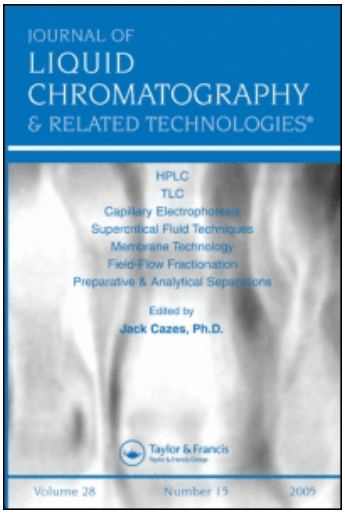

Journal of Liquid Chromatography & Related Technologies

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

Determination of Trimeprazine and Its Main Metabolites in Mouse Serum and Thyroid by Liquid Chromatography-Electrospray-Mass Spectrometry

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To cite this Article Sauvage, M. F. , Marquet, P. , Rousseau, A. , Buxeraud, J. , Raby, C. and Lachtre, G.(1998) 'Determination of Trimeprazine and Its Main Metabolites in Mouse Serum and Thyroid by Liquid Chromatography-Electrospray-Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 21: 20, 3173 — 3185

To link to this Article: DOI: 10.1080/10826079808001266

URL: http://dx.doi.org/10.1080/10826079808001266

J. LIQ. CHROM. & REL. TECHNOL., 21(20), 3173–3185 (1998)

DETERMINATION OF TRIMEPRAZINE AND ITS MAIN METABOLITES IN MOUSE SERUM AND THYROID BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY-MASS SPECTROMETRY

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ABSTRACT

A sensitive and highly specific method for the determination of a phenothiazine neuroleptic, trimeprazine, and three of its main metabolites in mouse serum and thyroid, using combined liquid chromatography-electrospray-mass spectrometry (LC-ES-MS) was developed. A Nucleosil C_{18} (150 × 1 mm I.D.) reversed-phase column was used for the chromatographic separation together with a mixture of 5 mM ammonium formate buffer

(pH 3) and acetonitrile (53/47; v/v) as mobile phase. Recoveries of the four analytes in serum were better than 60%, detection limits were 0.4 ng/mL, and limits of quantitation were 0.5 ng/mL for all analytes. Intra-assay precision, was close to 3% at 10 ng/mL and better than 18% at 2 ng/mL for the four analytes. Reproducibility was good (C.V. < 20%) and linearity excellent (r > 0.997) from 0.5 to 40 ng/mL. This sensitive technique allows the determination of serum and thyroid concentration of trimeprazine and metabolites in small tissue or serum samples.

INTRODUCTION

Trimeprazine (N,N-β-trimethyl-10H-phenothiazine-10-propanamine) is clinically widely used as a neuroleptic drug, and is often administered over lengthy periods. Main metabolic pathways of phenothiazines are: N-demethylation, sulphoxidation and hydroxylation. Long term treatment with this drug might lead to a decrease in serum thyroid hormones.[1,2] Based on previous work, an antithyroid mechanism based on molecular iodine complexation has been hypothesized.[3-5]

The further step to the elucidation of this complexation mechanism, is to investigate if this drug or its metabolites accumulate in thyroid, which can be evaluated by determining the mean concentration ratio between serum and thyroid. For this purpose, analysis of trimeprazine (TMP) and its main metabolites have to be performed in serum and in thyroid.

To date, few methods have been reported for the determination of trimeprazine in biological fluids. Separation techniques like Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) connected to different detection techniques (U.V., electrochemical detection, radioimmunoassay (RIA), or mass spectrometry (MS)), were previously proposed.[6-8] Moreover, to the best of our knowledge, only one metabolite, N-demethyltrimeprazine (NDT) has been quantified.[8] No quantitative determination of the other metabolites has been previously reported, nor qualitative or quantitative analysis of TMP itself in tissues.

In order to study the accumulation of trimeprazine and its major metabolites (N-demethyl trimeprazine -NDT-, trimeprazine sulphoxide -TSO-, and 3-hydroxy trimeprazine -3OHT-) in thyroid, this work intended to design a sensitive method for the quantitative analysis of these compounds in both mouse serum and thyroid. In that aim, mice received 2 mg/kg/day of trimeprazine in drinking water for three weeks, for a preliminary study.

