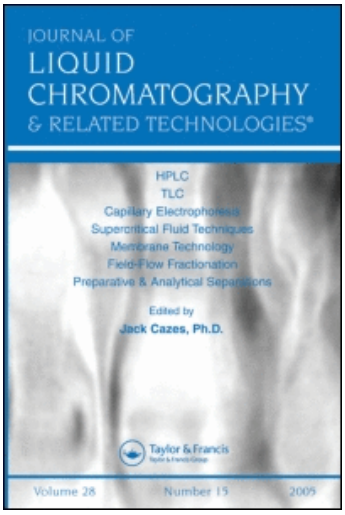

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Meulemans, A. , Manuel, C. , Ferriere, C. and Valpillat, M.(1980) 'Determination of Methimazole in Plasma by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 3: 2, 287 — 298
To link to this Article: DOI: 10.1080/01483918008060172
URL: http://dx.doi.org/10.1080/01483918008060172

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(2), 287-298 (1980)

DETERMINATION OF METHIMAZOLE IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The present paper describes a sensitive and precise method for the determination of methimazole in plasma of patients with hyperthyroidism under carbimazole therapy.

The method uses the 2-6 dichloroquinone chloroimide which forms a complex with methimazole sulfur group. After selective chloroform extraction of the complex, high performance liquid chromatography (HPLC) on a μNH_2 column was performed in the reconcentrated extract. The drug was detected with a spectrophotometer at a wavelength of 405 nm. The sensitivity of the method allowed the detection of a concentration as low as 5 ng ml^{-1} of plasma. No interference with other biological products was detected.

INTRODUCTION

Carbimazole and methimazole are widely used for hyperthyroidism. Some authors studying the ^{35}S-compounds (1,2) demonstrated that carbimazole is totaly hydrolysed as methimazole before being absorbed in

digestive tract (1). This reaction is very rapid ; hydrolysis is completely achieved in less than 10 minutes in vitro, at pH 4 or 8. So far, these drugs are the only treatment available for such a disease, and up to now there has been no possibility of evaluating the amount of methimazole present in blood. Skellern and al., have described a method, using HPLC, to assay the methimazole, but the sensitivity of this technique (0.3 $\mu g.ml^{-1}$) does not make its assay in plasma possible(3).

We previously attempted to develop various methods, namely a colorimetric procedure (4) using the 2-6 dichloroquinone chloroimide reaction, then a fluorimetric technique using o-phtalaldehyde. Both of these techniques as well as gas chromatography (0.1 $\mu g.ml^{-1}$) lack sensitivity and fail to provide reliable results in plasma analysis. The only test providing accurate results is the double isotopic dilution analysis with ^{14}C-methimazole (5 $ng.ml^{-1}$). But this assay is time consuming. Thus, we combined the colorimetric procedure and HPLC to obtain a sensitive and precise method for the determination of methimazole in plasma.

MATERIAL AND METHODS

Instruments

The instrument used for this work consisted of a high performance liquid chromatograph from Waters associates (Waters France, 19, Rue Goubet F. 75019 Paris) fitted with a model 6000A pump, a U6K injector, a μ-Bondapak-NH2 column (30 cm, 3.9 mm I.D., Waters) and M 440 UV absorbance detector operated at 405 nm.

Reagents

. methimazole and carbimazole from FLUKA A.G.

Chemische Fabrick. Buchs, Switzerland.

. 2-6 dichloroquinone chloroimide dissolved in methanol (1g/100 ml) in a 100 ml-volumetric flask.

. Buffer chloride solution, pH 8 : boric acid, 3.10 g, KCl, 3.75 g and NaCl, 100 g were dissolved in water. They were added to 40 ml NaOH, 0.1 molar. Volume was completed with water to 1 liter in a calibrated flask. pH was adjusted to pH 8 with 6 molar NaOH

The eluent consisted of chloroform (All reagents were analytical grade pure, Merck Darmstadt)

Sampling

In the preliminary study, venous blood was sampled from patients under carbimazole therapy (one to six 5 mg-tablets per day).

Each time the patients underwent a hormonal follow up test, an aliquot of the venous blood was sampled in a heparinized tube for a drug assay. Two ml of the supernatant plasma were pipetted, centrifuged and kept at -30°C until the time of the assay.

