Сравнение процедур пробоподготовки для определения этилглюкуронида и этилсульфата в сыворотке крови методом высокоэффективной жидкостной хроматографии с масс-селективным детектированием

Ризванова Л.Н.
БУ ХМАО-Югры «Нижневартовская психоневрологическая больница»

Москва, 17-18 мая 2018г.

МЕТАБОЛИЗМ ЭТАНОЛА

(Шабанов П.Д., Калишевич С.Ю., 1998; Маркова И.В. и др., 1999; Афанасьев В.В. и др., 2002; Маркизова Н.Ф и др., 2004)

Основные метаболические последствия потребления этанола

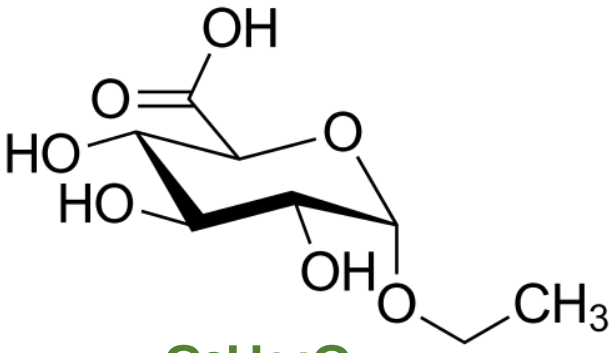
(Шабанов П.Д., Калишевич С.Ю., 1998; Маркова И.В. и др., 1999; Афанасьев В.В. и др., 2002; Маркизова Н.Ф и др., 2004)

- диспропорция в окислительно-восстановительных процессах;
- образование высокотоксичного ацетальдегида;
- отвлечение ферментов от нормального метаболизма эндогенных субстратов, содержащих спиртовые и альдегидные группы;
- накопление избыточных количеств ацетата, что приводит к усиленному образованию жирных кислот и холестерина

Ethyl glucuronide (ETG) and Ethyl sulfate (ETS) as biomarkers of alcohol consumption

Ethyl glucuronide (EtG)

is formed by the direct conjugation of ethanol and glucuronic acid through the action of a liver enzyme.

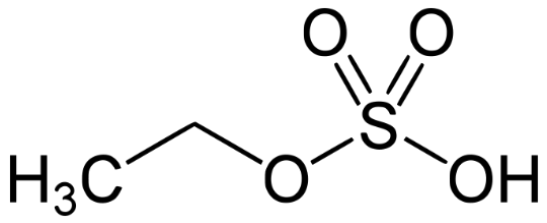


$C_8H_{14}O$

Ex. mass 222.07 g/mol

Ethyl sulfate (EtS)

is formed directly by the conjugation of ethanol with a sulfate group.



$C_2H_6O_4S$

Ex. mass 125.99 g/mol

EtG and EtS are non-volatile, acidic and water soluble phase II metabolites of alcohol, which are widely used for clinical, forensic and traffic cases. EtG and EtS has gained popularity due to their use as physiological indicators for screening previous alcohol consumption.

Цель:

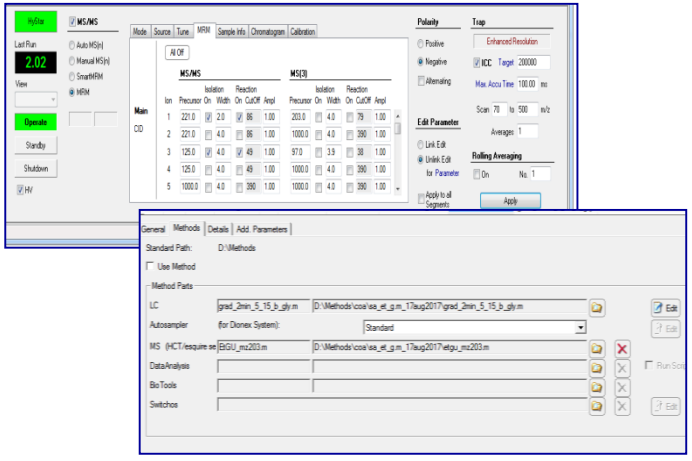
- Разработать ВЭЖХ-МС/МС метод для определения этилглюкуронида (ETG) и этилсульфата (ETS) в сыворотке крови для анализатора с масс-селективным детектором типа трёхмерная ионная ловушка.
- Сравнить процедуры пробоподготовки с целью подобрать наиболее оптимальную для определения EtG и EtS в сыворотке крови методом ВЭЖХ-МС/МС. Пробоподготовка для рутинного исследования должна быть простой, быстрой и недорогостоящей.

WHY triple quadrupole? WHY not an ion trap?

