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Measurement of ethyl methanesulfonate in human plasma and breast milk samples using high-performance liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry☆

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ABSTRACT

Ethyl methanesulfonate (EMS) is a mesylate ester, which is known to be a potent mutagen, teratogen, and possibly carcinogen. Mesylate esters have been found in pharmaceuticals as contaminants formed during the manufacturing process and may potentially pose an exposure hazard to humans. We have developed and validated a method for detection of trace amounts (ng/ml levels) of EMS in human plasma and breast milk. The samples were extracted by matrix solid-phase dispersion with ethyl acetate using Hydromatrix™ and the ASE 200 Accelerated Solvent Extractor. The extracts were separated by high-performance liquid chromatography (HPLC) using a HILIC column. The detection was performed with a triple quadrupole mass spectrometer (TSQ Quantum Ultra, Thermo Electron Corporation) using atmospheric pressure chemical ionization in negative-ion mode and multiple reaction monitoring. The use of a surrogate internal standard in combination with HPLC–MS/MS provided a high degree of accuracy and precision. The extraction efficiency was greater than 70%. Repeated analyses of plasma and breast milk samples spiked with high (100 ng/ml), medium (50 ng/ml) and low (5 ng/ml) concentrations of the analytes gave relative standard deviations of less than 12%. The limits of detection were in the range of 0.5–0.9 ng/ml for both matrices.

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1. Introduction

To drug regulators, residual mesylate ester impurities in pharmaceuticals—including methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and isopropyl methanesulfonate (IPMS)—have been a significant safety concern [1–4]. The presence of these impurities in pharmaceutical products could be the result of reactions between methanesulfonic acid and alcohols, both of which are in the synthesis mixture [3–5].

In 2007, measurable levels of EMS were found in Nelfinavir mesylate—the active pharmaceutical ingredient of Viracept® [6,7]. Viracept® is a protease inhibitor, which effectively blocks reproduction of HIV. It is used in combination with other antiviral medicines to treat adults, adolescents, and children infected with HIV.

Ethyl methanesulfonate (EMS) has been used in a wide variety of biological test systems in studies of mutation effects [8–11]. Evidence of carcinogenicity in experimental animals indicates that EMS is a possible human carcinogen [11]. EMS induces DNA damage by a direct mechanism, acting at various sites as a monofunctional ethylating agent of nucleotides [6].

Analytical methods using gas chromatography–mass spectrometry (GC–MS) have been reported for the measurement of mesylate esters, a contaminant in pharmaceutical products. GC–MS procedures using single-ion monitoring were developed to characterize and determine MMS and EMS in bismesylate salt, used in the treatment of heart failure [1] and in imatinib mesylate, used for the treatment of gastrointestinal stromal sarcoma, a rare form of cancer [12]. Recently, a GC–MS method was developed for the trace analysis of EMS in Viracept® 250 mg tablets, with a reported limit of detection (LOD) of 5 μg/ml [13].

The accidental contamination of Viracept® with EMS is problematic. Exposure to EMS in HIV infected patients participating in a clinical trial and to their nursing infants could result in potentially dangerous side effects. To evaluate whether exposures occurred in the patients or their infants and whether any resulting health effects ensued, methods for measuring EMS in biological matrices were needed.

We have developed a simple and efficient sample preparation method to extract EMS from plasma and from breast milk.

☆ The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the Centers for Disease Control and Prevention.

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Our method employs extraction by matrix solid-phase dispersion with ethyl acetate followed by a highly selective and sensitive analysis using high-performance liquid chromatography with atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC/APCI-MS/MS).

2. Materials and methods

2.1. Chemicals

We purchased the native standard of ethyl methanesulfonate from Fisher Scientific (Pittsburgh, PA). Cambridge Isotope Laboratories (Andover, MA, USA) custom-synthesized the surrogate internal standard methamidophos-dimethyl-d6 (MMP-d6).

All solvents were of analytical grade. Ethyl acetate was purchased from Fisher Scientific. Acetonitrile was purchased from Tedia Company, Inc. (Fairfield, OH), Formic acid was purchased from Acros Organics (Morris Plains, NJ) and Ammonium acetate was purchased from Sigma–Aldrich (St. Louis, MO). Deionized water was organically and biologically purified by use of an Aqua Solutions water system (Jasper, GA).