Drug kinetics were studied twice on three volunteers, following oral administration of 30 mg of carbimazole. Seven samples were drawn over a 24 h. -period (2,4,6,8, 10,12,24 hours).

Procedure

Standard solutions were prepared using drug-free plasmas. One milliliter of the standard was pipetted into labeled 50 ml-screw-cap tubes. Then 4 ml of the buffer chloride solution were added to each tube and thoroughly mixed. 0.2 ml of the 2-6 dichloroquinone chloroimide reagent were poured into each tube (for low concentrations 0.02 ml were sufficient) and were mixed thoroughly during 1 minute with a Vortex-type mixer.

The tubes were kept at room temperature for five minutes to allow the reaction to set in. Then 2 ml of chloroform

were added. The tubes were mixed and vortexed during five minutes and finally they were centrifuged (10 min,1000g). Most of the dark aqueous (upper) layer was removed. The remaining material from each tube was filtered through a phase separating filter paper (Whatman IPS 11cm). The chloroform extract (1.5ml) was evaporated to dryness and reconstituted with 50 µl of chloroform ; 20 µl of this solution were injected into the chromatograph. The mobile phase consisted of chloroform flowing at the rate of 1.5 ml/min. The absorbance detector was set to monitor the column effluent at 405 nm.

RESULTS

Chromatograms of blank (drug-free) and of patient's plasmas showed different peaks.

The peak occuring at 5 minutes (K' = 1) was identified as methimazole :

Three other peaks : t_1 = 4.5 min, k' = 0.8 ; t_3 = 6.5 min; k'= 1.6 ; t_4 = 7.7 min, k' = 2.08, occured for low concentrations of methimazole and corresponded to the 2-6 dichloroquinone chloroimide. These peaks were similar to those

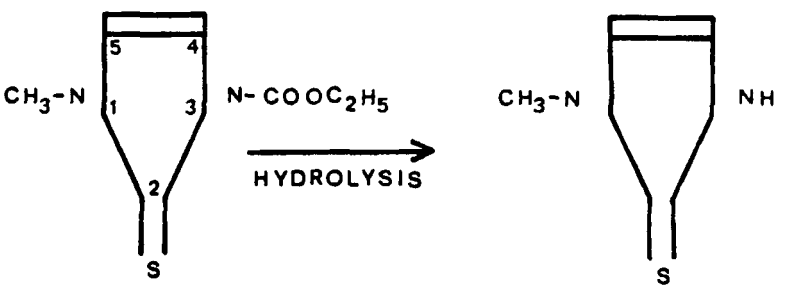

Figure 1

Hydrolysis of carbimazole in methimazole. The latter is the only form present in plasma after oral adminis-tration. At pH 7.4,in plasma and distilled water,hydrolysis is totaly achieved after 2 hours. It is inhibited by freezing at -80°C.