Аналитическое оборудование и условия ВЭЖХ-МС/МС

The same equipment
for drugs/NPS analysis and EtG/EtS analysis

HPLC-MS/MS (ion trap) Bruker Toxtyper



Dionex UltiMate 3000 HPLC system coupled to an AmaZon speed Bruker mass spectrometer	
Capillary voltage, 4500V. Drying gas, 159°C. Nebulizing gas pressure, 29.3 psi	
Column: Acclaim® RSLC 120 C18 2.1 x 100 mm (Dionex) Particle size 2.2 µm. Pore diameter 120A. Surface area 340 m2/g.	
Mobile phase A: 2mM ammonium formate, 0.1% formic acid, 1% acetonitrile in deionized water	
Mobile phase B: 2mM ammonium formate, 0.1% formic acid, 1% deionized water in acetonitrile	
Flow rate, 0.5 ml/min.	
Column oven: 40 °C. Autosampler: 12 °C.	
Method for drugs and NPS markers	**Method for EtG and EtS**
Gradient mode: 0-1 min 1% eluent B, 1-8 min gradient up to 95% eluent B, 8-9 min 95% eluent B, Final for 2 min 1% eluent B	**Isocratic mode:** 5% eluent B
MS1, MS2, MS3 **(full scan)** detection mode; mass range, 70–800 m/z. Simultaneous registration of **positive and negative** ions.	The detection using **MRM in negative registration mode:** EtG m/z 221->203 EtS m/z 125->97

ИССЛЕДУЕМЫЕ ОБРАЗЦЫ

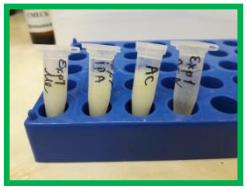
СЫВОРОТКА

Blood in vacutaners with an accelerator for coagulation was taken from a volunteer who had not consumed alcohol for several months.

Serum was separated by blood centrifugation for 10 minutes at 3000 rpm.

EtG 5000 ng/ml and EtS 5000 ng/ml were spiked to serum. Serum without spiked EtG and EtS was analysed as a negative sample.

Serum samples were prepared using protein precipitation technique (PPT) with an organic solvent: methanole (MeOH), isopropanole (IPA), acethone (Ac), acetonitrile (AcN). Dry spots technique (DSP) was approbated too.



ЦЕЛЬНАЯ КРОВЬ

To approbate the sample preparation procedures, blood with heparin was taken from patients with alcohol intoxication.

Blood in vacutaners with a heparin was taken from a volunteer who had not consumed alcohol and was analysed as a negative sample.

Sample preparation procedures (PPT)