2.2. Standard and internal standard preparation

A stock solution of the native EMS was prepared in acetonitrile to give concentrations ranging from 3 to 4 mg/ml and stored at −70 °C. Seven working standard solutions, covering a range of 10–8000 ng/ml, were prepared by performing serial dilutions in acetonitrile. The working standard solutions were stored at −20 °C. Seven calibration standards covering a range from 0.5 to 100 ng/ml were made by adding the working standard solutions to blank plasma (pooled heparinized plasma—Interstate Blood Bank, Memphis, TN) or blank breast milk (pooled breast milk—UC Berkeley, Berkeley, CA). The calibration standards were made fresh before each analytical run.

The internal standard stock solution (MMP-d6) was prepared by weighing approximately 1.5 mg of the internal standard into a 10-ml volumetric flask and dissolving in acetonitrile. The stock solution was stored at −70 °C. An internal standard working solution containing the labeled MMP was prepared at 800 ng/ml in acetonitrile and stored at −20 °C.

2.3. Quality control (QC) materials

The blank plasma or blank breast milk pool was diluted 1:3 in deionizer water, vortexed and divided into four pools. The first pool (QCL), the second pool (QCM), and the third pool (QCH) were spiked with the native standard stock solution to yield concentrations of 5, 50, and 100 ng/ml, respectively. The fourth pool was not spiked, but was used as blank working matrix material for calibration standards and blanks.

2.4. Sample preparation

To ensure homogeneity, samples were thawed and vortex-mixed. The plasma or breast milk sample (250 μl) was pipetted into a 10-ml Kimble® disposable glass conical centrifuge tube, and 750 μl of deionized water was added to the sample. To denature proteins present into the matrix, 10 μm of formic acid were added to the sample. To give a matrix concentration of 20 ng/ml, samples were spiked with 25 μl of the labeled internal standard working solution. The samples were then vortex-mixed and loaded into a screw-cap vial (23 mm × 85 mm, BGC vials) that contained 2.5 g of Hydromatrix (Varian, Inc., Walnut Creek, CA). The Hydromatrix with the adsorbed sample was vortex-mixed and placed into stainless steel extraction cells that contained a cellulose filter (Restek, Bellefonte, PA) placed at the cell bottom and packed with an additional 2.5 g of Hydromatrix. Two ml of ethyl acetate was added on top of the Hydromatrix in the extraction cell. A filter was added on top of the surface of the Hydromatrix and the extraction cell was capped. The extraction cells were placed on the accelerated solvent extractor ASE 200 (Dionex Corporation, Sunnyvale, CA). The analyte was statically extracted for 4 min (at 25 °C and 1500 psi) with ethyl acetate, and the eluate was collected in 60-ml collection vials (Restek, Bellefonte, PA). Each extraction used 40 ml of solvent. The samples were evaporated in a Turbovap LV (Zymark, Hopkinton, MA) at 35 °C and 10 psi of nitrogen until 100 μl of solvent remained. Samples were transferred to auto-injection vials.

2.5. Chromatography and mass spectrometry conditions

The analytes were separated by HPLC using Agilent 1100 Series autosampler and pump (Agilent, Santa Clara, CA). The column used was an Atlantis® HILIC Silica, 4.6 mm × 100 mm, 3.0 μm (Waters Corporation, Milford, MA). The analytes were separated with isocratic elution by using 93% of acetonitrile and 7% of 100 mM ammonium acetate in deionized water. The total run time was 6 minutes, the flow rate was 400 μl/min, and the injection volume was 5 μl. for the HPLC pump, the maximum pressure was set at 400 bar.

The TSQ Quantum Ultra-triple quadrupole mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) was operated in the multiple reactions monitoring (MRM) mode, with an APCI (atmospheric pressure chemical ionization) source. The run was divided into two distinct, timed segments: 0–4.30 min and 4.31–6.00 min. In the first segment (0–4.30 min), the source was operated in negative-ion mode. In the second segment (4.31–6 min), it was switched to positive mode. In the APCI source, the vaporizer temperature was set at 450 °C, the corona discharge was set at 4.0 kV, and the capillary temperature was set at 225 °C. The sheath and auxiliary nitrogen gas pressures were set to 17 and 5 psi, respectively, and collision gas pressure at 1.5 mTorr.

2.6. Method validation

2.6.1. Extraction efficiency

The extraction recovery of the method was determined at two concentrations, 20 and 100 ng/ml, by analyzing five blank working matrix samples spiked with the appropriate standard concentration. Five additional blank, unspiked working matrix samples were processed concurrently. Before the evaporation steps, the unspiked samples were then spiked with the appropriate native standard to serve as control samples representative of 100% recovery. In addition, to correct for instrument variation, all of the samples were spiked with a known amount of labeled internal standard. After evaporation until 100 μl solvent remained, the samples were analyzed. The recovery was calculated by comparing the responses of the blank plasma and breast milk samples spiked before extraction with the responses of the blank plasma and breast milk samples spiked after extraction.

