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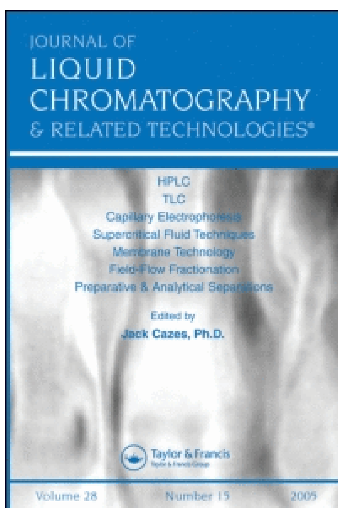

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Abstract: A method based on on-line solid phased extraction capillary liquid chromatography-electrospray ionization-mass spectrometry (SPE-capLC-ESI-MS) has been developed for the determination of oxomemazine in human plasma. Prior to injection, 0.5 mL of plasma spiked with metronidazole (internal standard) was mixed with ammonium formate buffer and methyl orange, which served as an ion pair reagent for effective chloroform liquid–liquid extraction. The employment of methyl orange as an ion pair reagent doubled the extraction efficiency, as compared to not using methyl orange. In preliminary experiments, conventional LC-UV instrumentation was employed. However, it was found that employing a capillary column with an inner diameter of 0.3 mm increased the sensitivity by a factor of $\sim$100, when injecting the same mass of analyte. Incorporating an easily automated reversed phase column switching system with SPE made it possible to inject up to 100 μL of solution, and the total analysis time was 5 minutes. The method was validated in the range 3 to 30 ng/mL oxomemazine, yielding a correlation coefficient of 0.99 (r^2). The within-assay and between-assay precisions were between 6.7 and 12% and 6.8 and 7.4%, respectively. The method was used to determine the amount of oxomemazine in a healthy female 20 hours after an intake of 1 teaspoon (approximately 1 mL) of the cough syrup Toplexil®, which contains 0.033 g oxomemazine per 100 mL syrup. Oxomemazine was detected, and the concentration was calculated to 2.0 ng/mL plasma.

Keywords: Capillary liquid chromatography, Column switching, Oxomemazine, Liquid–liquid extraction, Mass spectrometry

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INTRODUCTION

Oxomemazine (Figure 1), a phenothiazine derivative, is an antihistamine used for the symptomatic relief of hypersensitivity reactions. It is also an ingredient of preparations for the symptomatic treatment of coughs and the common cold. It is given orally in doses equivalent to 10 to 40 mg of oxomemazine daily and may also be administered rectally in form of suppositories. Different methods have been reported for its determination including HPLC,[1,2] GC,[3,4] and TLC.[5] However, to our knowledge, no methods have been reported for the determination of oxomemazine in plasma, which may be necessary for pharmacokinetic studies and in cases of side effects and adverse events. The concentration of oxomemazine in plasma is expected to be low and, hence, a highly sensitive method is needed. Several instrumental steps can be made to improve sensitivity; reducing the inner diameter of the analytical column (e.g., replacing a 4.6 mm I.D LC column with a 0.3 mm I.D capillary LC column) will reduce chromatographic band dilution and will, therefore, increase sensitivity when employing concentration sensitive detectors.[6] However, if a narrower column is used (using a standard pump→injector→column set up), the injection volume must be significantly smaller than what can be injected on to a regular column. To overcome the problem of potentially not being able to inject enough sample for analyte detection/quantification, a column switching system (on-line SPE-capLC,[7–10]) can be employed. The principle of column switching is that a weak mobile phase is used for transfer of the sample to an enrichment column. The mobile phase must be weak enough so that the analyte(s) are retained on the enrichment column. When a reversed phase system is used, solvent, salts, and less hydrophobic compounds, which are not retained, will pass through the enrichment column and to waste. When the entire sample has been loaded on to the enrichment column, the easily automated plumbing of the system is set so that a second mobile phase (from a second

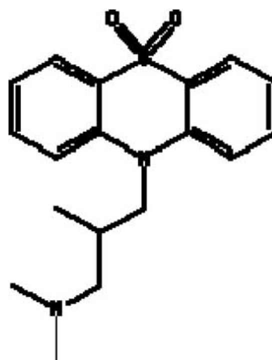

Figure 1. Structure of oxomemazine.