Experience 1

250 µl serum,
1000 µl organic solvent

Vortex mix 1 min

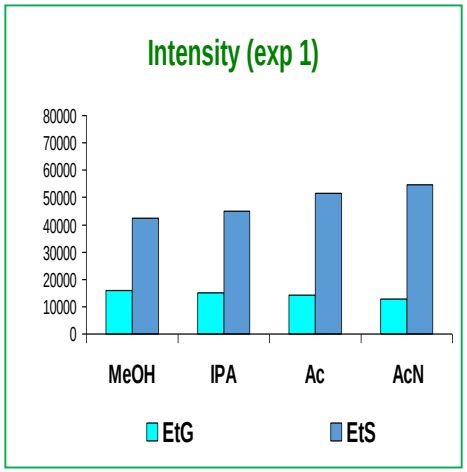
Centrifuge
15000 rpm, 10 min

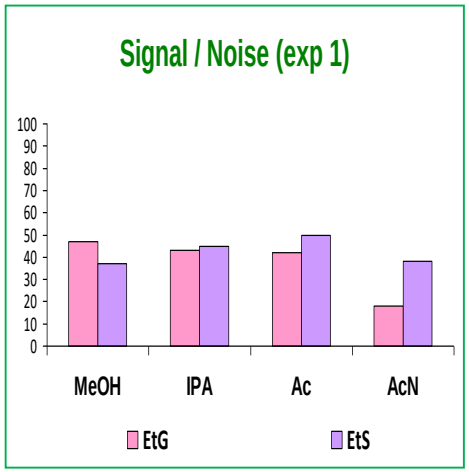
Transfer 1000 µl
supernatant

Evaporation at 60 C

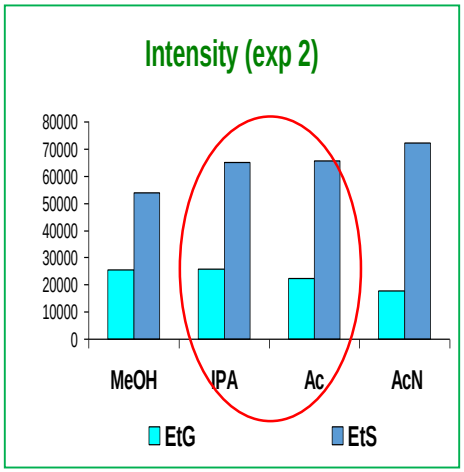
Reconstraction
250 µl water

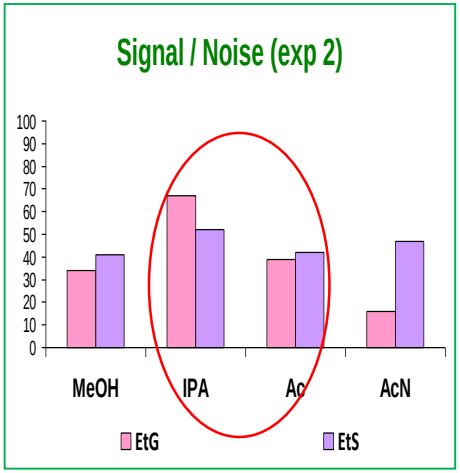
Injection 10 µL





Experience 2





250 µl serum,
1000 µl organic solvent

Vortex mix 1 min

Centrifuge
15000 rpm, 10 min

Transfer 1000 µl
supernatant

1000 µl organic solvent
to sediment

Vortex mix 1 min

Centrifuge
15000 rpm, 10 min

Combine 1000 µl
supernatant with
previous one

Evaporation at 60 C

Reconstraction
250 µl water

Injection 10 µL

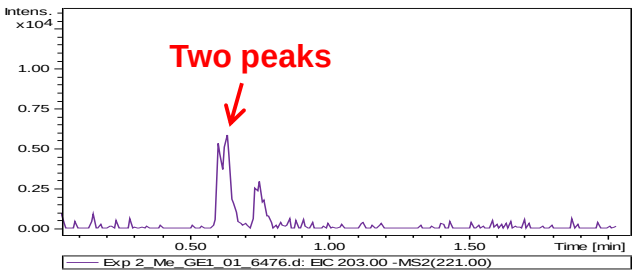
Double precipitation with isopropanol or acetone
gives the best results of intensity and signal/noise ratio.
However, acetone extract evaporates faster than isopropanol extract.

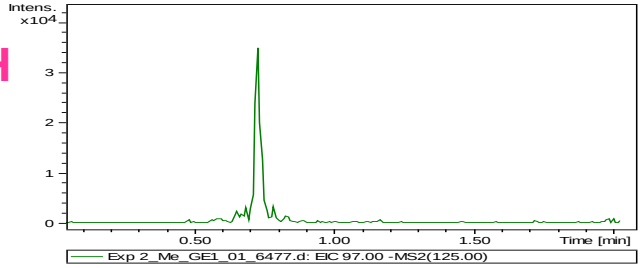
Chromatograms of EtG and EtS (PPT)

