thermoscientific

Quantitative analysis of 40 Fentanyl, its Precursors, Analogues and Metabolites in Urine, Oral fluids and Blood using LC-MS/MS for forensic use TP189

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ABSTRACT

Introduction: Fentanyl and its analogues have become of considerable interest recently due to their increased potency, increased risk of abuse and their potential for life-threatening harm. They are synthetic opioids that are used for pain medication and anesthesia and in this study over 30 of the fentanyl analogues, their metabolites and isoforms were evaluated. A sensitive, robust and specific LC-MS/MS analytical method was developed for the quantitation of the fentanyl analogues, their metabolites and isoforms using simple sample preparation techniques in urine, oral fluid and blood. The method was developed and optimized for accurate and robust analysis of these drugs while demonstrating the analytical issues associated with investigating multiple compounds of subtle varying structures and physicochemical properties.

Methods: A Thermo Scientific™ TSQ Quantis™ tandem mass spectrometer in positive Electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 100 µl of urine, oral fluid and blood were used and various columns and mobile phases were evaluated and initially a Thermo Scientific™ Accucore™ C18 50 x 2.1 mm, 2.6 µm column with a water:methanol mixture containing 0.01% formic acid and 2.5 mM ammonium formate gradient was used achieving baseline chromatographic separation in 10 minute run times. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive mode. The accuracy of these methods were verified using reference materials from UTAK.

Preliminary Data: Good linearity and reproducibility were obtained with the concentration range from 10 pg/ml to 1000 ng/ml for the various fentanyl analogues, precursors and metabolites with a coefficient of determination R2>0.98 or better for all drugs in the various matrices over multiples batches. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined for each compound and the method could easily achieve the pg/ml levels of detection required in all matrices as well as baseline separation of the various isoforms analyzed. Excellent reproducibility of precision and accuracy was observed (CV < 10%) for all compounds in all matrices across all calibration levels and QC material. A sensitive, simple, specific and accurate LC-MS/MS method was developed and verified for the simultaneous measurement of fentanyl, its analogues and metabolites. The sample preparation techniques are quick and easily applied for high throughput analysis in urine, oral fluids and blood for forensic toxicology and included dilute and shoot, protein precipitation and liquid-liquid extraction. The analytical method achieved the best and most sensitive results possible for the analysis of these new and expanding compounds that have subtle structurally differences and demonstrated the versatility of the instrument to consistently quantitate drugs at pg/ml levels.

INTRODUCTION

Fentanyl is an opioid that has rapid onset and effects and can be injected, absorbed through a skin patch, or mouth tissues. It activates the μ-opioid receptors and depending on the analogue may be as much as 10,000 times more potent than morphine. Fentanyl can have serious side effects which have results in many deaths.

In this research study, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of Fentanyl, Its Analogues, Precursors and metabolites. The sample preparation choices were kept simple and included protein precipitation for serum, dilution for urine and oral fluid.. The methodologies were developed on a TSQ Quantis tandem mass spectrometer in positive and negative Electrospray ionization modes with a Vanquish HPLC system for a 10 minute analytical gradient.

MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from Cerilliant, Inc. (Round Rock, TX)

Alfentanil		Isobutyryl Fentanyl	
Acetyl Fentanyl	Acetyl Fentanyl-13C6	cis-3-Methyl Fentanyl	
Acetyl Norfentanyl	Acetyl Norfentanyl-13C6	Norfentanyl	Norfentanyl-D5
4-ANPP	4-ANPP-D5	Norcarfentanil	
Acryl Fentanyl		Ocfentanil	
Butyryl Fentanyl		Remifentanil	
Carfentanil	Carfentnail-D5	Remifentanil Acid	
Cyclopropyl Fentanyl		Sufentanil	Sufentanil-D5
Fentanyl	Fentanyl-D5	Valeryl Fentanyl	Valeryl Fentanyl-D5
Furanyl Fentanyl			
para-Fluorobutyryl Fentanyl			
para-Fluorofentanyl			
4-Fluoro-isobutyryl Fentanyl			
β-Hydroxythiofentanyl			
Methoxyacetyl Fentanyl		N-Methyl Cyclopropyl Fentanyl	
N-Methyl Norcarfentanil		Cyclopropyl Norfentanyl	
Cis-3-Methyl Norfentanyl		Despropionyl Meta-Fluorofentanyl	
Despropionyl Ortho-Fluorofentanyl		Despropionyl Para-Fluorofentanyl	
Norsufentnail	Norsufentanil-D3	Despropionyl 2-Fluoro-Ortho-Fluorofentanyl	
Despropionyl Ortho-Methylfentanyl		Butyryl Norfentanyl	
4-Anilino-1-Benzylpiperidine			

