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Short communication

MMSPE-RP-HPLC method for the simultaneous determination of methimazole and selected metabolites in fish homogenates

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Abstract

A simple and rapid high-performance liquid chromatography method with ultraviolet detection was developed for the determination of the thyreostatic compound methimazole (1-methyl-2-mercaptoimidazole, MET) and metabolites 2-mercaptoimidazol (SHMET), *N*-methylthiourea (MTU) and *N*-methylhydantoin (MEH) from zebrafish (*D. rerio*) whole body homogenates using mixed mode solid phase extraction technique for sample pre-treatment. The highly polar compounds were separated on a difunctionally bonded silica based reverse phase column using gradient elution. Retention factors ranged between 1.53 and 5.66. The method was linear between 0.1 and 30 μg/ml, the detection limits were 0.4 ng for MET and SHMET, 0.6 ng for MTU and 2.6 ng for MEH. Extraction method was exhibited average recovery rates of 85.2–97.6%.
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Keywords: Mixed mode solid phase extraction; RP-HPLC; Polar substances; Zebrafish; Methimazole and metabolites

1. Introduction

Chromatographically correct quantitative and qualitative determination of small molecular weight polar substances, e.g. amines, heterocyclic compounds is still one of the most problematic tasks in the reversed phase liquid chromatography. The hydrophobic stationary gives unsatisfactory retention of the polar analytes although silanol groups can retain polar compounds due to the silanophyl interaction. In this case peak broadening or tailing can decrease the effectivity of the separation. Even ionisation of ionisable group containing substances and silanol groups of the stationary phase can make the separation more difficult. Using reversed phase it is recommended to keep all ionisable groups of the analytes in undissociated form but in case of, e.g. basic molecules this means high pH values of the mobile phase which is not suited with silica based phases. Normal phase separation is a possible choice for retaining polar analytes but the most common used reversed phase methods are preferred. In this paper we present a short exemplar, how to solve this problem. We used methimazole and its metabolites as low molecular weight heterocyclic and basic substances. Methimazole (1-methyl-2-mercaptoimidazole), an antihormone, is widely used in medicine for treatment of hyperthyroidism and even as model substance for endocrine (thyroid axis) disruption in physiological and genomic studies. Its action is to inhibit iodide integration into thyrosine and thus inhibits the production of thyroid hormones. Various techniques have already been reported to analyse residues or thyreostatics in meat [1], urine [2–4], blood [5–8], vegetable samples [9]. Most of these methods are capable for identification of methimazole, methylthiouracil, prophylthiouracil and other thiourea derivates. Techniques used were thin layer chromatography [10], high-performance liquid chromatography with UV and/or electrochemical detection [11] and flow injection analysis [12]. These methods were capable to determine the compounds separately but there is an urgent need to develop an innovative, fast and effective method for the determination of methimazole and its metabolites simultaneously from bi-

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ological samples. We now present a method for the simultaneously determination of methimazole (MET) and selected metabolites as *N*-methylthiourea (MTU), *N*-methylhydantoin (MEH), 2-mercaptoimidazole (SHMET) (Fig. 1) employing reversed-phase HPLC with UV detection. The metabolites were selected to represent all types of main metabolising steps (oxidation, demethylation, bond-cleavage) in vertebrates.

2. Experimental

2.1. Chemicals and materials

Methimazole (medicine, inhibits thyroid function, possible teratogen), 2-mercaptoimidazole (harmful), 1-methylhydantoin (harmful) and *N*-methylthiourea (possible teratogen) were obtained from Sigma–Aldrich GmbH, Steinberg, Germany. Oasis MCX 3 ml/60 mg cartridges for the solid phase extraction were obtained from Waters GmbH, Eschborn, Germany. The HPLC grade Methanol, 85% *o*-phosphoric acid, sodiumdihydrogenphosphate monohydrate and disodiumhydrogenphosphate anhydrous were purchased from Merck GmbH. The Osmonics Cameo 17F syringe filters 0.45 μm, teflon, 17 mm were purchased from Sigma–Aldrich GmbH, Steinberg, Germany. 300 μg/ml MET, SHMET, MEH and MTU stock solutions were prepared in water and later mixed and diluted by water in seven different concentration for external calibration.