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pump) passes through the enrichment column in the backflush mode, and is strong enough to elute the analyte(s) off of the enrichment column and on to an analytical reversed phased column for chromatographic separation. In addition to being a tool for loading large injection volumes on to a narrow column, column switching also replaces time consuming off-line SPE steps.

The sensitivity will most often be further increased by replacing a UV detector with a mass spectrometer with an electrospray interface, which provides high sensitivity when operated with low flow rates, as those used with capillary LC.

Here, we describe a method for determining oxomemazine in plasma using capLC-MS. The decision to employ such instrumentation instead of conventional HPLC-UV instrumentation was based on expected improvements with regards to analysis time, selectivity, and sensitivity and these points are addressed in this paper. In order to avoid possible ion suppression during MS detection, the method includes a selective sample preparation based on ion-pairing and liquid–liquid extraction (LLE). To lower the demands on the operator, the method also includes a column switching system, and the performance and limitations of this system are described. The performance of the method is demonstrated by quantifying oxomemazine in plasma from a volunteer who had taken one dose of oxomemazine containing cough syrup 20 hours prior to sampling.

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EXPERIMENTAL

Chemicals, Buffers, and Materials

HPLC grade acetonitrile (ACN) was obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). Gradient quality water and reagent grade ammonium formate were obtained from Fluka (Buchs, Switzerland). Chloroform and 4-dimethylaminoazobenzene-4′-sulfonic acid (methyl orange) were purchased from Merck KGaA (Darmstadtt, Germany). Oxomemazine hydrochloride was purchased from Amriya For Pharmaceutical Industries (Alexandria-Egypt). Metronidazole was purchased from Sigma-Aldrich (Steinheim, Germany). Nitrogen (99.99%) was obtained from AGA (Oslo, Norway). Ammonium formate buffer pH 3.0 (0.05 M) was prepared as follows: 3.15 g of ammonium formate was dissolved in 950 mL water, pH was adjusted to value 3.0 ± 0.1 with formic acid (diluted with water in ratio 1:5), the buffer was diluted to 1000 mL with water and then was filtered through a 0.45 μm (HVLP, Germany) membrane filter.

Drug free human plasma was purchased from Ullevaal University Hospital (Oslo, Norway). Plasma samples from volunteers who had taken oxomemazine containing cough syrup were frozen within 1 h of collection, and kept frozen until analyzed.

All fused silica capillaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA). Zorbax C_{18} columns (0.3 mm I.D × 50 mm) were purchased from Agilent (Palo Alto, CA, USA). For preconcentration of the analytes, HotSep® Tracy trace enrichment (SPE) columns (G & T Septech, Kolbotn, Norway) were employed. The dimensions were 1 × 5 mm, and the columns were packed with 5 μm Kromasil C_{18} particles, with a pore size of 100 Å. The enrichment column's inner dimensions were a compromise between column capacity and system dead volumes. The stationary phase particle size of the enrichment column was larger than that of the analytical column to assist refocusing on the column and for minimizing back pressure. A particle size larger than 5 μm was not used due to concern that larger particles would not retain the analytes well enough during the enrichment stage.

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Chromatographic System

A Hitachi L-7110 isocratic LC pump from Merck (Darmstadt, Germany) was used for sample loading (pump 1 in Figure 1). An Agilent Series 1100 capillary gradient pump with an incorporated on-line vacuum degasser was used to deliver the mobile phase (pump 2 in Figure 1) providing back flushed desorption from the precolumn and elution on to the analytical column. The back flush mode is used to avoid analyte band broadening, which can occur if the analyte(s) pass through the entire enrichment column before entering the analytical column.