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used-

HPLC grade Water	Formic Acid
Methanol	Ammonium Formate
Acetonitrile	

The standards and internal standards were made up in Methanol.

Sample Preparation- Urine Dilution

- 100 mL of urine sample, calibrators, controls were added to 1.5 ml eppendorf tubes and 10 mL of Fentanyl ISTD at 100 ng/mL were added to each tube and vortexed briefly
- 890 mL of HPLC grade water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators were prepared in drug-free urine (Golden West Biological, Inc, Temecula, CA)

Sample Preparation- Oral Fluid Dilution

- 100 mL of oral fluid sample (50 mL of oral fluid and 50 mL of buffer), calibrators, controls were added to 1.5 ml eppendorf tube and 5.0 mL of Fentanyl ISTD at 1000ng/mL were added to each and vortexed briefly
- 395 mL of HPLC grade water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators and controls were prepared in negative oral fluid (Oral-Eze™ -Thermo Fisher Scientific)

Sample Preparation- Blood Protein crash

- 100 mL of blood sample, calibrators, controls added to 1.5 ml eppendorf tubes and 10 mL of Fentanyl ISTD at 100 ng/mL were added to each and vortexed briefly
- 100 mL of HPLC grade Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators were prepared in drug-free blood (Golden West Biological, Inc, Temecula, CA)

Data Analysis

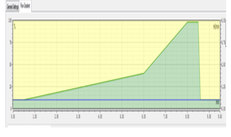
The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Altis Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

METHOD

HPLC Conditions-

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 50 x 2.1 mm, 2.6 µm
Column Temperature:	50 °C
Injection Volume:	5 µL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.01% Formic Acid + 2.5mM Ammonium Formate
Mobile Phase B:	Methanol
Flow Rate:	0.5 ml/min
Gradient:	0.0 min- 90%A:10%B
	0.5 min- 90%A:10%B
	6.0 min- 40%A:60%B
	8.0 min- 2%A:98%B
	8.5 min- 2%A:98%B
	8.6 min- 90%A:10%B
Run time:	10 mins