2.6.2. Limits of detection

The LOD was defined as three times the standard deviation of the noise at zero concentration ($3S_0$), where S_0 was estimated as the y-intercept of a linear regression analysis of a plot of the standard deviation of the three lowest standards versus the expected concentration from 7 runs [14]. Furthermore, the LOD was compared with the results of the calibration standard samples and low-level spiked samples. This was to ensure that the calculated values agreed with the peak observed, and that a minimum signal-to-noise ratio of 3 was present at these low levels.

2.6.3. Accuracy

The accuracies, sometimes called relative recoveries, were calculated by spiking blank working matrix material samples at different concentrations and then calculating the concentration. A linear regression analysis was performed on a plot of the measured concentrations versus the expected concentrations. A slope of 1.00 was considered 100% accuracy.

2.6.4. Precision

The method precision was determined by calculating the relative standard deviations (RSDs) of repeat measurements of the QC materials at three different concentrations (5, 50, and 100 ng/ml). To determine the method RSDs for each analyte, at least 17 repeat measurements of QC materials were made.

3. Results and discussion

Preliminary experiments were conducted to find the best atmospheric pressure ionization (API) mass spectrometer source type that gave the highest sensitivity and best specificity for EMS at trace levels. The polar character of EMS suggested using an ESI (electrosprayionization) source type. The acid dissociation constants (pK_a) for EMS are below 1 and above 14; a buffer system that produces ions in solution would likely cause corrosion in the mass spectrometer ion source. Because of the corrosion problem, we tried using an atmospheric pressure chemical ionization (APCI) source. In APCI, the ions are formed in the source and not necessarily in the mobile phase. We found that for our analysis, APCI was quite acceptable.

In optimizing the mass spectrometry conditions, we first performed a full scan analysis in Q1MS in the negative-ion mode. The spectrum showed a precursor molecular ion at m/z 95, instead of the expected precursor molecular ion at m/z 123 $[M-H]^-$. The most abundant ion transition in MS/MS mode for m/z 95 was just one product ion with an m/z 80. From the EMS chemical structure, we concluded that the m/z 95 precursor ion was formed by the loss of the ethyl group, and the m/z 80 product ion was formed by the loss of the methyl group producing the sulfate ion—a common artifact in negative-ion mode mass spectrometry.

The precursor/product ion pairs, the collision off-set energy, the ion mode, and the timed segment for the target compound are summarized in Table 1. To improve selectivity of the analysis, we used the transition 95 → 80 as a quantification ion and the transition 95 → 95 as the confirmation ion. The organophosphate pesticide methamidophos (MMP) (Fig. 1b), labeled with six deuterium atoms, was used as a surrogate internal standard. Labeled MMP was chosen because it could be extracted by matrix, solid-phase dispersion along with EMS, and it could be separated on the same column as EMS. Fig. 2 shows a typical ion chromatogram of a plasma extract spiked with 100 ng/ml of the analyte sample.

Fig. 3 shows recoveries obtained at two different spiking levels for both matrices. Notably good recoveries were obtained for serum and breast milk, with a range between 72% and 97%. The difference in recovery efficiency between the higher (100 ng/ml) and the lower (20 ng/ml) concentration is automatically corrected by using the surrogate internal standard. In that way, variable extraction recoveries do not negatively affect the method's accuracy.

Over the entire range, calibration curves were linear in either serum or breast milk matrices. Fig. 4 shows a typical calibration curve for EMS quantification in breast milk. The slope average of a linear regression analysis of seven calibration standards in seven runs was calculated; the data are shown in Table 2. For both matrices the R^2 values were greater than 0.990; the accuracy of the method was greater than 99%. The calculation was based on a slope average of linear regression analyses of plots of spiked-sample calculated concentrations versus the expected concentration of the same samples from seven runs.

Analysis of fortified samples determined the accuracy and precision of the method, expressed as the relative standard deviation of repeated analyses. These analyses were performed at three different concentration levels (5, 50 and 100 ng/ml) in seventeen runs. For both matrices, the relative standard deviation of the quality control (QC) samples was below 12% (Table 2).