Elution of the analytes was conducted isocratically (30% ACN/70% 0.05 M ammonium formate buffer, v/v). The mobile phase was delivered at a constant flow of 5 μL/min through the analytical column. The sample loading solution consisted of ACN/0.05 M ammonium formate buffer (3/97, v/v). The loading flow rate was 200 μL/min. Valco Cheminert C2 six-port valves (Cotati, CA, USA) were used for manually injecting the samples and manual column switching. A schematic drawing of the system is presented in Figure 2.

The on-line solid phased extraction-liquid chromatography-mass spectrometry (SPE-LC-MS) system times were as follows: 100 μL of sample was injected, and loaded at a flow rate of 100 μL/minute. After 1.1 minutes, the SPE retained compounds were back flushed on to the analytical column. At the time of the void volume (2.5 minutes), valve 2 was switched to reequilibrate the enrichment column with loading solvent, which took only approximately 10 seconds. At the time where the analyte began to elute from the analytical column (3.1 minutes), a new sample could be loaded on to the SPE column (see Figure 3). Hence, immediately after completed data collection of one sample, data collection of the next sample could begin.

The analytical column was connected to a Micromass LCT TOF-MS (Micromass, Manchester, UK). The TOF-MS was equipped with a Z-spray

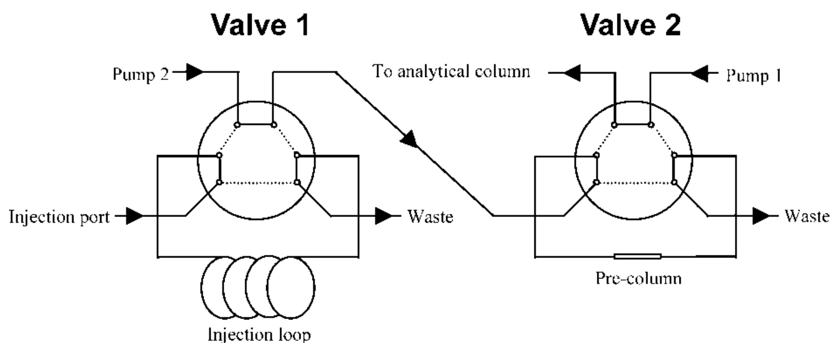

Figure 2. Scheme of the switching system employed.

atmospheric pressure ionization source for ESI, which was modified to handle flow rates in the low μL/min range. Ionization was performed in positive ion mode and oxomemazine was observed at $m/z = 331.34$ and metronidazole was observed at 172.44 ($[M+H]^+$ for both compounds). The following voltages were used: 3.2 kV on the capillary, 20 V on the sample cone, and

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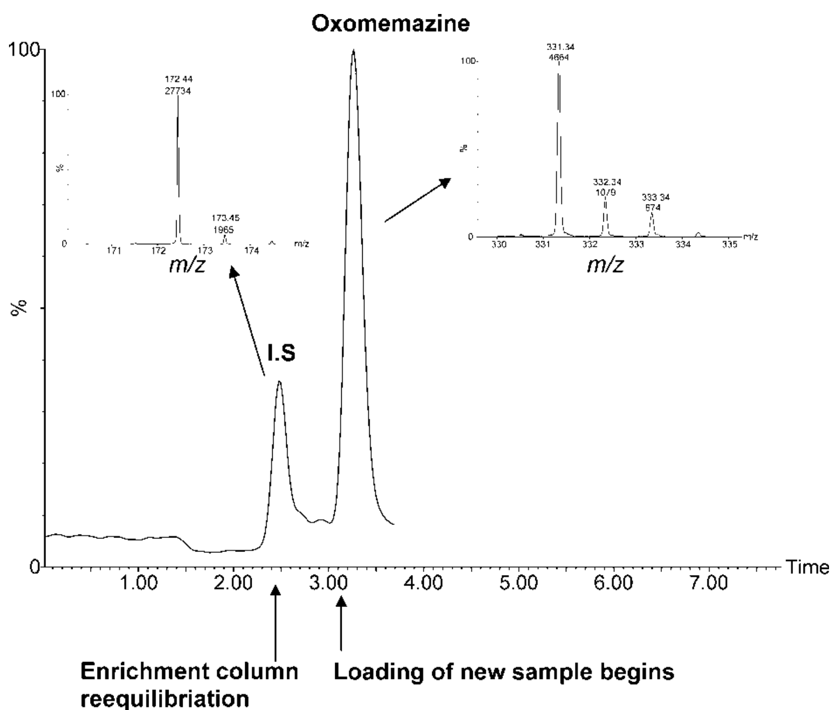

Figure 3. Chromatogram of oxomemazine (analyte) and metronidazole (I.S).