MS and Ion Source Conditions

TSQ Quantis triple quadrupole mass spectrometer

Ion mode:	Positive Electrospray (H-ESI) Mode
Vaporizer Temperature:	475 °C
Ion Transfer Tube Temperature:	300 °C
Sheath Gas:	70
Aux Gas:	10
Sweep Gas:	0
Spray Voltage:	Positive Ion (V):500 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle time (sec):	0.6
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
NPP	0.55	204.16	77.1/105	45/22	170
4-Anilinopiperidine	0.62	177.08	55/84.1	28/12	93
Methoxyacetyl Fentanyl	0.73	249.11	55/84.1	34/16	124
Acetyl Norfentanyl	0.83	219.16	55.1/84.1	33/18	126
Norfentanyl	2.41	233.18	55/84.1	34/18	128
Furanyl Norfentanyl	2.58	271.12	55.1/84.1	35/16	134
N-Methyl Cyclopropyl Norfentanyl	3.01	259.11	70.1/98	29/20	134
N-Methyl Norcarfentanil	3.12	305.13	245.1/273.2	15/10	119
Cyclopropyl Norfentanyl	3.19	245.16	84.1/177.1	18/17	131
Cis-3-Methyl Norfentanyl	3.22	247.14	69.1/98	29/18	132
Norcarfentanil	3.29	291.18	231.1/259.1	16/10	124
Remifentanil Acid	3.68	363.12	113/331.1	29/13	167
Remifentanil	3.80	377.12	113/317.1	30/17	177
Butyryl Norfentanyl	3.88	247.16	84.1/177.1	18/16	133
4-Anilino-1-Benzylpiperidine	3.98	267.21	90.9/174.1	31/16	132
Acetyl Fentanyl	4.20	323.11	105/188.1	36/23	169
β-Hydroxythiofentanyl	4.31	359.18	192/341.1	23/17	175
Ocfentanil	4.37	371.13	105/188.1	38/23	198
4-ANPP	4.47	281.16	105/188.2	31/17	143
Despropionyl m-Fluorofentanyl	4.80	299.14	105/188.1	31/18	152
Despropionyl o-Fluorofentanyl	5.02	299.16	105/188.1	31/18	146
Despropionyl p-Flourofentanyl	5.10	299.18	105/188.1	31/18	149
Norsufentanil	4.75	277.16	96/128.1	22/14	122
Acryl Fentanyl	5.10	335.12	105/188.1	36/23	188
Fentanyl	5.33	337.25	105/188.1	37/23	190
Despropionyl 2-Fluoro-o-Fluorfentanyl	5.42	317.21	123/206.1	32/18	150
p-Fluorofentanyl	5.48	355.11	105/188.1	38/24	200
Furanyl Fentanyl	5.56	375.19	105/188.1	38/22	197
Despropionyl o-Methylfentanyl	5.59	295.13	105/188.2	32/17	142
Isobutyryl Fentanyl	6.27	351.11	105/188.1	38/24	195
Cis-3-Methyl Fentanyl	6.09	351.16	105/202.1	37/25	205
Butyryl Fentanyl	6.39	351.25	105/188.1	38/24	197
Cyclopropyl Fentanyl	5.86	349.17	105/188.1	38/24	206
Carfentanil	5.95	395.26	113/363.2	31/14	184
4-Fluoro-Isobutyryl Fentanyl	6.40	369.11	105/188.1	39/25	204
p-Fluorobutyryl Fentanyl	6.53	369.13	105/188.1	39/25	201
Alfentanil	6.38	417.28	197.2/268.2	26/18	185
Sufentanil	6.88	387.13	110.9/238.1	37/20	178
Valeryl Fentanyl	7.2	365.16	105/188.1	39/25	204

RESULTS

Linearity/Sensitivity

The linear range of the 40 Fentanyls, its analogues, precursors and metabolites was from 1 to 500 ng/ml for Urine, Oral Fluid and Blood. The linearity of each matrix was determined in triplicate over 3 days and the results are shown with LOD and LOQ being determined as 3:1 and 10:1 of signal to noise respectively where possible and the mean coefficient of determination (R2) > 0.99 for each matrix and the %CV for each calibration point were all <10%.

Accuracy

The accuracy was determined by the analysis of UTAK control material as the percentage deviation from the targeted mean and the results were <10% for all levels in each matrix. Therefore, the analytical method can achieve the required accuracy for the analysis of the Fentanyls, its analogues, precursors and metabolites in urine, oral fluid and blood.

Precision/Specificity

The intra–assay precision (%CV) of the Fentanyls in each matrix were determined by extracting and quantifying three replicates of the pooled sample control material. The inter-assay precision was determined over 3 consecutive days and was found to have a %CV <10% for each Fentanyl for the three levels of pooled sample control material respectively in urine, oral fluid and blood. Therefore, the analytical method can achieve the required precision for the analysis of the Fentanyls in urine, oral fluid and blood.

Figure 1: Chromatograms

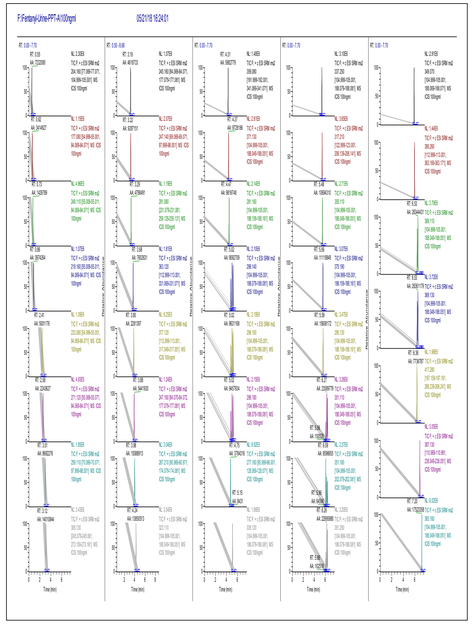


Table 2- Sensitivity for the extractions (pg/ml)