EXPERIMENTAL

Chemical and Reagents

TMP, TSO, NDT, and 3OHT were gifts from Rhone-Poulenc (Vitry-sur-Seine, France). Protriptyline (internal standard) was from Merck-Sharp and Dohme (Paris, France). The other chemicals used for the preparation of the solutions or for the extraction procedure were: diethyl oxide (Prolabo, Paris, France), dichloromethane (Prolabo), acetonitrile (Carlo Erba, Milan, Italy), formic acid and ammonium formate (Sigma, St. Louis, MO), all of chromatographic purity. Deionized water was prepared in a MilliQ laboratory plant (Millipore, Bedford, MA).

The extraction solvent was a diethyl oxide/dichloromethane mixture (v/v; 70/40). A NH_4Cl saturated solution in deionized water was adjusted to pH 9.5 with 0.25 M NH_4OH.

For each of the four analytes and for the internal standard (I.S.), a 1 g/L stock solution was prepared in methanol and kept at +4°C to ensure stability. A 10 mg/L solution of I.S. was prepared every week and kept at +4°C, from which a 1 mg/L working solution was prepared in deionized water, daily. The other working solutions were prepared daily by suitable dilutions in deionized water.

Apparatus

Chromatography

The HPLC system consisted of a dual-piston syringe pump (Brownlee Labs, Santa Clara, CA), an autosampler series 200 (Perkin-Elmer, Norwalk, CT, USA) including a Rheodyne model 7725 injection valve equipped with a 5 µL internal loop (Rheodyne, Cotati, CA). Chromatographic separation was performed on a Nucleosil C_{18} 5 µm (150 × 1 mm I.D.) reversed-phase column (LC-Packings, Touzart & Matignon, Courtaboeuf, France) using a mixture of 5 mM ammonium formate (adjusted to pH 3 with formic acid) and acetonitrile (53/47, v/v) as mobile phase, delivered at a flow rate of 45 µL/min. All chromatographic solvents were filtered (0.46 µm) prior to mixing and degassed with helium thereafter.

Mass spectrometry

An API-100 atmospheric pressure ionization mass spectrometer, equipped with an electrospray-type Ionspray® ionization device was used (Sciex, Toronto,

ON, Canada). Medical compressed air was used for nebulization of the chromatographic effluent and ultra high-purity nitrogen was used as curtain gas. Calibration of the mass spectrometer analyzer was performed by infusion (5 μL/min) of a commercial mixture of PPGs (polypropylene glycols, Applied Biosystems, Saint-Quentin-en-Yvelines, France) using a Harvard model 11 syringe pump (Harvard Scientific, South Natick, MA, USA) and monitoring m/z ratios in the 50 to 400 a.m.u. mass range.[9]

For optimization of the detection of trimeprazine and its metabolites, the four compounds were diluted in mobile phase in order to obtain a 10 mg/L concentration for each of them, then individually infused into the ion source with a flow rate of 10 μL/min. Characteristic ions of each drug were identified using full scan acquisition (m/z 60-500, step-size 1 a.m.u) and with an orifice voltage of 30 V. The main parameter settings of the mass spectrometer were as follows: nebulization gas 1.16 L/min; curtain gas 0.96 L/min; ionspray voltage 5000 V; electron multiplier 2500 V; dwell time 250 ms. The orifice voltage was thereafter optimized for each mass-to-charge ratio acquired in the selected ion monitoring (SIM) mode according to the method previously described by Hoja.[10] We selected two ions for each analyte: the pseudomolecular ion and the main fragment ion, of which the most abundant was chosen as quantifying ion, the other one being used as confirmation ion.