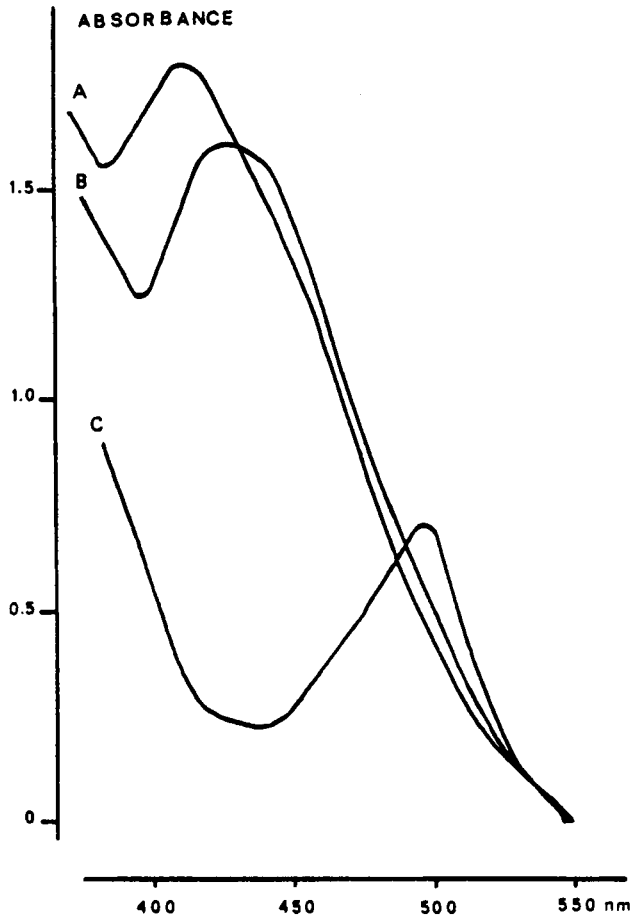

Figure 2

Absorption spectra of color yielded by the product of condensation of 2-6 dichloroquinone chloroimide with methimazole (A:100 ug/ml),with propylthiouracil (B: 10 ug/ml),or with carbimazole (C:100ug/ml). methimazole maximum absorbance at 410 nm;propylthiouracil at 435 nm; carbimazole at 498 nm.

in the blank chromatogram. When the assay was performed with samples containing artificially high concentrations (10 $\mu g.ml^{-1}$), only the methimazole peak could be detected at 405 nm. The detection limit for methimazole was 5 ng.

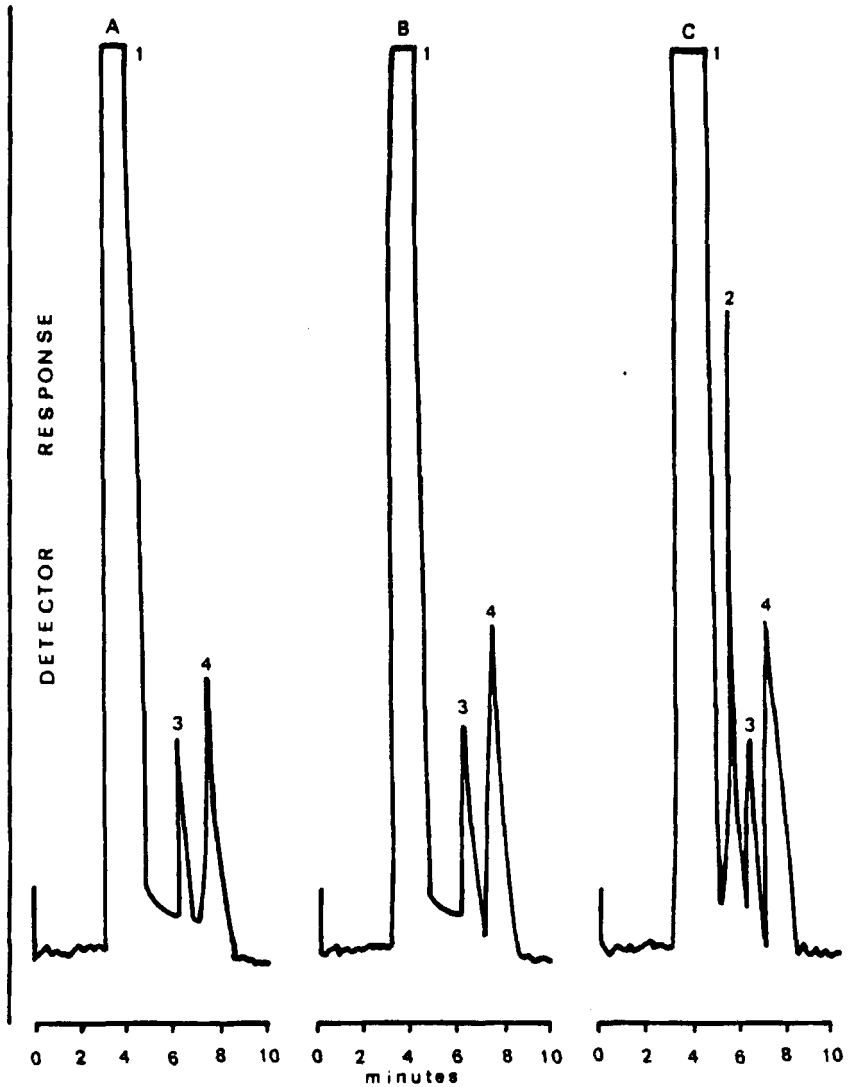

Figure 3

Chromatogramms of an aqueous drug-free solution (A); a drug free plasma (B); and a plasma with 15 ng/ml of methimazole (C, peak 2). Other peaks correspond to the 2-6 dichloroquinone chloroimide. Solvent: chloroform; column: u-NH_2; flow rate: 1.5 ml/mn; wavelength: 405 nm; sensitivity: 0.005 AUFS.