Compound	LOQ Urine	LOQ Oral	LOQ Blood
NPP	25	25	10
4-Anilinopiperidine	50	25	10
Methoxyacetyl Fentanyl	50	25	25
Acetyl Norfentanyl	50	25	5
Norfentanyl	25	10	10
Furanyl Norfentanyl	25	10	25
N-Methyl Cyclopropyl Norfentanyl	50	50	10
N-Methyl Norcarfentanil	250	100	100
Cyclopropyl Norfentanyl	100	50	25
Cis-3-Methyl Fentanyl	50	50	25
Norcarfentanil	50	50	50
Remifentanil Acid	250	100	25
Remifentanil	250	100	100
Butyryl Norfentanyl	50	25	50
4-Anilino-4-piperidine	25	25	50
Acetyl Fentanyl	50	50	50
B-Hydroxythiofentanyl	50	100	100
Ocfentanil	25	50	50
4-ANPP	25	25	25
Norsufentanil	50	25	25
Despropionyl-p-Fluorofentanyl	50	25	25
Despropionyl-o-Fluorofentanyl	100	100	50
Acryl Fentanyl	100	50	50
Despropionyl-m-Fluorofentanyl	50	25	25
Fentanyl	50	25	25
Despropionyl-2-Fluoro-o-Fluorofentanyl	50	50	50
p-Fluorofentanyl	50	50	50
Furanyl Fentanyl	100	50	50
Despropionyl-o-Methyfentanyl	50	25	50
Cyclopropyl Fentanyl	50	50	50
Carfentanil	50	100	100
Cis-3-Methylffentanyl	50	50	100
Alfentanil	50	25	50
4-Fluoro-Isobutyryl Fentanyl	50	25	25
Butyryl Fentanyl	50	25	25
Isobutyryl Fentanyl	25	25	25
p-Fluorobutyryl Fentanyl	50	25	25
Sufentanil	25	25	10
Valeryl Fentanyl	25	10	5

CONCLUSIONS

- Baseline separation of 40 Fentanyls, its analogues, precursors and metabolites in 10 minutes with good LOQ in positive mode in three matrix types.
- Excellent linearity of calibration curves with good accuracy, precision and reproducibility across the calibration curve from 50 pg/ml to 500 ng/ml for each respective fentanyl
- Future work is needed to eliminate potential matrix or drug interferences to this analytical method, and for additional synthetics to be included when they are discovered, and standards made

For Forensic Use Only.

REFERENCES

1. A comprehensive LC-MS-based quantitative analysis of fentanyl-like drugs in plasma and urine Journal of Separation Science Volume33, Issue17-18 Pages 2654-2662
2. LC-MS/MS-Based Method for the Multiplex Detection of 24 Fentanyl Analogues and Metabolites in Whole Blood at Sub ng mL–1 Concentrations ACS Omega, 2018, 3 (1), pp 514–523

TRADEMARKS/LICENSING

thermoscientific

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Methods: A Thermo Scientific™ TSQ Quantis™ tandem mass spectrometer in positive Electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 100 µl of urine, oral fluid and blood were used and various columns and mobile phases were evaluated and initially a Thermo Fisher™ Accucore™ C18 50 x 2.1 mm, 2.6 µm column with a water:methanol mixture containing 0.01% formic acid and 2.5 mM ammonium formate gradient was used achieving baseline chromatographic separation in 10 minute run times. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive mode. The accuracy of these methods were verified using reference materials from UTAK.