EtG 5000 ng/ml m/z 221->203

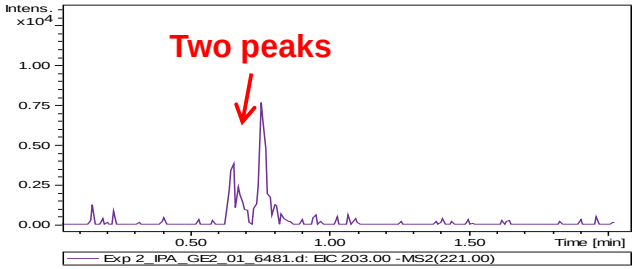
EtS 5000 ng/ml m/z 125->97

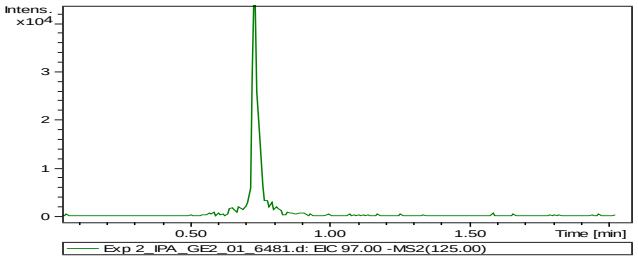
MeOH



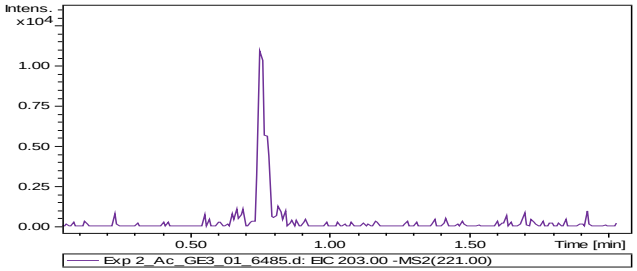


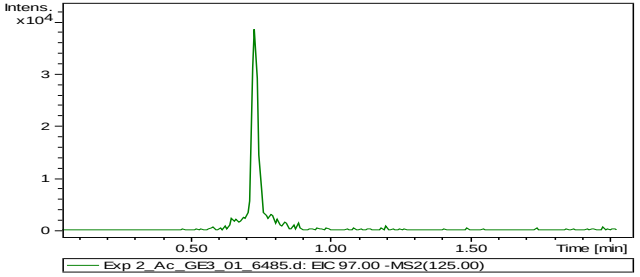
IPA



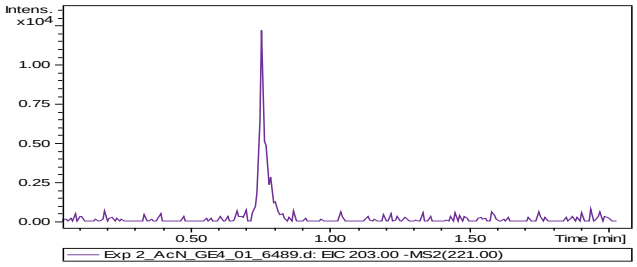


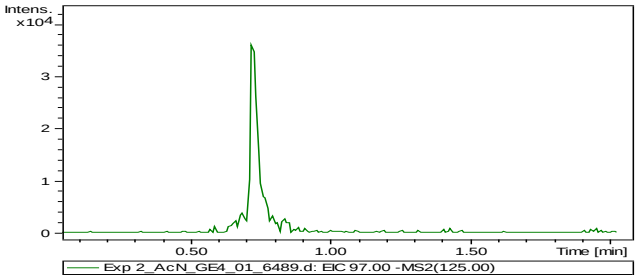
Ac



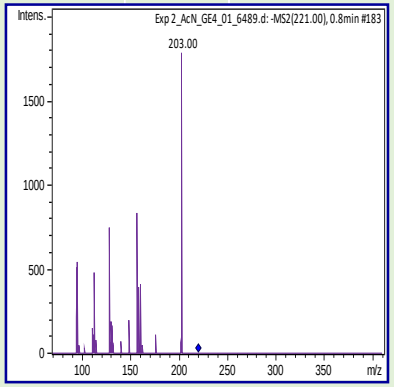


AcN

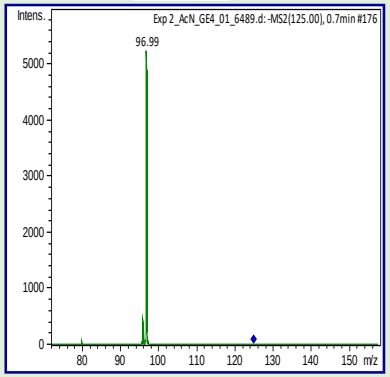




EtG



EtS



Sample preparation procedures (PPT)