Sample Preparation

Serum

In a 15 mL glass tube were successively added, 1 mL of serum, 50 μL of I.S. (1 mg/L), 1 mL of pH 9.5 ammonium acetate buffer and 8 mL of a diethyloxide/dichloromethane mixture (70/40, v/v). The tubes were shaken for 25 min on a Laboral oscillating agitator (Prolabo, Paris, France), then phase separation was accomplished by centrifugation (18°C, 900 g, 10 min). The organic phase was then transferred to a 10 mL conical-bottomed glass tube and evaporated to dryness under a gentle stream of nitrogen at 37°C. The resultant residue was redissolved in 25 μL mobile phase, of which 3 μL were injected into the chromatograph.

Thyroid

Pools of eight mice thyroids (22 mg ± 2.8) were crushed in 1 mL of 0.1 N HCl, sonicated at ambient temperature for 10 min and agitated for 15 min on a Laboral oscillating agitator. Analytes were extracted from the supernatant following the same procedure as from the serum.

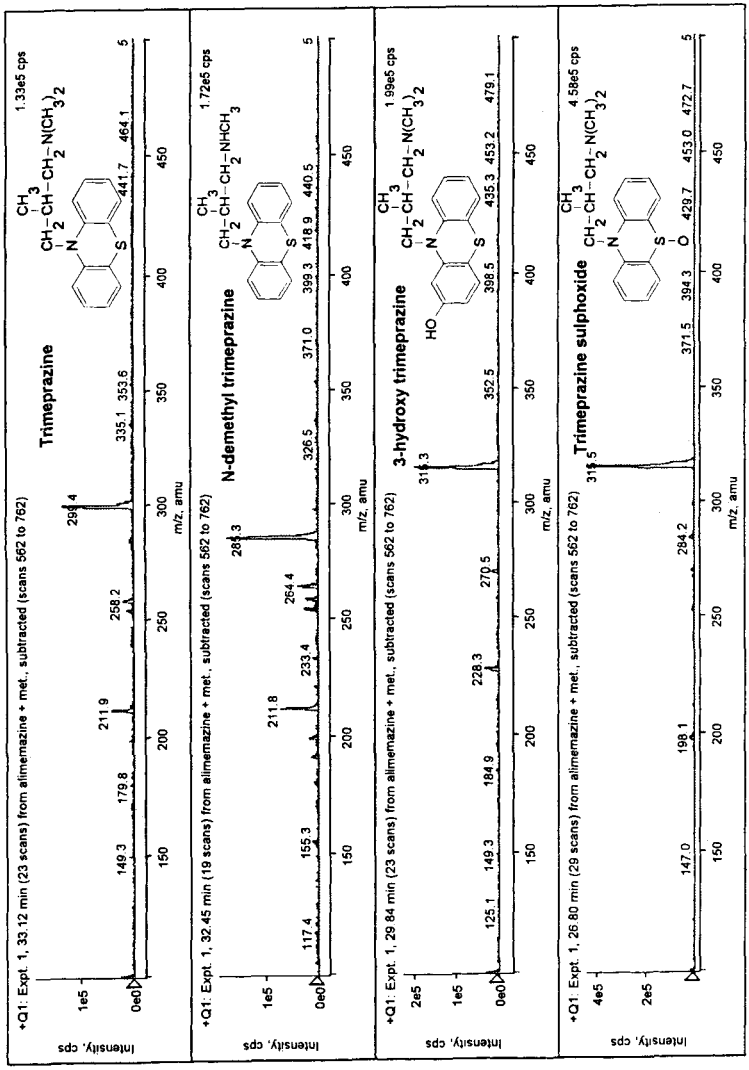

Figure 1. Mass spectra and structures of trimeprazine and metabolites.

Table 1

Retention Times and Selected m/z Values

	Retention Time (min)	Selected Ion (a.m.u.)	Orifice Voltage (V)
Trimeprazine	7.9	299.4	90
		212.0	60
Trimeprazine sulphoxide	3.5	**315.2**	40
		286	40
N-Demethyl trimeprazine	7	285.2	95
		212.0	60
3-Hydroxy trimeprazine	4.7	315.2	40
		228.0	60
Protriptyline (I.S.)	6.7	**191.0**	80

Quantifying ions are in bold characters.

