 783, all from Kratos (Weiterstadt, Germany), and the integration system EZchrom from Scientific Software (San Ramon, CA, USA). The centrifuge 5403 supplied with a rotor for 1.5 ml vials was from Eppendorf (Hamburg, Germany).

2.2. Conditions

For sample preparation 60 μl trichloroacetic acid (TCA; 30%) was added to 300 μl human serum. The sample was immediately vortexed and centrifuged for 10 min at 23 000 *g* at 4°C. A 200 μl volume of supernatant and 45 μl 1 *M* $NaHCO_3$ were mixed and briefly centrifuged. Aliquots of 50 μl were injected on a Kromasil 100 C_4 column (5 μm, 100×3 mm I.D.) at room temperature using a flow-rate of 0.6 ml/min. The mobile phase consisted of 9.534 g sodium tetraborate and 6.4625 g dibutylamine per liter adjusted to pH 8 (using conc. HCl)–acetonitrile (75:25, v/v). The detection wavelength was 291 nm.

2.3. Test solutions

Spiked serum samples were prepared from 90% serum and 10% of the corresponding 10-fold PTH concentration in acetonitrile–water. Serum samples of each final concentration (18.5, 36.3, 90.75, 181.5, 363, 726, 1452, 2178, 2904, and 3630 μg/l) were prepared to determine accuracy and precision. The same concentrations were used for calibration.

3. Results and discussion

Based on our experience in the determination of analogous compounds we established an analytical system for the quantification of PTH, using a Kromasil 100 C_4 column, acetonitrile–sodium tetraborate pH 8–dibutylamine as mobile phase, and detection at 291 nm. An acetonitrile content of 25% (v/v) produced a symmetric peak at a retention time of about 5 min. After serum protein precipitation by acetonitrile the separation of PTH was unsuccessful. Therefore TCA was selected. However, the application of the acidic sample showed deformed peaks with low reproducibility, in a range from getting no peak to only small deformation depending on the buffer capacity of the mobile phase. PTH is protonated by TCA and the cationic form retained in the unprecipitable matrix components. This phenomenon can be reversed by higher molar buffer concentration in the mobile phase or by titration of the sample before injection (thus preserving the HPLC system). A neutralization step using 1 *M* $NaHCO_3$ eliminated the problem. Fig. 2 shows the overlaid chromatograms of a spiked (181.5 μg/l) and a blank serum sample. Another problem was sample storage. Whereas PTH was stable for at least 4 days in samples stored at 4°C and decreased by only 10% within 4.5 h in human serum at 37°C, the determination in frozen serum samples was not reproducible. The concentration of PTH decreased dramatically during the storage time and freeze–thaw steps. This effect was not due to the stability, but to the very low solubility of PTH in water, it precipitated partly. Whereas in aqueous samples the precipitate can be dissolved by warming up and intense

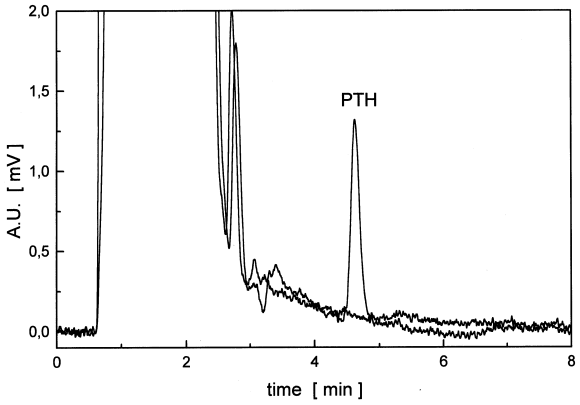

Fig. 2. Overlaid chromatograms of blank and spiked serum samples. Sample spiked with 181.5 μg/l PTH. A 300 μl sample vortexed with 60 μl TCA, centrifuged (10 min, 23 000 *g*, 4°C), 200 μl supernatant mixed with 45 μl 1 *M* $NaHCO_3$. Aliquots of 50 μl were separated on Kromasil 100 C_4 (5 μm, 100×3 mm I.D.) using 75% sodium tetraborate pH 8–dibutyl amine–25% acetonitrile at 0.6 ml/min and room temperature.

vortexing, this procedure was ineffective in serum samples. Therefore we prefer for a short time storage (3–4 days) a sample temperature of 4°C. For longer storage periods the serum has to be first precipitated by TCA and centrifuged and only the supernatant should be frozen. By this procedure the precipitation of PTH is avoided, as its solubility in acidic solutions is much higher. After thawing the samples are neutralized as usual. Further stability investigations are described in Ref. [5].

For validation and calibration five sample sets described in Section 2.3 were analyzed at different times. Concentrations above 200 μg/l can be easily described by a simple linear regression without weighting (amount=0.01193×area+53.758). This linearity was confirmed in single tests up to 15 mg/l. These data (only ''between days'') are shown in the lower part of Table 1. Whereas the valid range (181.5 μg/l–3630 μg/l) shows a good accuracy, the calculation of lower concentrations results in extreme errors. However, the whole concentration range can be described by a quadratic function with $1/x$ weighting (amount$=-4.06\cdot10^{-9}\times\text{area}^2+1.31\cdot10^{-2}\times\text{area}+7.37$). The upper part of Table 1 shows the validation data using this calibration function. The precision and the accuracy are in an acceptable range. Therefore it is important to select a calibration function describing the investigated concentration range. The statistical estimation of the lower limit of quantification [6–8] considering the whole calibration range results in 27 μg/l for the described method.