Experience 1

250 µl serum,
1000 µl organic solvent

Vortex mix 1 min

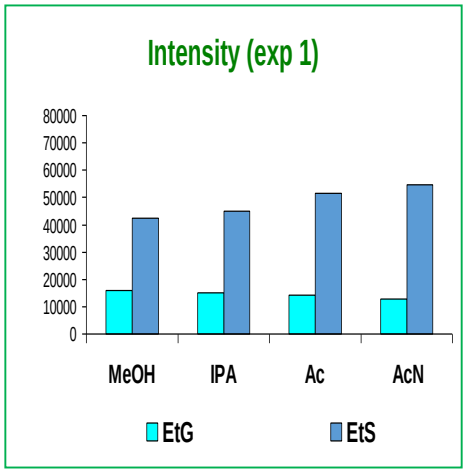
Centrifuge
15000 rpm, 10 min

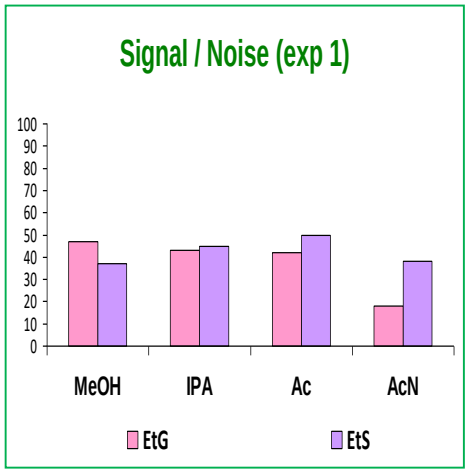
Transfer 1000 µl
supernatant

Evaporation at 60 C

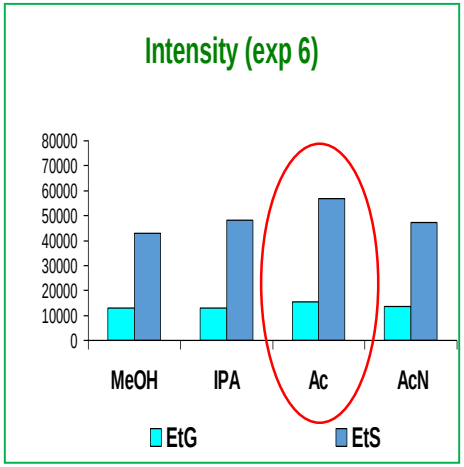
Reconstraction
250 µl water

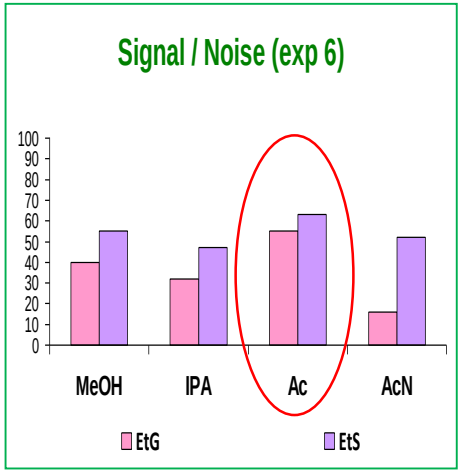
Injection 10 µL





Experience 6





250 µl serum,
500 µl Hexane

Vortex mix 1 min

Centrifuge
15000 rpm, 10 min

Waste
500 µl organic phase

250 µl serum,
1000 µl organic solvent

Vortex mix 1 min

Centrifuge
15000 rpm, 10 min

Transfer 1000 µl
supernatant

Evaporation at 60 C

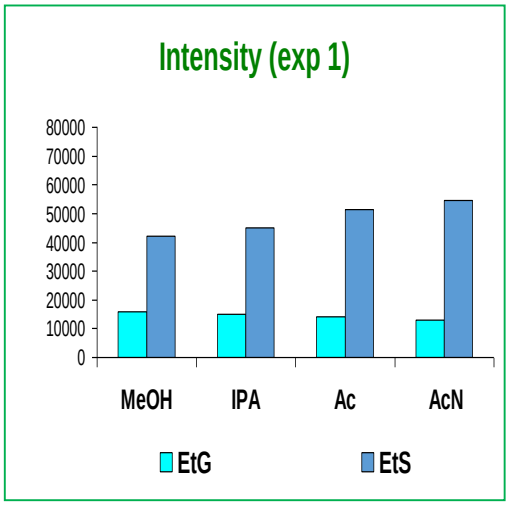
Reconstraction
250 µl water

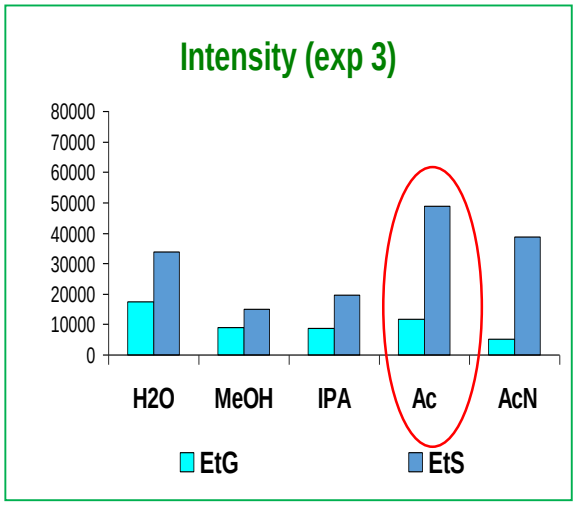
Injection 10 µL

Pre-extraction and wasting of phospholipids with hexane gives a slight advantage of the signal-to-noise ratio.

Sample preparation procedures (serum, DST)

Experience 3

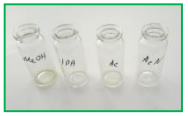




250 µl serum

Drying 100 C 15 min

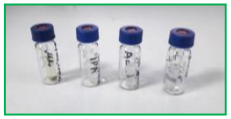
Adding (layering) 250 µl water or organic solvent



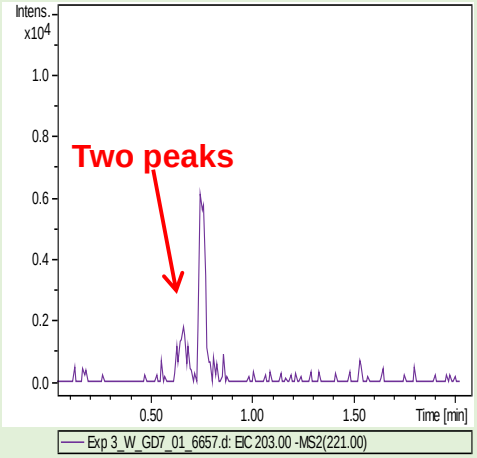
Ultrasound 10 min

200 µl supernatant to vial

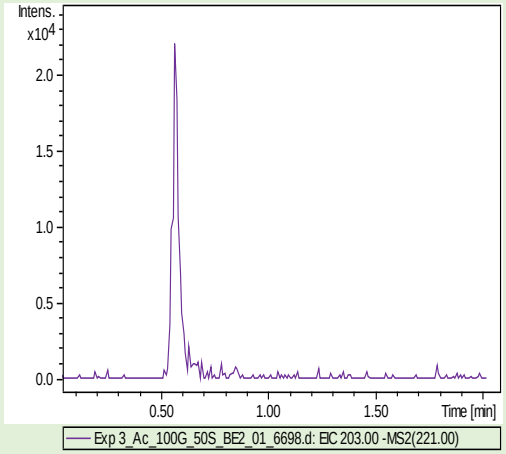
Injection 10 µL



EtG, extracted by water (exp 3)