5 V on the extraction cone. In order to obtain a stable spray performance and aid solvent vaporization, nebulizer gas and desolvation gas were applied at 50 L/hour and 300 L/hour, respectively. The TOF-MS instrument was controlled by MassLynx v4.0 software, and mass spectra were acquired in the m/z range 100–1000. The instrument was calibrated with a sodium iodide and cesium iodide solution. For preliminary experiments, a Spectra 100 UV detector (Spectra Physics, Mountain View, CA, USA) modified for miniaturized LC was employed.

Ion suppression studies were conducted by chromatographing a blank plasma sample with the conditions described above, and mixing the eluent by a t-coupling with a 1 μg/mL standard solution of oxomemazine, pumped with a direct infusion pump at 5 μL/minute. The mixed eluent/analyte solution was introduced to the TOF-MS, and the analyte signal was monitored at $m/z = 331.34$.

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Preparation of Stock and Working Solutions

Stock solutions of 0.1 mg/mL were made by dissolving, respectively, 100 mg free base in 100 mL of methanol. Aqueous working solutions and calibration standards were prepared by appropriate dilution of the stock solutions with water. Spiked plasma samples (working, calibration, and validation samples) were prepared by adding diluted solutions of the compounds to drug free plasma in volumes not exceeding 10% of the plasma volume. Aqueous solutions of 10, 50, and 100 μg/mL of both internal standard and analyte were used for recovery studies with LC-UV instrumentation. Aqueous calibration solutions used with the column switching method were prepared to the levels 3, 15, 30, and 100 ng/mL of analyte. Plasma calibration samples at 3, 15, and 30 ng/mL concentration levels and validation plasma samples at 3, 6, 15, 20, and 30 ng/mL were used. All samples and solutions used with the switching system were spiked to 15 ng/mL of internal standard. The stock solutions and plasma samples were stored at −18°C, and the aqueous solutions were stored at +4°C.

Sample Preparation

Plasma (0.5 mL) spiked with oxomemazine (analyte) and metronidazole (I.S.) was mixed with 1 mL 0.1 mg/mL methyl orange (ion pair reagent) and 1 mL 0.05 M ammonium formate buffer. Chloroform of 5 mL was added and the solution was shaken for 5 minutes. The solution was removed and centrifuged for 10 minutes (10 krpm). The organic layer was evaporated with nitrogen, and the residue was dissolved in 1 mL of ammonium formate buffer. Of this solution, 100 μL was injected on to the column switching system.

Recovery and Accuracy

For the liquid–liquid extraction step, the recovery (%) of the target compounds was defined as: (The chromatographic peak areas of the peak areas of the aqueous calibration solutions subject to the extraction procedure/aqueous calibration solutions) $\times$ 100%.

For the column switching step, the recovery was defined as: (The peak areas injecting 5 μL of 10 μg/mL analyte + I.S. on to the switching system/the chromatographic peak areas injecting 5 μL of 10 μg/mL analyte + I.S. directly on to the analytical column) $\times$ 100%.

The total recovery of the method was defined as: (The chromatographic peak areas of plasma calibration samples/the peak areas of aqueous calibration solutions) $\times$ 100%.

The accuracy (%) of the (total) method was defined as the ratio of a known amount of analyte spiked to a plasma sample and the amount found, multiplied by 100%.

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Calibration Curves

The calibration curves were obtained by weighted linear regression, using the formula $(\text{Concentration}_{\text{analyte}}/\text{Concentration}_{\text{I.S}}) = \text{constant} \times (\text{Area}_{\text{analyte}}/\text{Area}_{\text{I.S}})$.