2.2. Instrumentation

The liquid chromatographic process was performed using a PE Series 200 pump with a PE ISS 200 autosampler (Perkin-Elmer Inc., USA). The separation was carried out on an Atlantis dC18 column (150 mm × 4.0 mm, 5 μm Waters Corp., Milford, Massachusetts, USA) with a LiChrospher RP 18, 4–4 guard column (Merck KGaA, Darmstadt, Germany) and was detected by a PE 235 C diode array detector (Perkin-Elmer Inc., USA). Pallas software was used to predict chromatographical conditions and Turbochrom3 (Perkin-Elmer Inc., USA) for recording chromatogramms and to calculate the peak area. A Beckman L-60 Ultracentrifuge (Beckman Coulter GmbH, Germany) was used to ultracentrifuge samples.

2.3. Chromatographic conditions

Methimazole and metabolites were eluted at a flow rate of 1 ml/min. The mobile phase was phosphat buffer (pH 6.7): methanol gradient starting from 100% phosphate-buffer for 2 min, increased to 10% MeOH in 2 min and was kept at 10% MeOH for 4 min The system returned to starting condition in 2 min and was operated at 100% buffer for 4 min to re-equilibrate the column. The eluents were monitored by UV detection at wavelength of 255 nm for methimazole and 2-mercaptoimidazol and at 220 nm for *N*-methylthiourea and *N*-methylhydantoin. The total run time was 14 min. The chromatographic analysis was performed at room temperature. The injection volume was 10 μl. Void time was determined by injecting phosphate ions (eluent: water) detected at 255 nm. For the identification of analytes the UV spectrum analysis was applied.

2.4. Sample preparation

The sample pre-treatment method was investigated for fish whole body homogenates. Adult zebrafish (*D. rerio*) species were sacrificed by placing them on ice and then stored at −70 °C until processing. Weight of each fish was determined. Fishes were homogenized in four times the body weight of water by a Schütt-Homgen homogenator at 4 °C. Homogenates were kept on ice. Samples were centrifuged at 4.000 × *g* for 10 min and than supernatant at 80.000 × *g* for 60 min. Supernatant was used for the next, solid phase extraction, step. The solid phase extraction was carried out on a mixed mode mean cationic exchange cartridge. The cartridges were placed onto a vacuum system. MCX cartridges were conditioned with 2 ml MeOH and equilibrated with 3 ml water. One milliliters of centrifuged homogenates were acidified by adding 10 μl 50% H_3PO_4/water to the homogenate. This solution was placed on the top of the cartridge. Cartridges were eluted with 3 ml 90% MeOH:H_2O, pH 12 (adjusted with NH_4OH). Eluat was filtered through a Cameo 17F syringe filter and evaporated under a stream of nitrogen and dissolved in 200 μl water.

3. Results and discussion

3.1. Chromatogram

The chromatographic conditions were optimized in order to provide good performance of the assay. Chromatographic profiles were obtained for standard, blank and spiked fish whole body homogenates (Figs. 2 and 3) after solid phase extraction using OASIS MCX cartridge at HPLC conditions as descried above. Retention times were 8.93, 3.66, 3.57

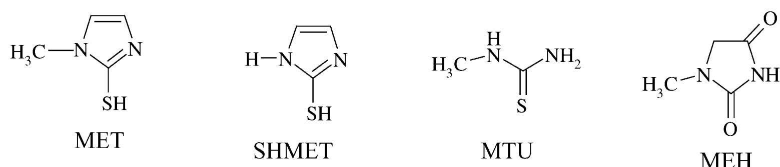

Fig. 1. Chemical structure of Methimazole (MET), 2-mercaptoimidazole (SHMET), *N*-methylthiourea (MTU) and *N*-methylhydantoin (MEH).