The LODs of the method for both matrices are also shown in Table 2. In plasma, the LOD for EMS was 0.548 ng/ml, and in breast milk was 0.861 ng/ml. The LOD calculations were based on seven runs of calibration curves. The LODs reported here for EMS in both matrices are lower in magnitude than are the LODs previously published for EMS methods in pharmaceutical products using gas chromatography–mass spectrometry [12,13]. The LODs for EMS methods in pharmaceutical products, including Viracept® tablets, were in the 0.3–5 μg/ml range. Over 3 months in the spring of 2007, the Viracept® tablets were contaminated by EMS in the manufacturing process at levels up to 0.055 mg/kg/day [6]. We can probably infer from the high level of contamination and the low magnitude

Table 1
The precursor and product ions, the collision energy, the ion mode and the timed segment for the native analyte and the labeled surrogate internal standard.

Analyte	Precursor → product	Collision energy (V)	Ion mode	Timed segment (min)
EMS-Q	95 → 80	21	–	0–4.30
EMS-C	95 → 95	1	–	0–4.30
d6-MMP	148 → 97	19	+	4.31–6.00

"Q", quantification ion. "C", confirmation ion. d6-MMP, Internal standard.

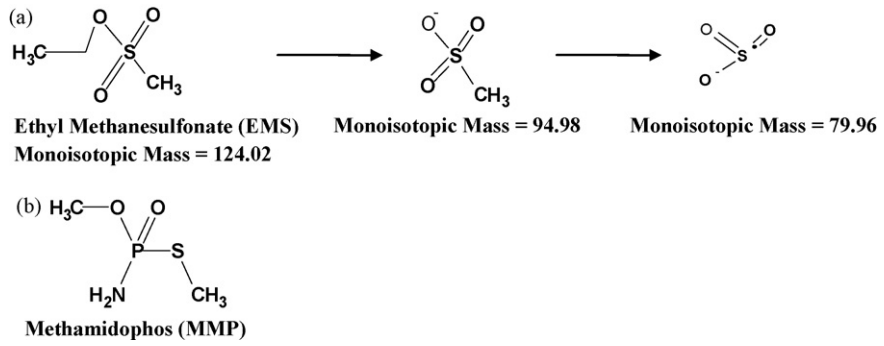

Fig. 1. (a) MS/MS fragmentation for EMS and (b) MMP chemical structure.