Limit of Quantification (cLOQ) and Limit of Detection (cLOD)

The cLOQ was defined as the analyte concentration that would produce a peak height of $\sim$10 times the intensity of the baseline noise. The cLOD was defined as the concentration that would provide a signal-to-noise ratio of $\sim$3.

RESULTS AND DISCUSSION

Sample Preparation

The use of methyl orange as an ion pair reagent for the extraction of target compounds was based on an earlier report using a similar approach in a spectrophotometric method for quantification of oxomemazine.[11] The peak areas of the compounds in solutions subjected/not subjected to the liquid–liquid extraction procedure were compared using a t-test, and the difference in mean was insignificant at $p = 0.05$ for all three concentration levels using UV-detection (see Experimental), implying a recovery of 100%. Without the use of ion pair reagent, the extraction efficiency was between 42 and 45% for the analyte and internal standard, illustrating that the ion pair formation was crucial to obtain high extraction efficiency.

The extraction recovery of the analyte and internal standard was 72 ± 5 and 91 ± 6%, respectively, when spiked plasma samples were ion pair extracted (n = 3, three levels).

Large Volume Injection SPE-capLC-MS

In preliminary experiments, conventional LC-UV instrumentation utilizing a 4.6 mm bore C_{18} column was employed and was intended to be used throughout the study. However, it was found that employing capillary columns (0.3 mm I.D) enhanced the sensitivity of the method by a factor of ~100, due to reduced radial dilution of the chromatographic band over the column. Although, it was feared that this approach could be less robust than a conventional HPLC system, the capillary LC system performance was satis factory. The back pressure over the 0.3 mm analytical column was 65 bar (±1 bar) during the entire study. The retention times of the internal standard and the analyte were stable throughout the study, at 2.71 (0.8% RSD) and 3.41 (0.56% RSD) for metronidazole and oxomemazine, respectively. The peak areas of the target compounds were unaltered by including a switching system, tested by injecting equal amounts on to the column directly or by a switching system (n = 3 for each system), and comparing the mean and deviations of the two sets by Student's t-test (p = 0.05). No reduction of chromatographic performance was observed when utilizing the switching system. The chromatographic performance and recovery of the target compounds were also unaffected by the loading flow rate, tested up to 200 μL/minute. Injecting volumes up to 100 μL did not affect the performance of the system or recovery, but for injection volumes of 150 μL, peak broadening was observed. Hence, 100 μL was considered to be the maximum allowable injection volume. The back pressure of the enrichment column during sample loading was stable at 32 (±1) bars throughout the study at a flow rate of 200 μL/minute.

When employing capillary LC with UV detection, a buffer/ACN gradient was employed to separate the internal standard from system void volume peaks and other early eluting compounds. This approach, although successful separation wise, required a reconditioning of the system for 7 minutes before another sample could be injected. When employing a TOF-MS, coelution issues were eliminated by monitoring single ion masses, allowing the use of isocratic elution, with no reconditioning step and no ion suppression was observed (see below).

With the on-line SPE-LC-MS system times described in Experimental, the target compounds can be subject to different eluting conditions depending on the sequence, since late eluting compounds from one injection may elute at the same time as the target compounds eluting from a second injection and possibly give ion suppression. However, analysis of a blank plasma sample when monitoring the signal of a constant supply of

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analyte (using a direct injection pump) showed no intensity drops in the analyte's extracted ion monitoring (EIM) signal during this experiment, showing that analyte suppression did not occur for plasma samples (Figure 4).

The TOF-MS did not require recalibration during the study, since the measured masses of the target compounds did not drift more than 0.01 Da.

Method Validation

The correlation coefficient of the analyte (r-squared) was 0.99 for aqueous calibration curves, plasma sample calibration curves, and plasma validation samples. For spiked plasma samples, the lower limit of quantification (the lowest concentration of spiked plasma standard employed) was ~3 ng/mL. The cLOD of the method was approximately 0.8 ng/mL.