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INTRODUCTION

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MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from-

Cerilliant, Inc, Round Rock, TX-

Alfentanil		Isobutyryl Fentanyl	
Acetyl Fentanyl	Acetyl Fentanyl-D5	cis-3-Methyl Fentanyl	
Acetyl Norfentanyl	Acetyl Norfentanyl-13C6	Norfentanyl	Norfentanyl-D5
4-ANPP	4-ANPP-D5	Norcarfentanil	
Acryl Fentanyl		Ocfentanil	
Butyryl Fentanyl		Remifentanil	
Carfentanil	Carfentanil-D5	Remifentanil Acid	
Cyclopropyl Fentanyl		Sufentanil	Sufentanil-D5
Fentanyl	Fentanyl-D5	Valeryl Fentanyl	Valeryl Fentanyl-D5
Furanyl Fentanyl		para-Fluorobutyryl Fentanyl	
para-Fluorofentanyl		4-Fluoro-isobutyryl Fentanyl	
β-Hydroxythiofentanyl			

Cayman Chemicals, Inc., Ann Arbor, MI-

NPP	4-Anilinopiperidine

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used-

HPLC grade Water	Acetic Acid
Methanol	7N Ammonia in Methanol
Acetonitrile	Ethyl Acetate
Hydrogen chloride - 1-butanol solution	

The standards and internal standards were made up in 0.1N Ammonia in Methanol to prevent iodine migration.

Total Sample Preparation- Protein Crash

- 200 µL of Serum/HSA mixture calibrators, controls and serum sample were added to 1.5 ml Eppendorf tubes and 20 µL of Thyroid ISTD mixture at 200 ng/mL were added to each tube and vortexed briefly
- 400 µL of Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators were prepared in thyroid depleted serum and water (Golden West Biological, Inc, Temecula, CA)

Total Sample Preparation- Liquid-Liquid Extraction

- 200 µL of Serum/HSA mixture calibrators, controls and serum samples were added to a test tube and 20 µL of Thyroid ISTD mixture at 200 ng/mL were added to each and vortexed briefly
- 200 µL of Acetonitrile was added to each tube and vortexed for 1 min
- 1.2 mL of Ethyl Acetate was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature
- The extract was reconstituted in 200 µL of 3:1 water and methanol
- The supernatant was transferred to an MS vial and capped.

Free Sample Preparation- Ultracentrifugation

- 400 µl of serum sample, calibrator matrix, controls was added to a Millipore Amicon Ultra 0.5 ml, Ultracel 10 membrane, 10 KDa Centrifugal Filter unit prior to centrifugation for 60 min at 13200 rpm at 37C..
- 200 µl of the filtrate was removed to a new tube and 20 µl ISTD at 1000 pg/ml were added to each tube and the calibrators were spiked with standards to the desired concentration and vortexed briefly
- The extraction process was continued as described above for the Total sample preparation- Protein crash and Liquid-Liquid Extraction protocol.

Derivatization Sample Preparation-Butyl Esterification

- The dried extracts produced following liquid-liquid extraction were derivatized using 50 µL of Butanol in 3M HCl for 15 minutes at 65˚C
- Due to the acidity of the reagent, the liquid was removed by heated nitrogen flow at 40˚C.
- The samples were reconstituted in 200 µL of 3:1 water and methanol
- The supernatant was transferred to an MS vial and capped.

The calibration curves ranged from 1 pg/mL to 1000 ng/mL and various pooled samples were used as control material.

Data Analysis

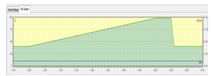
The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Altis Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

METHOD

HPLC Conditions-

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 50 x 2.1 mm, 2.6 µm
Column Temperature:	50 °C
Injection Volume:	20 µL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.1% Acetic Acid
Mobile Phase B:	Methanol
Flow Rate:	0.5 ml/min
Gradient:	0.0 min- 60%A:40%B
	0.5 min- 60%A:40%B
	4.5 min- 2%A:98%B
	5.0 min- 2%A:98%B
	5.1 min- 60%A:40%B
Run time:	6 mins



MS and Ion Source Conditions-Underivatized

TSQ Altis triple quadrupole mass spectrometer

Ion mode:	Positive and Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	300 C
Ion Transfer Tube Temperature:	275 C
Sheath Gas:	42
Aux Gas:	15
Sweep Gas:	0
Spray Voltage:	Positive Ion (V):3925 V Negative Ion (V):3750 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle time (sec):	0.8
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