ml^{-1} with a signal to noise ratio equal to two. Linearity was verified on the standard concentration curve and in a series of dilutions of high concentration plasmas. Plasmas, A, B, C, were diluted with drug-free plasma. Each value was determined by the corresponding peak height and is the mean of four replicates.

TABLE I

LINEARITY FOR PLASMA A,B,C

methimazole ng/ml

	Dilution	measured	expected	% of recovery
A	1	154	154	100 %
	1/2	75	77	97.4 %
	1/4	36	38.5	93.5 %
	1/8	20	19.25	104 %
B	1	132	132	100 %
	1/2	65	66	98.5 %
	1/4	32	33	97%
	1/8	17	16.5	103
C	1	116	116	100 %
	1/2	57	58	98.3 %
	1/4	28	29	96.5 %
	1/8	15.5	14.5	107

The large dispersion of the concentrations (5 to 150 ng. ml^{-1}) required the plotting of several standard curves using different sensitivities for the detector.

Precision of the HPLC method was evaluated with replicates of pooled plasmas

TABLE II

REPRODUCIBILITY

Pooled sera	Number of determinations	Mean (ng/ml)	Coefficient of variation
A	10	25.3	4.3
B	10	44.2	3.6
C	10	115.3	1.2

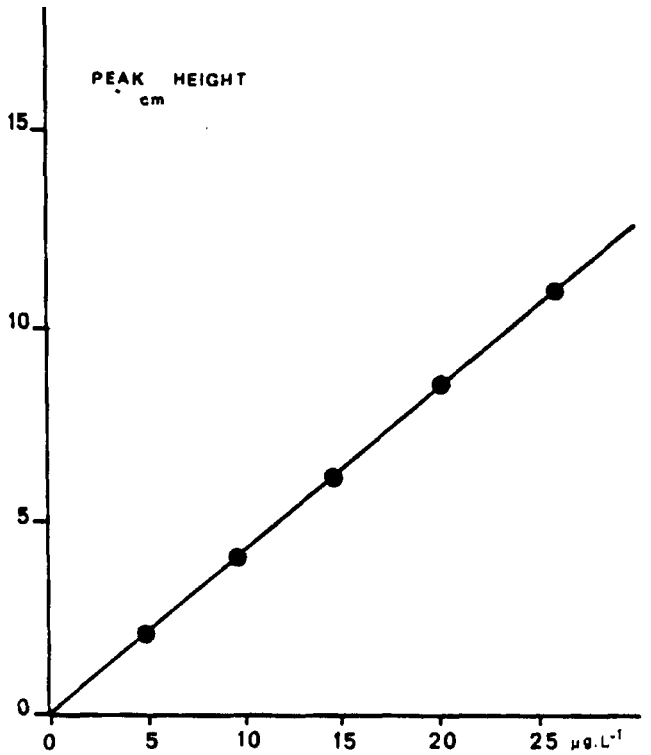

Figure 4

Standard curve for a range of concentration of 5 to 25 ng/ml methimazole. Sensitivity of the detector 0.005 AUFS at 405 nm. Peak heights measured on chromatogramms from 25 cm recorder for full-scale detection.

For several concentrations of the drug, within-run and between run (day to day) assays were performed. Each value expressed in ng/ml is the mean of four determinations. Plasmas were thawed at the time of the assay.

TABLE III

PRECISION

Plasma	Within-run assays mean (ng/ml)	C.V.	Between-run assays mean (ng/ml)	C.V.
1	13	6.1	15.2	7.5
2	28.3	3.7	29	7.2
3	52.3	3.9	48	6.5
4	96	3.6	98	5.7
5	112	2.7	120	5.3

Coefficients of variation were $\leq$ 6,1 % and 7,5 % respectively. Accuracy, tested through the recovery of methimazole added to pooled sera remained within the range 93 % - 107 %.