The analysis of human serum 2 h after application of a combination of 300 mg rifabutin, 400 mg ofloxacin, 500 mg PTH, 100 mg clofazimine, and 150 mg thiacetazone is demonstrated in Fig. 3. The peak area represents a PTH concentration of 1.16 mg/l. In consideration of these experiments the determination of PTH even in combination therapy is possible.

Table 1
Data of precision and accuracy for PTH in human serum

Day	Statistical parameter	Theoretical concentration (μg/l)									
		18.15	36.3	90.75	181.5	363	726	1452	2178	2904	3630
1	Mean (n=4)	17.81	33.70	85.35	169.41	354.42	709.97	1470.29	2216.79	2908.75	3597.77
	% Of R.S.D.	2.32	3.47	2.62	1.07	1.86	0.30	0.41	1.05	0.32	0.06
	% Of theory	95.35	92.84	94.05	93.34	97.64	97.79	101.26	101.78	100.16	99.11
2	Mean (n=4)	19.81	33.03	87.30	174.71	356.12	717.46	1478.12	2205.96	2905.29	3611.95
	% Of R.S.D.	0.50	6.59	1.07	1.68	1.05	1.15	0.43	0.36	0.18	0.55
	% Of theory	109.15	90.99	96.20	96.26	98.10	98.82	101.80	101.28	100.04	99.50
	Mean (n=4)	20.79	35.67	88.22	171.67	359.18	723.09	1480.47	2224.04	2924.99	3612.69
3	% Of R S.D.	3.93	3.70	4.35	2.14	1.10	0.86	0.80	0.54	0.87	0.72
	% Of theory	114.55	98.26	97.21	94.58	98.95	99.60	101.96	102.11	100.72	99.52
4	Mean (n=4)	21.98	37.21	86.86	171.25	358.45	713.39	1465.95	2197.91	2895.07	3589.61
	% Of R.S.D.	7.54	4.83	3.32	1.69	2.20	0.62	1.37	0.32	0.52	0.45
	% Of theory	121.08	102.50	95.71	94.38	98.75	98.26	100.96	100.91	99.69	98.89
5	Mean (n=4)	20.07	39.69	85.85	168.78	355.28	704.95	1475.08	2206.90	2901.02	3602.71
	% Of R.S.D.	12.29	10.24	1.29	3.53	2.57	0.86	1.05	0.62	1.40	1.11
	% Of theory	110.59	109.34	94.60	92.99	97.87	97.10	101.59	101.33	99.90	99.25
Between	Mean (n=20)	19.99	35.86	86.71	171.17	356.69	713.77	1473.98	2210.32	2907.02	3602.95
days	% Of R.S.D	10.03	9.04	2.79	2.30	1.73	1.15	0.87	0.70	0.79	0.65
	% Of theory	110.14	98.78	95.55	94.31	98.26	98.32	101.51	101.48	100.10	99.25
The following data were calculated using linear regression (amount=0.01192836×area+53.75792 valid in the range from 181.5 μg/l to 3630 μg/l											
Between	Mean (n=20)	65.20	79.59	125.79	202.77	372.94	704.86	1431.90	2165.34	2889.29	3645.62
days	% Of R.S.D.	2.79	3.69	1.75	1.77	1.52	1.09	0.88	0.73	0.84	0.71
	% Of theory	359.22	219.26	138.62	111.72	102.74	97.09	98.62	99.42	99.49	100.43

The concentrations were calculated using a quadratic function with $1/x$ weighting (upper part) and a simple linear regression without weighting (lower part). Both functions are described in Section 3. For sample preparation see Section 2.2.

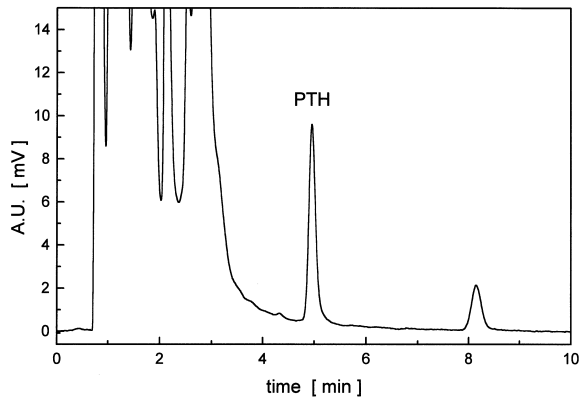

Fig. 3. Chromatogram of human serum. Sample obtained 2 h after application of a combination of 300 mg rifabutin, 400 mg ofloxacin, 500 mg PTH, 100 mg clofazimine, and 150 mg thiacetazone. The peak area represents a PTH concentration of 1.16 mg/l.

4. Conclusion

The technique described above allows the rapid, simple, and sensitive quantitative determination of PTH in human serum. However, the sample preparation step is decisive for a successful analysis and last but not least the history of sample storage is important to get the proper concentration.

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