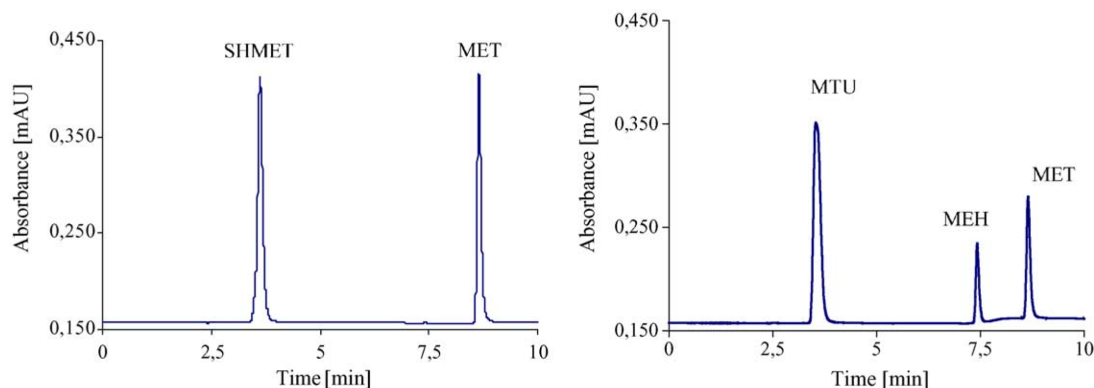

Fig. 2. Chromatograms of 10 μg/ml standard solutions detected at 255 nm (left) and 220 nm (right).

and 7.47 min for MET, SHMET, MTU, MEH, void time was 1.41 min. Intra-day precision of retention times for the substances are shown in Table 1. Retention factors (k) were 5.33; 1.60; 1.53 and 4.30 for MET, SHMET, MTU and MEH, according to the chromatographical requirement $1 < k < 10$. Selectivity factors were determined according to equation $\alpha = k_2/k_1$ and show values of 3.31; 3.48; 1.23 for MET against SHMET, MTU and MEH. Values as 2.68 and 2.81 were found for MEH against SHMET and MTU and 1.04 for SHMET against MTU. Selectivity values show good separation of MET, MEH and SHMET or MTU, but the value lower than 1.05 of selectivity shows no satisfactory separation of MTU and SHMET. For real samples the quantification and identification of both analytes was carried out by simultaneous detection at 255 nm for SHMET and 220 nm for MTU. MTU and SHMET peaks minimal overlapped at both wavelengths; the peak area ratio of SHMET at 255 nm was 90 ± 1.8% and of MTU at 220 nm was 87 ± 1.2%. Additionally ultraviolet spectra were recorded and analysed. Other peaks were thoroughly separated during the total run time of 14 min. According to the spectral analysis of blank fish samples there was no interference found from endogenous substances in biological matrices by using MCX cartridges, neither at 255 nm nor at 220 nm (Fig. 3).

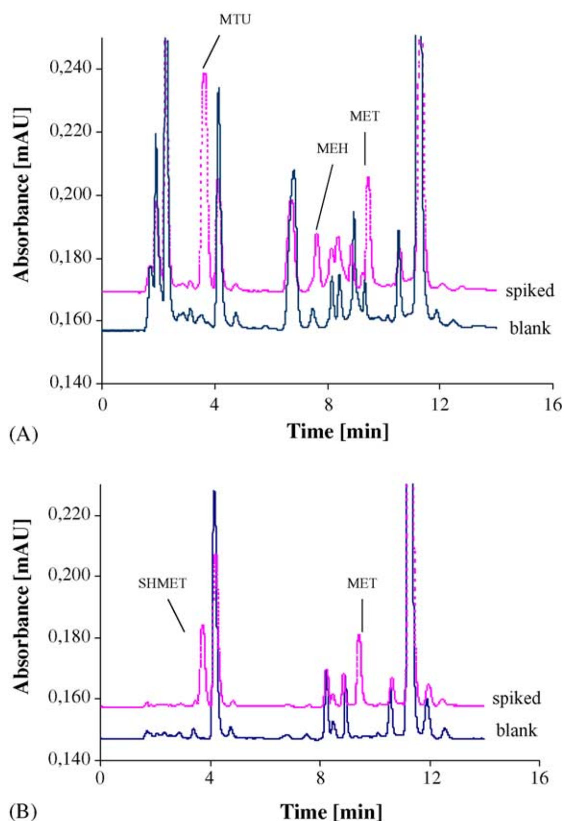

Fig. 3. Typical chromatograms of extracted blank and spiked (5 μg/ml each substances) fish homogenates obtained by extraction method described at detection wavelength of 220 nm (above) and 255 nm (below).