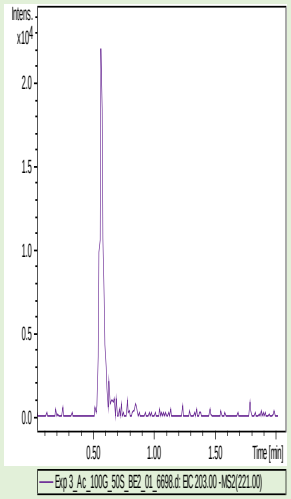
EtG, extracted by acetone (exp 3)



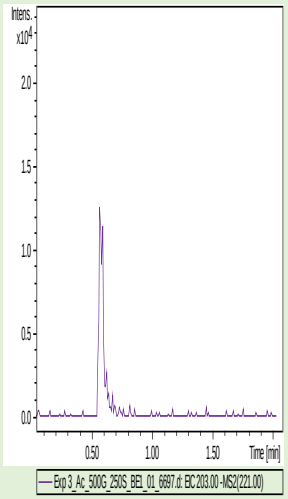
This sample preparation procedure is the most simple, fast and inexpensive.

Chromatograms of EtG and EtS (serum, DST)

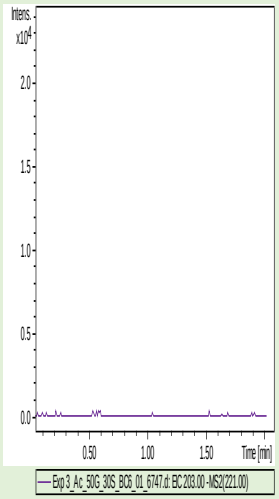
m/z 221->203



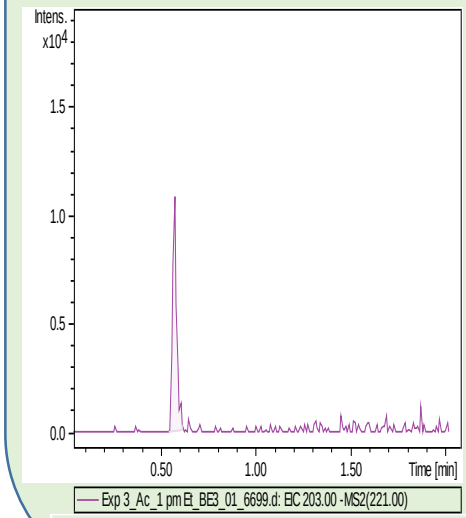
EtG 500 ng/ml



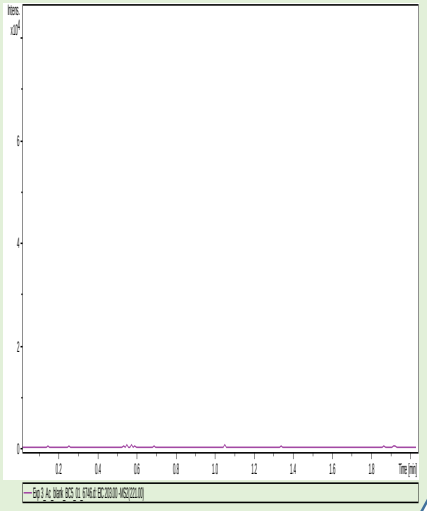
EtG 100 ng/ml



EtG 50 ng/ml

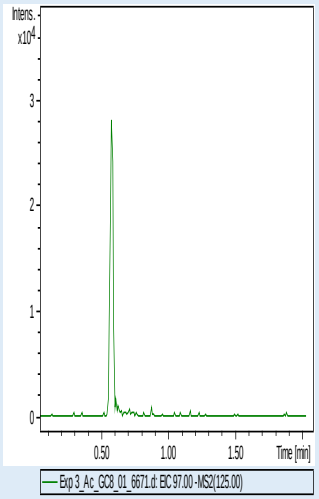


EtG in serum (1,4 g/l ethanol)