VALIDATION PROCEDURES AND STANDARD CURVES

All validation procedures were performed using spiked drug-free serum. Extraction recoveries were determined in triplicate at concentrations of 5, 10, and 20 ng/mL by extraction of samples fortified with the four analytes (TMP, TSO, NDT, and 3OHT) but not I.S. After the extraction procedure, the dry extract was dissolved in 25 µL mobile phase containing I.S. (1 mg/L). The peak area ratios obtained were then compared with those of unextracted mixed standard solutions of TMP, TSO, NDT, 3OHT, and I.S. representing 100% recovery.

For the analytical validation, the guiding principles established during a conference on "method validation for the quantitation of drugs in biological media" were followed.[11]

Intra-assay precision (i.e. repeatability) was assessed as follows: six standards were prepared, extracted, analyzed, on the same day, for each of three concentrations levels (5, 10, 20, ng/mL). Intermediate precision and accuracy were assessed at seven concentrations of TMP, TSO, NDT, and 3OHT (0, 0.5, 1,

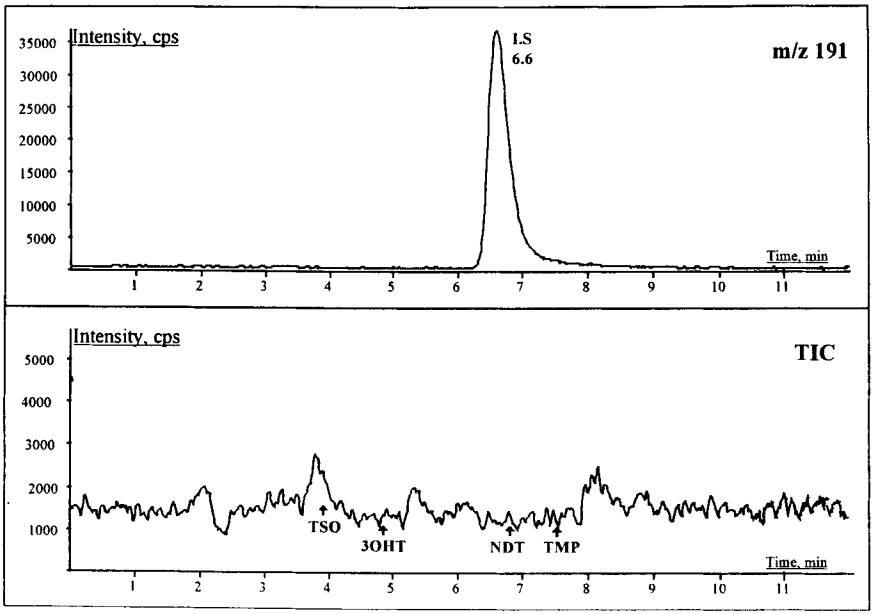

Figure 2. Total ion current from a blank serum extract.

Table 2

Mean Recoveries of Trimeprazine and Metabolites in Plasma

Added Conc. (ng/mL)	Trimeprazine Mean Rec. (n=3)	CV (%)	Trimeprazine Sulphoxide Mean Rec. (n=3)	CV (%)	N-Demethyl Trimeprazine Mean Rec. (n=3)	CV (%)	3-Hydroxy Trimeprazine Mean Rec. (n=3)	CV (%)
5	60.9	9.6	79.2	11.6	86.4	3.9	65.9	6.7
10	70.5	3.8	84.9	1.9	76.0	6.5	70.7	4.7
20	74.9	5.5	95.3	9.1	73.7	5.5	76.2	8.5

2, 5, 10, 20, and 40 ng/mL) each day for six days. Calibration graphs were constructed using a 1/x weighted least square linear regression of the drug-to-internal standard peak-area ratios of the quantitation ions versus theoretical drug concentrations.[12]