3.2. Calibration, extraction efficiency and recovery

Linearity was achieved at concentration range between 0.1 and 30 μg/ml for MET, SHMET and MTU and a concentration range between 0.5 and 30 μg/ml for MEH using seven points calibration. Slope, intercept, correlation coefficient of each calibration curve are shown in Table 1. The linearity of standard calibration curves is confirmed by the high values of the correlation coefficients. The calibration curve was also determined using preparation blank fish homogenates and no matrix effect was observed. Limits of detection were calculated from a peak signal to noise ratio of 3:1. The resulting detection limits were 0.06, 0.06, 0.10 and 0.43 μg/ml for MET, SHMET, MTU and MEH, respectively. Limit of detection values expressed in mass units were: 0.6 ng for MET; 0.6 ng for SHMET; 1.0 ng for MTU and 4.3 ng for MEH, respectively. Limits of quantification (noise ratio of 5) were determined to be 0.13; 0.13; 0.20 and 0.87 μg/ml from fish whole body homogenate for MET, SHMET, MTU and MEH, respectively. The average extraction recoveries and relative standard deviations of peak area were determined at concentrations ranging between 0.5 and 30 μg/ml for MCX (Table 2). Spiked whole body homogenates were extracted by extraction method described above and analysed for each concentration in five replicates. Average recoveries and relative standard deviations with cationic exchange cartridge (MCX) were 87.8 ± 5.8% for methimazole, 85.2 ± 8.7%

Table 1
Calibration parameters of external calibration processes for methimazole and metabolites

	Conc. range (μg/ml)	Slope	Intercept	Corr. coeff.	Ret. factor	Retention	% R.S.D. retention		LOD (μg/ml)	LOQ (μg/ml)	% R.S.D. quantification	
							Intra-day	Inter-day			Intra-day	Inter-day
MET	0.1–30	59174	1378	0.9995	5.33	8.93	1.3	5.6	0.06	0.13	2.5	7.9
SHMET	0.1–30	68132	1050	0.9998	1.60	3.66	1.4	2.1	0.06	0.13	2.4	6.3
MTU	0.1–30	46956	−85	0.9999	1.53	3.57	0.7	1.6	0.10	0.20	2.6	8.5
MEH	0.5–30	15323	967	0.9994	4.30	7.47	2.5	3.6	0.43	0.87	3.0	9.5

Table 2
Percentage recovery values of spiked fish homogenates extracted by MCX cartridge in different concentrations

	MET	SHMET	MTU	MEH
0.5 μg/ml	79.9 ± 6.9	87.4 ± 6.6	88.7 ± 5.9	93.4 ± 5.4
3 μg/ml	86.2 ± 4.8	89.8 ± 6.7	94.2 ± 7.3	101.3 ± 6.2
10 μg/ml	97.4 ± 5.6	78.2 ± 12.7	102.1 ± 8.6	98.2 ± 7.6

Values are expressed as mean recovery ± %RSD ($n = 5$).

for 2-mercaptoimidazole, 95.0 ± 7.3% for *N*-methylthiourea and 97.6 ± 6.4% for *N*-methylhydantoin. Average errors of the method show good results, all average errors were less than 10%. In this study recoveries ranged between 78.2 and 102.1%. This range lies within the reported values in the literature. In previous studies recovery of MTU ranged between 70 and 93% rat plasma [5]. Recovery of MET from serum and urine samples was found between 20 and 1112% [2,8] in the literature. Recoveries of solid phase extraction methods for MEH and SHMET from biological matrices were not found in the previous publications. Observed recovery values of methimazole and metabolites were suitable for the simultaneous sample pre-treatment of these compounds from fish whole body homogenate. The advantage of the solid phase extraction compared to liquid–liquid extraction is saved time and solvent. In this method, we simultaneously determined four analytes, which is another advantage of the pre-treatment method.

4. Conclusion

The present study reports on the development of reversed phase chromatographic determination and solid phase extraction method for the simultaneous separation and quantitative analysis of methimazole and its metabolites in fish body homogenates. The novel chromatographic method is capable to qualify and quantify the polar parent compound and its the more polar metabolites on a reversed phase chromatographical column in one simultaneous run. Sample preparation using new approaches is also new in our study. SPE methods for simultaneous determination of these compounds were not published yet. Extraction method described is efficient and show higher recovery rates than, e.g. liquid–liquid extraction methods. Simultaneous analysis of parent compound and metabolites are cost efficient and save time for sample preparation. Owing to the sample preparation procedures and chromatographic separation optimisation, the proposed method is selective and specific enough for the determination of methimazole and metabolites from fish. Relative standard deviations, which are lower than 10% demonstrate the reproducibility and precision of method presented and also the accuracy of the method is confirmed. It can be concluded, that method with MCX extraction is simple and able to determine methimazole and its metabolites simultaneously from fish whole body homogenates. The method has potential as a base for development of clinical applications too.

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