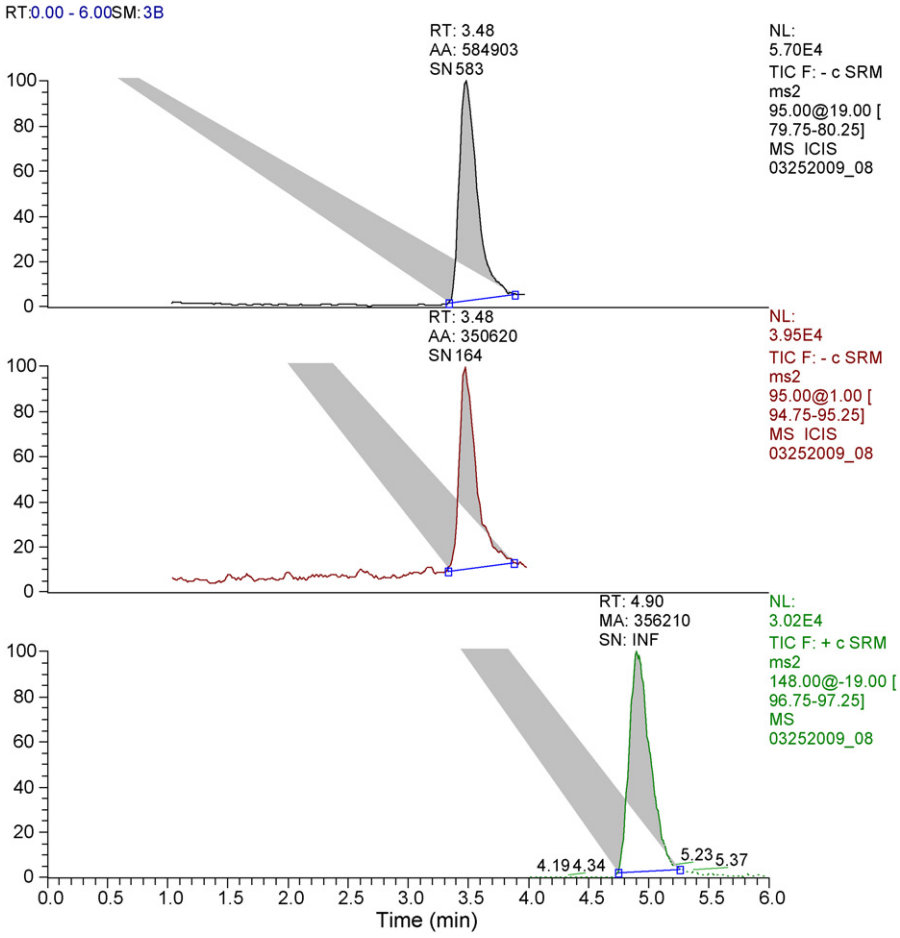

Fig. 2. A typical ion chromatogram of native (100 ng/ml) and isotopically labeled MMP (20 ng/ml) in spiked plasma.

Table 2
Summary of method specifications.

Matrix	LOD ng/ml (ppb)	Standard curve R^2	Accuracy (%)	QC values			RSD		
				QCL	QCM	QCH	QCL	QCM	QCH
Plasma	0.548	0.994	99.2	4.88	47.65	97.10	8.25	6.56	3.42
Breast milk	0.861	0.990	99.9	5.07	49.76	99.88	11.89	8.49	5.16

LOD: Calculated as $3S_0$. Standard deviation at zero concentration (S_0) was estimated as the y-intercept of a plot of the standard deviation of the three lowest calibration standards from 7 runs versus the expected concentration. Standard Curve: Slope average of a linear regression analysis of ten calibration standard from 7 runs. Accuracy: Expressed as the percentage of the expected concentration that was quantified from 7 runs. QC (Quality Control) values: Average of QCL (low), QCM (medium) and QCH (high) from 6 runs. Blank plasma or breast milk pools were spiked with the native standard stock solution to yield a concentration of 5 ng/ml (QCL), 50 ng/ml (QCM) and 100 ng/ml (QCH). RSD: Relative standard deviation of the QC values from 17 runs.

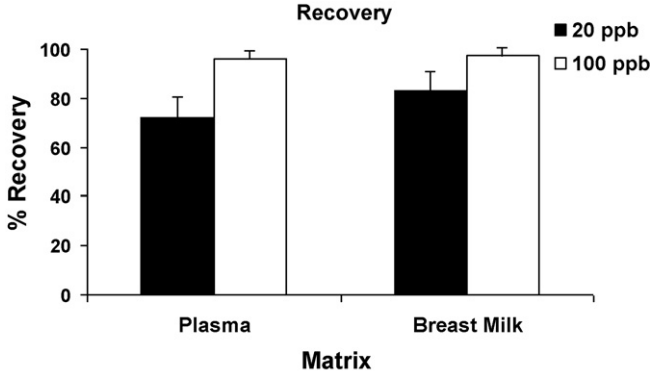

Fig. 3. Percentage of recovery of the analyte EMS in two concentrations of spiked plasma and breast milk after the standard clean-up procedure.

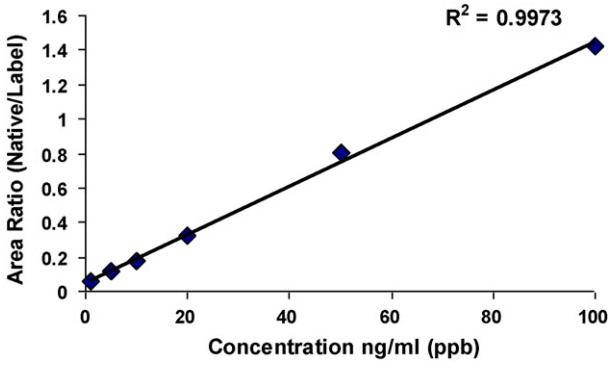

Fig. 4. A typical calibration curve for EMS quantification ion in breast milk matrix.

level of our LOD that we will have a satisfactory identification and quantification of exposure to the contaminant EMS measured in plasma or breast milk.

Insufficient information is available regarding the levels at which EMS are harmful to humans. Using linear dose response relations, estimates were that for humans, the adverse effects of EMS in Viracept® tablets would be very small [6,7]. We do not address in this paper the risk of adverse health effects to patients as a consequence of their exposure to EMS by taking Viracept®. Rather, we emphasize that this method is accurate, sensitive, and can be applied to epidemiological studies of possible health effects in HIV patients who were administered Viracept®.

Conflicts of interest

The authors declare no competing financial interests.

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