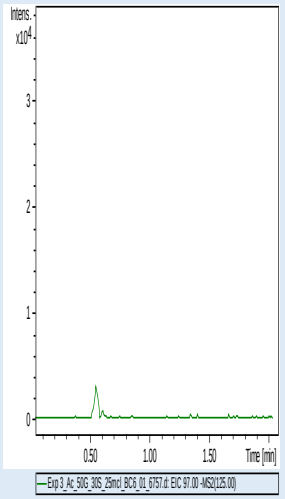


EtG in negative serum

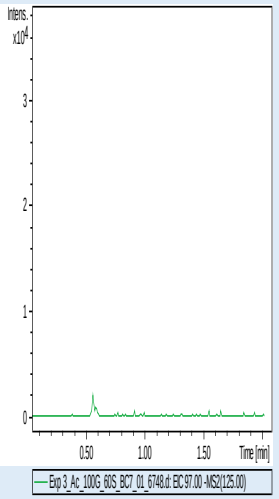
m/z 125->97



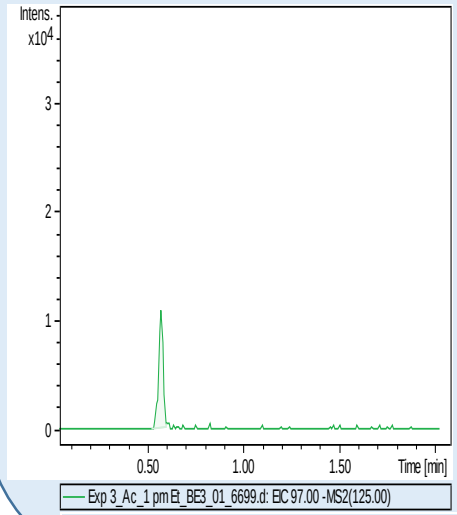
EtS 250 ng/ml



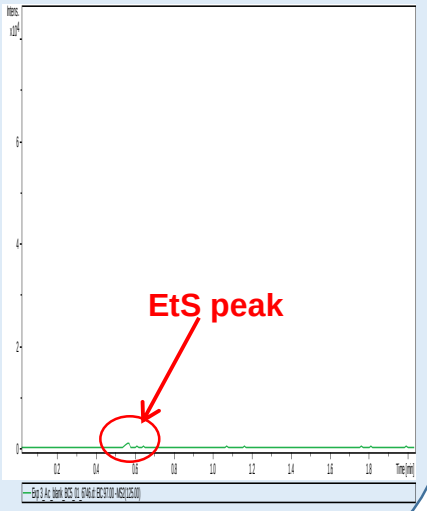
EtS 60 ng/ml



EtS 30 ng/ml



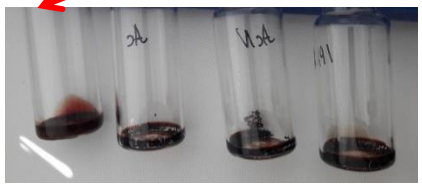
EtS in serum (1,4 g/l ethanol)



EtS in negative serum

Sample preparation procedures (whole blood, DST)

Water is not a good choice, because hemoglobin will be extracted



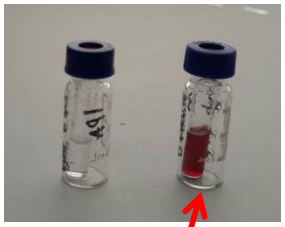
250 µl whole blood. Drying 100 C 25 min.

Adding (layering) 250 µl organic solvent after cooling to room temp

Ultrasound 10 min

200 µl supernatant to vial. Centrifugation is not required. 10 µL aliquot was injected into the LCMS.