MS and Ion Source Conditions-Derivatized

TSQ Altis triple quadrupole mass spectrometer

Ion mode:	Positive and Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	300 ˚C
Ion Transfer Tube Temperature:	275 ˚C
Sheath Gas:	42
Aux Gas:	15
Sweep Gas:	0
Spray Voltage:	Positive Ion (V):2000 V Negative Ion (V):2500 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle time (sec):	0.8
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
3,3',5-Triiodothyronine-T3	3.31	Positive	651.84	605.7/478.9	21.9/34.4	99
		Negative	649.84	126.9/632.8	45.3/19.1	108
3,3',5-Triiodothyronine-13C6	3.31	Positive	657.67	611.7/484.9	22.1/34.4	99
		Negative	655.84	126.9//638.8	45.1/19.4	111
3,3',5'-Triiodothyronine-rT3	3.63	Positive	651.83	605.8/507.8	22.8/23.8	103
		Negative	649.98	126.9/478.9	51.9/22.1	130
3,3',5'-Triiodothyronine-13C6	3.63	Positive	657.8	611.8/513.9	22.7/37.2	104
		Negative	655.8	126.9/484.9	54.9/22.4	125
Thyroxine-T4	3.82	Positive	777.74	731.7/604.8	24.8/39.2	116
		Negative	775.31	126.9/604.8	47.3/20.3	140
Thyroxine-13C6	3.82	Positive	783.77	737.4/610.8	24.7/39.2	116
		Negative	781.54	126.9/610.9	46.1/20.6	135
3,3',5-Triiodothyronine-T3-Butyl	2.83	Positive	707.83	605.8/478.9	24.3/38.3	102
		Negative	705.79	126.9/448.7	41.9/26.2	110
3,3',5-Triiodothyronine-13C6-Butyl	2.83	Positive	713.83	611.8/484.9	18.4/38.4	98
		Negative	711.83	126.9/454.8	42.9/26.2	115
3,3',5'-Triiodothyronine-rT3-Butyl	3.16	Positive	707.85	605.8/507.9	24.4/24.6	98
		Negative	705.83	126.9/575.7	55/31.6	128
3,3',5'-Triiodothyronine-13C6-Butyl	3.16	Positive	713.85	611.8/513.9	23.9/25.1	96
		Negative	711.85	126.9/581.7	53.1/31.7	112
Thyroxine-T4-Butyl	3.17	Positive	833.75	777.8/731.8	19.8/27.3	132
		Negative	831.71	126.9/574.8	47.9/31.5	122
Thyroxine-13C6-Butyl	3.17	Positive	839..75	783.8/737.8	20.1/26.2	109
		Negative	837.75	126.9/580.8	52.8/31.4	120

RESULTS

Linearity/Sensitivity

The assays were linear over the calibration curve for the total and free Thyroids in thyroid depleted serum/water mixture as shown in the table with their mean of coefficient of determinations (R^2) for positive and negative mode and all sample preparation techniques. The linearity of each extraction was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R^2) > 98 for each sample extraction technique and the %CV for each calibration point were all <10% in order to be accepted. The analysis of total and free Thyroid hormones underivatized and derivatized by positive mode electrospray using the LC and source conditions shown were found to be more sensitive than negative mode with a 5 to 10 fold difference in mass spectral response.

Precision/Specificity

The inter–assay precision and accuracy for total and free T4, rT3 and T3 was determined by extracting and quantifying in-house control material resulting in %CV for T4, rT3, T3 of <10% deviation form the targeted mean.

Therefore, the analytical method was determined to work best for the liquid-liquid extraction in positive mode for total and free thyroid hormones and butyl derivatization of these compounds resulted in a further 10 fold increase in sensitivity. The use of either analytical technique can achieve the laboratory required accuracy for the analysis of total and free thyroid hormones in serum.