TABLE IV

RECOVERY OF METHIMAZOLE (M)

Serum	Sample volume (ml)	Added M (ng/ml)	Total recovered (ng/ml)	M originally present (ng/ml)	mean $\pm$ SD ng/ml
A	1	25.3	47.5	22.2	
A	1	12.5	37.0	24.5	23.6 $\pm$ 4,4%
A	1	00.0	24.3	24.3	
B	1	51.0	95.3	44.3	
B	1	25.0	64.5	42.0	43.1 $\pm$ 2.2%
B	1	00.0	43.0	43.0	
C	1	25.3	135.2	109.9	
C	1	12.5	122.8	110.3	110.7 $\pm$ 0.8%
C	1	00.0	112.0	112.0	

A good correlation was found between double isotopic dilution analysis results and those obtained with the HPLC method. Each value expressed in ng/ml is the mean of 2

determinations in duplicate. The regression analysis indicates :

$A = 0.999\ B + 0.02$; $r = 0.999$. $n = 32$ $p < 0.001$

TABLE V

CORRELATION BETWEEN HPLC AND DOUBLE ISOTOPIC DILUTION ANALYSIS (DIDA)

Plasma	A DIDA methimazole (ng/ml)	B HPLC methimazole (ng/ml)
1	14	14.2
2	19.2	18.7
3	26	26.5
4	48	48.5
5	69	68.1
6	89	87.5
7	110	112.5
8	132	131

DISCUSSION

The method selected for the present study is simple sensitive, and specific.

Simplicity :

we use the same solvent for both the mobile phase and the unique extraction.

Sensitivity :

Sensitivity is enhanced by the colorimetric reaction which occurs between the drug and the 2-6 dichloroquinone chloroimide : it amplifies the absorbance of methimazole and limits interferences with other non-sulfur compounds.

Selectivity :

Selectivity was obtained by using chloroform for the extraction at pH8, since this procedure removes the other SH-reacting groups from plasma (4). However, two com-

pounds may interfere : prophylthiouracil and carbimazole. Both of them elute from the column at the same retention time as methimazole, after they have reacted and complexed with the 2-6 dichloroquinone chloroimide, and absorb at 405 nm. (see fig 2, curve B,C).
But in clinical practice, prophylthiouracyl is no longer prescribed and carbimazole is completely hydrolysed to methimazole at the time of the analysis.

It is of interest when studying carbimazole intravenous kinetics, to follow this hydrolysis, and to separate carbimazole from methimazole on the chromatogram. As we have demonstrated previously, carbimazole and methimazole may be separated with different solvent systems on silica thin layer plates, and it is possible to transpose this separation to a μ-silica column.
During carbimazole therapy, such a method may be successfully used. A large range of methimazole concentrations (9 to 130 $ng.ml^{-1}$) is then observed that is possibly explained at least by two factors :
-patients were not given the same dose of drug (5 to 30 mg).
-blood has been sampled at various times after the oral administration of the drug. A kinetic study of methimazole has been performed twice in three volunteers after a single oral administration of 6 tablets containing 5 mg of methimazole. The plasmatic peak of the drug occured at the 8-9th hour (23 to 149 $ng.ml^{-1}$). This figure is in agreement with those found in litterature concerning the ^{35}S-carbimazole effect in man. Our data agree with the approximated maximum concentrations calculated from the ^{35}S-carbimazole decay curve (100 to 350 $ng.ml^{-1}$)(1,2). Values found for one volunteer (1,81 m, 79 kg) are represented in:
In summary, the method we describe readily allows the investigation of the absorption of carbimazole after oral administration. In three patients investigated

TABLE VI

Sampling time	Measured Methimazole ng/ml
2 h	86
4 h	97
6 h	133
8 h	147
9 h	133
10 h	119
12 h	118
24 h	98

twice, methimazole peak values differed widely from one patient to another : 149 and 128 ; 48 and 38 ; 31 and 23 $ng.ml^{-1}$ respectively. methimazole was not detectable in urine by this technique.

The method makes a study possible of the correlation of metabolism of carbimazole with its clinical effect.

ACKNOWLEDGEMENTS

We are specialy indebted to Claude DALDOSS and Mrs Annie-Christine POIDEVIN for their technical assistance.

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