For spiked levels of 3, 15, and 30 ng/mL, inter day variation (n = 6) for oxomemazine was 6.8, 11, and 7.4% RSD, respectively, and the intra day variation (n = 5) was 6.7, 11, and 12%, respectively. The accuracy at the three levels was 108, 108, and 99%, respectively.

The method was used to determine the amount of oxomemazine in a healthy female 20 hours after an intake of 1 teaspoon (approximately 1 mL) of the cough syrup Toplexil®, which contains 0.033 g oxomemazine per 100 mL syrup. Oxomemazine was detected (Figure 5), and the concentration was calculated to 2.0 ng/mL plasma.

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CONCLUSIONS

The on-line SPE-capillary LC-ESI-MS method described in this paper provided selective, fast, and sensitive determination of oxomemazine in

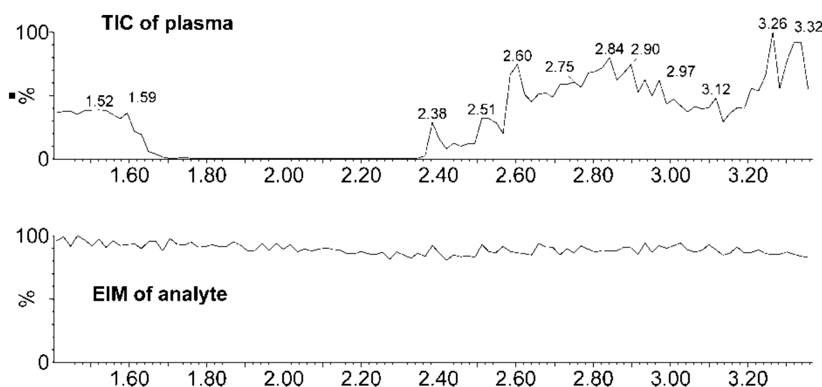

Figure 4. TIC of blank plasma and EIM of analyte recorded for ion suppression monitoring.

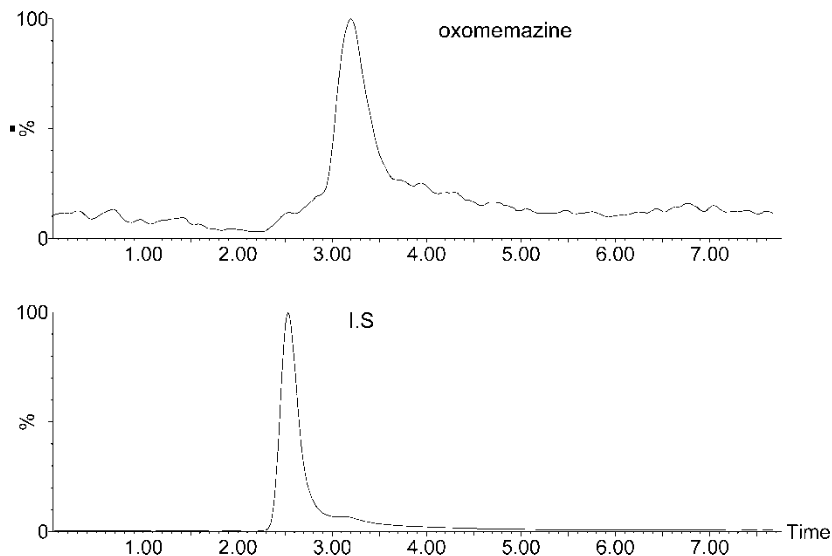

Figure 5. Oxomemazine detected in plasma of volunteer who took oxomemazine containing cough syrup 20 hours prior to sampling. The concentration was determined to be 2 ng/mL.

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human plasma with acceptable precision and accuracy. This easily automated system was found to be robust; the retention times and back pressures were constant throughout the study. This approach is, thus, an attractive alternative to off-line SPE procedures prior to LC injection. Miniaturizing the system for increased sensitivity was a useful step, since the level of oxomemazine in a volunteer's plasma 20 hours after exposure was as low as 2 ng/mL, and this concentration is almost impossible to determine employing a larger column diameter.

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