Figure 1: Chromatograms Underivatized, derivatized, negative and positive mode

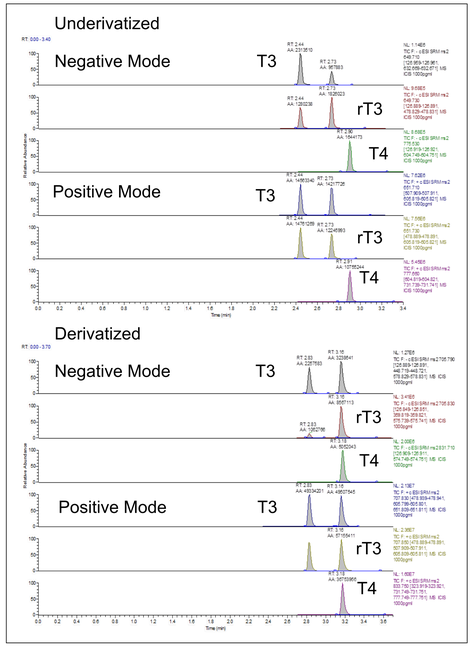


Table 2- Linearity and Sensitivity for the extraction methodology-PPT/LLE

Compound	Linearity Total	LOQ (pg/ml)	Linearity Free	LOQ (pg/ml)
3,3',5-Triiodothyronine-PPT	5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10	5 - 1000 pg/ml (+) 10 – 1000 pg/ml (-)	2.5 10
3,3',5'-Triiodothyronine-PPT	5 pg/ml – 1000 ng/ml (+) 25 pg/ml – 1000 ng/ml (-)	5 25	5 – 1000 pg/ml (+) 25 – 1000 pg/ml (-)	5 25
Thyroxine-PPT	5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10	5 – 1000 pg/ml (+) 10 – 1000 pg/ml (-)	2.5 5
3,3',5-Triiodothyronine-LLE-No	1 pg/ml -1000 ng/ml (+) 5 pg/ml -1000 ng/ml (-)	1 5	1 – 1000 pg/ml (+) 5 – 1000 pg/ml (-)	1 5
3,3',5'-Triiodothyronine-No	2.5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10	2.5 – 1000 pg/ml (+) 10 – 1000 pg/ml (-)	2.5 10
Thyroxine-LLE-No	1 pg/ml – 1000 ng/ml (+) 5 pg/ml – 1000 ng/ml (-)	1 5	1 – 1000 pg/ml (+) 5 – 1000 pg/ml (-)	1 5
3,3',5-Triiodothyronine-LLE-Butyl	0.25 pg/ml – 1000 ng/ml (+) 2.5 pg/ml – 1000 ng/ml (-)	0.5 5	0.25 – 1000 pg/ml (+) 2.5 – 1000 pg/ml (-)	0.25 2.5
3,3',5'-Triiodothyronine-LLE-Butyl	0.5 pg/ml – 1000 ng/ml (+) 5 pg/ml – 1000 ng/ml (-)	1 5	0.5 – 1000 pg/ml (+) 5 – 1000 pg/ml (-)	0.5 5
Thyroxine-LLE-Butyl	0.25 pg/ml – 1000 ng/ml (+) 2.5 pg/ml – 1000 ng/ml (-)	0.5 5	0.25 – 1000 pg/ml (+) 2.5 – 1000 pg/ml (-)	0.25 2.5

CONCLUSIONS

- Baseline separation of thyroid hormones in 6 minutes with good LOQ in positive and negative mode with the butyl derivatized hormones resulting in the better LOQ levels.
- A clean serum matrix is required to achieve the desired calibration curve and LOQ as the thyroid hormones bind to proteins and albumin within serum that can result in interfering responses
- Excellent linearity of calibration curves with better accuracy, precision and reproducibility in positive mode than in negative mode by a factor of 5 fold and again the butylated hormones gave the best results in positive mode
- Derivatization of the thyroid hormones resulted in a potential 5 fold improvement in sensitivity that underivatized but also resulted a high degree of interferences demanding the use of thyroid free serum for analysis
- Further evaluate other mass spectrometer platforms such as the Thermo Scientific™ TSQ Quantis™ tandem mass spectrometer as to its suitability for the analysis of total and free butylated Thyroid determinations and maximize the efficiency of the method as well as evaluate the potential for the analysis of other thyroid hormone metabolites using butylation.

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TRADEMARKS/LICENSING