At the bottom of the vial should be slightly cracked film

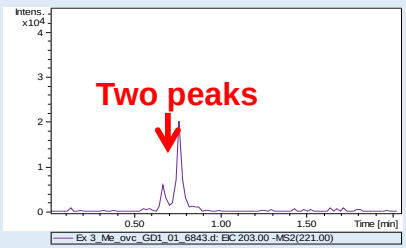
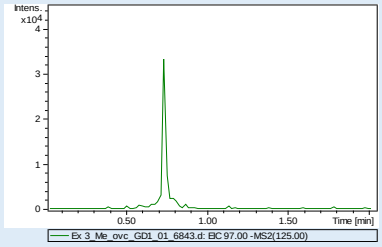
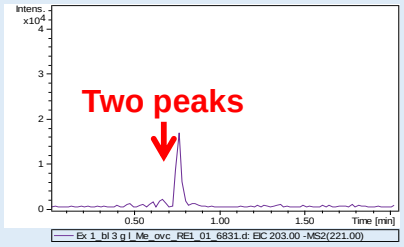
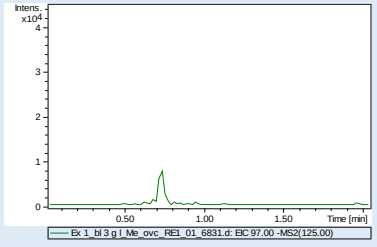
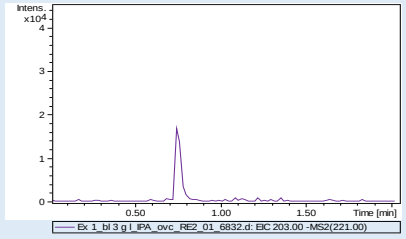
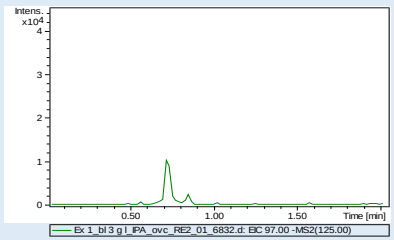
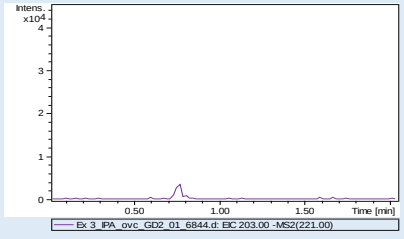
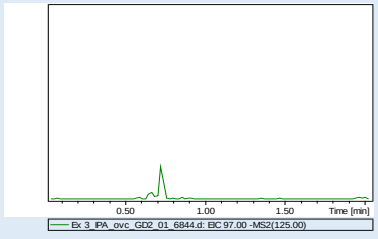
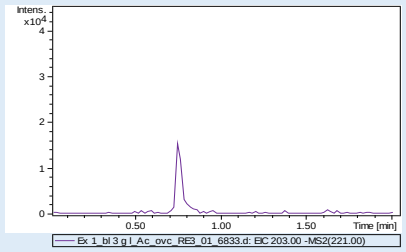
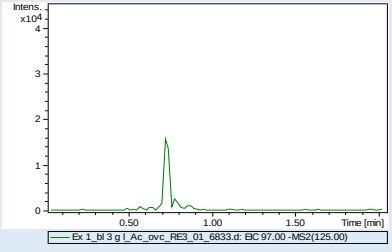
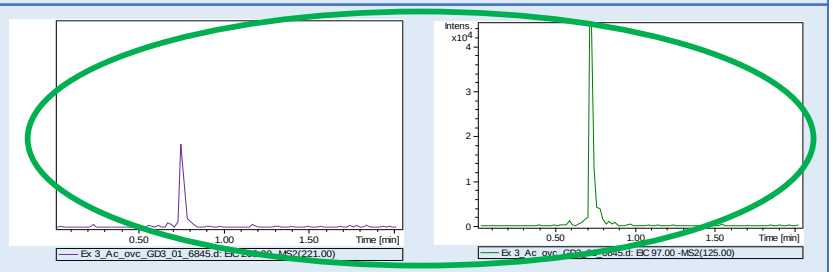
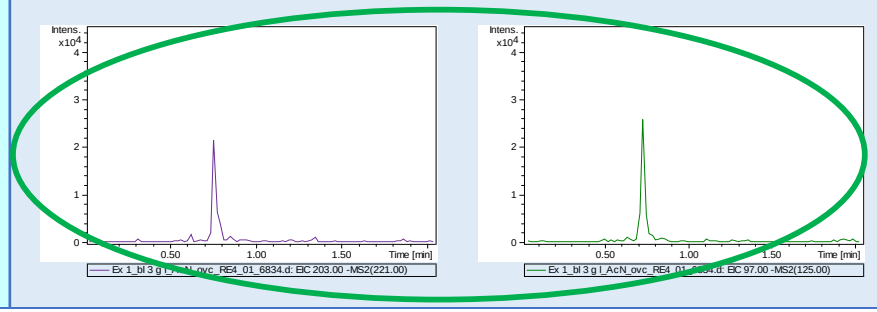
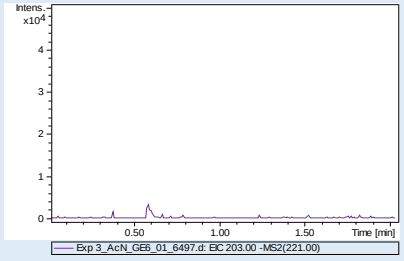
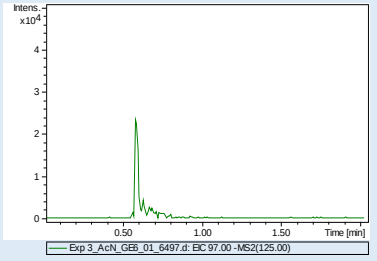


If not, hemoglobin will be extracted

Chromatograms of EtG and EtS (whole blood, DST)

Whole blood with ethanol 3.4 g/l

EtG m/z 221->203 EtS m/z 125->97

	Protein Precipitation Technique		Dry Spots Technique	
MeOH				
IPA				
Ac				
AcN				

ВЫВОДЫ

- Проведено сравнение процедур пробоподготовки сыворотки крови для определения этилглюкуронида и этилсульфата методом высокоэффективной жидкостной хроматографии с масс-селективным детектированием трёхмерной ионной ловушкой.

- Предложены и протестированы две наиболее простые и быстрые процедуры пробоподготовки сыворотки крови (осаждения белка и сухих пятен).

- Дальнейшая работа будет сосредоточена на повышении чувствительности внедряемого метода, разработке и валидациии метода определения этилглюкуронида и этилсульфата с применением калибровки по внутреннему стандарту, документированию процедуры рутинного анализа.

**Автор выражает признательность
д.х.н.Сергею Александровичу Савчуку
и Максиму Викторовичу Овчарову
за помощь в разработке методик**

Благодарю за внимание!

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