K40 Extraction of Selected Barbiturates, Primidone, and Phenytion From Blood Using Supported Liquid Extraction Columns With Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

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After attending this presentation, attendees will understand: (1) the process used for developing a new method for the simultaneous analysis of butalbital, pentobarbital, secobarbital, phenobarbital, primidone, and phenytoin extracted from blood; and, (2) the issues encountered during method validation.

This presentation will impact the forensic science community by developing a method for the extraction and analysis of selected barbiturates, primidone, and phenytoin by GC/MS. Three different extraction schemes were evaluated: Liquid-Liquid Extraction (LLE), Solid Phase Extraction (SPE), and Supported Liquid Extraction (SLE).

This study determined, based on peak area counts, chromatography, and the amount of sample used, that SLE was the best extraction scheme for all analytes of interest. In addition, the SLE columns sped up the extraction process, used less solvents, and used less sample volume when compared to other more traditional extraction schemes (SPE and LLE). The deuterated internal standards used consisted of butalbital-d5, secobarbital-d5, phenobarbital-d5, and phenytoin-d10. Due to either isotopic impurity of the deuterated analog and/or fragmentation characteristics of the parent compound, the secobarbital internal standard was eliminated from this method. The linear dynamic range of each analyte was determined and the calibration curve of each analyte spans this range.

Five hundred microliters of blood containing an internal standard was pretreated with 350µL of 0.1% formic acid and 0.2% ammonium formate in water, then loaded onto the supported liquid extraction columns. A pulse of positive pressure was used to initiate flow, and the sample was allowed to absorb onto the column for five minutes. The sample was then eluted with 2.5mL of dichloromethane and allowed to flow under gravity for five minutes before adding an additional 2.5mL of dichloromethane. The extraction was completed with a short burst of positive pressure. The extraction solvent was evaporated to dryness and the extract was reconstituted in 25µL of ethyl acetate and 25µL of MethElute Trimethylanilinium Hydroxide (TMAH). The reconstituted extract was heated at 70°C for ten minutes, cooled to room temperature, then transferred to auto sampler vials containing inserts. The analysis was performed on a Shimadzu® GC-2010 equipped with an AOC-20i auto injector and fitted with a DB-5MS column (30m x 0.25mm i.d., 0.25µm film thickness). The gas chromatograph was interfaced with a Shimadzu® GC-MS-QP2010S mass selective detector. The injection port and interface temperatures were 250.0°C and 280.0°C, respectively. The oven temperature program was as follows: initial temperature of 95.0°C for one minute and then ramped at 25.0°C per minute to 265.0°C and held for 2.5 minutes.

Method validation followed the Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. Bias and precision, calibration model, carryover, interference studies, Limit Of Detection (LOD), Limit Of Quantitation (LOQ), dilution integrity, and stability were documented during the validation experiments. Total bias was determined as a mean bias from the target value at three different concentration levels (differing in concentration from calibrator concentrations) over five runs. Total precision was determined as the mean Coefficient of Variation (CV) at three different concentration levels over five runs. The calibration model was determined by the analysis of five replicates each analyzed over five days at six different non-zero calibrator concentrations for each analyte. The data was analyzed using residual plots and the calibration model was determined to be a weighted least squares model. Eight multi analyte interference mixes containing more than 90 compounds at a concentration of 1,000ng/mL were analyzed by the method and no interference was observed. No carryover above the LOD of each analyte was observed at 30,000ng/mL. Additional validation data is summarized in the following table.

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Analyte	Avg (%) Bias	Avg %CV	LOD/LLOQ ng/mL	Cal Range ng/mL
Butalbital	0.4	2	200	200-10,000
Pentobarbital	-1.5	3	100	100-5,000
Secobarbital	0.2	8	100	100-2,500
Phenobarbital	-0.2	2	100	200-10,000
Primidone	-0.9	4	500	500-10,000
Phenytoin	-0.8	2	100	200-10,000

Supported Liquid Extraction, Barbiturates, Validation

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