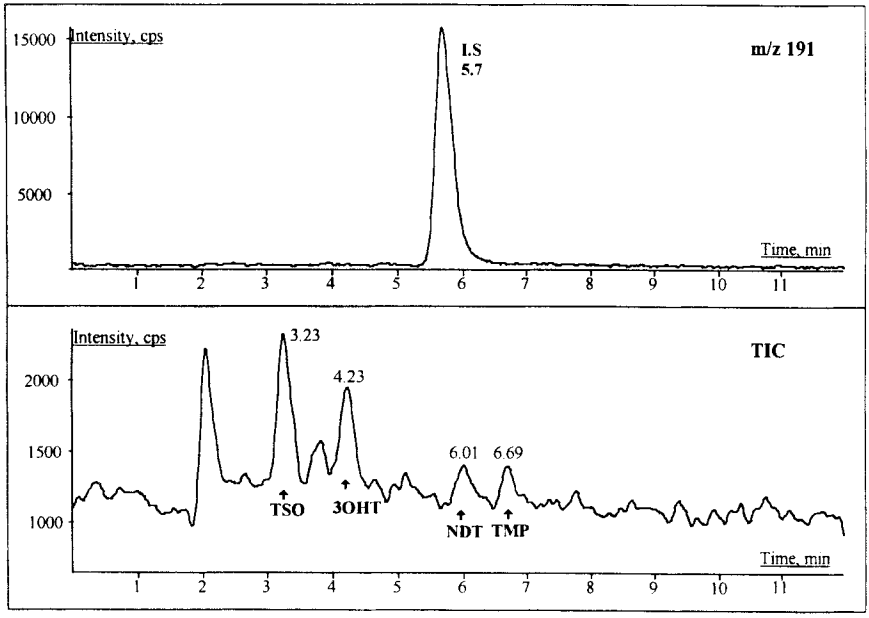

Figure 3. Total ion current of 1 ng/mL spiked serum extract.

RESULTS AND DISCUSSION

Figure 1 shows background-subtracted mass spectrum of TMP, TSO, NDT, and 3OHT acquired in full scan with an orifice voltage of 30 V. This parameter was found to significantly modify the absolute and relative abundances of the pseudomolecular and fragment ions; thus, optimum settings for each ion were used for data acquisition. The m/z ratios of the pseudomolecular ions and of the principal fragment ion selected for each compounds as well as the corresponding optimized orifice voltage are reported in Table 1. Figures 2 and 3 depict chromatograms obtained from blank serum and serum spiked at 1 ng/mL of TMP, TSO, NDT, and 3OHT. Analysis of blank serum samples showed that no unknown substances resulting from the extraction procedure interfered with trimeprazine and its three metabolites. Mean retention times were, 3.5 min for TSO, 4.7 min for 3OHT, 6.7 min for I.S., 7 min for NDT, and 7.9 min for TMP.

The validation of this method in thyroid appeared impossible, because trimeprazine is spontaneously and rapidly converted into trimeprazine sulphoxide in the matrice. The mean recoveries obtained for the four analytes in

Table 3

Intra-Assay Precision and Accuracy (n=6) of the Determination of Trimeprazine and Metabolites in Plasma

Added Conc. (ng/mL)	Mean Found Conc. (ng/mL)	Precision CV (%)	Accuracy (%)
		Trimeprazine	
2	2.13	10.9	106.4
5	5.14	3.6	102.7
10	10.17	2.8	101.6
		Trimeprazine Sulphoxide	
2	2.15	15.5	107.3
5	4.98	9.8	99.6
10	9.84	6.6	98.4
		N-Demethyl Trimeprazine	
2	2.04	17.1	102.1
5	4.93	4.4	98.6
10	10.40	3.1	103.9
		3-Hydroxy Trimeprazine	
2	1.93	11.5	96.7
5	4.93	4.2	98.6
10	10.51	2.9	105.1

serum, at the three concentrations studied (5, 10, 20 ng/mL) were satisfactory, and always better than 60% (Table 2). The method was shown repeatable since intra-assay coefficients of variation (C.V.) were between 2 and 17% (Table 3). The intermediate precision and accuracy were also satisfactory over the whole range of concentrations studied (Table 4): the C.V's were lower than 20% in all cases; deviation from the nominal values were less than 8% for concentrations between 1 and 40 ng/mL. These results allow a limit of quantitation of 0.5 ng/mL for TMP, TSO, NDT and 3OHT. An analysis of variance of the results obtained from the six different calibration curves showed the absence of any significant non-linearity over the range from 0.5 to 40 ng/mL. Correlation coefficients were higher than 0.997 for the four compounds. The detection limits were determined as lower than 0.4 ng/mL for all the analytes. These detection limits were among the lowest published to date for trimeprazine in

Table 4

Intermediate Precision and Accuracy of the Determination of Trimeprazine and Metabolites in Plasma (n=6)

Added Conc.	Mean Found Conc.	Precision CV (%)	Accuracy (%)
		Trimeprazine	
0.5	0.54	12.9	107.6
1	1.02	15.4	101.7
2	1.87	19.6	93.6
5	4.69	15.5	93.8
10	9.88	11.9	98.8
20	20.94	5.2	104.7
40	39.87	4.8	99.7
		Trimeprazine Sulphoxide	
0.5	0.53	16.2	99.5
1	1.00	19.8	99.9
2	2.11	12.9	105.4
5	5.15	14.3	103.1
10	9.39	10.8	93.9
20	18.88	5.8	94.4
40	41.44	3.8	103.6
		N-Demethyl Trimeprazine	
0.5	0.61	14.6	121.1
1	0.97	12.8	96.8
2	1.91	7.9	95.0
5	4.64	4.8	92.7
10	9.20	5.6	92.0
20	20.04	3.7	100.2
40	41.15	3.0	102.9
		3-Hydroxy Trimeprazine	
0.5	0.63	13.1	125.2
1	0.95	19.4	93.1
2	1.96	14.6	97.8
5	4.27	14.6	85.4
10	9.73	5.1	97.3
20	19.47	4.2	97.4
40	41.53	2.7	103.8

Table 5

Concentration of Trimeprazine and Metabolites in Mouse Serum and Thyroid

	Serum ng/mL	Thyroid ng/mL
Lot 1-8		
TMP	4.2	24.4
TSO	15.2	8.4
NDT	3.47	28.5
3OHT	3.4	11.6
Lot 9-16		
TMP	4.6	16.3
TSO	12.7	15.1
NDT	4.3	58.2
3OHT	3.7	7.5
Lot 17-24		
TMP	1.5	34.2
TSO	6.9	17.8
NDT	6.3	22.5
3OHT	<0.5	6.7

body fluids.[8] The present assay requires only 1 mL of serum, or approximately 20 mg of tissue, so this method appears suitable to characterize pharmacokinetic disposition of these compounds in cases of limited amounts of serum or tissue.

The present method has been used successfully to determine TMP, TSO, NDT, 3OHT concentrations in serum and thyroid of mice treated with 2 mg/kg/day of trimeprazine added in drinking water. This dose was chosen in accordance to therapeutic human doses, taking in account human and mice body weights or surfaces. After a 3 week treatment, mice were anesthetized with diethyl ether, then sacrificed.

Blood samples were obtained by cardiac puncture, they were centrifuged and serum was stored at -80°C until assayed. Thyroid was rapidly removed and stored at -80°C until analysis. Table 5 reports the mean observed concentrations.

As already mentioned, different techniques were described for the identification of trimeprazine in biological samples but very few dealt with its metabolites.[6-8] A gas chromatography-mass spectrometry technique allowed the determination of trimeprazine and three of its metabolites in urine of rats but the thermodegradation of sulphoxide into the parent drug was pointed out as a real hindrance to the precision of the method.[13] In the present study, as no part of the chromatographic device and electrospray interface was heated, no thermodegradation of trimeprazine sulphoxide to trimeprazine occurred, as verified by the determination of trimeprazine-sulphoxide alone.

In conclusion, the described method, using LC-ES-MS allows the sensitive and specific determination of trimeprazine and its metabolites (NDT, TSO, 3OHT) in serum and in thyroid; its applicability was demonstrated in a preliminary study in mouse.

ACKNOWLEDGMENT

This work was supported, in part, by a PDZR find from the Conseil Régional du Limoiusin.

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Received December 10, 1997
Accepted February 18, 1998
Manuscript 4681