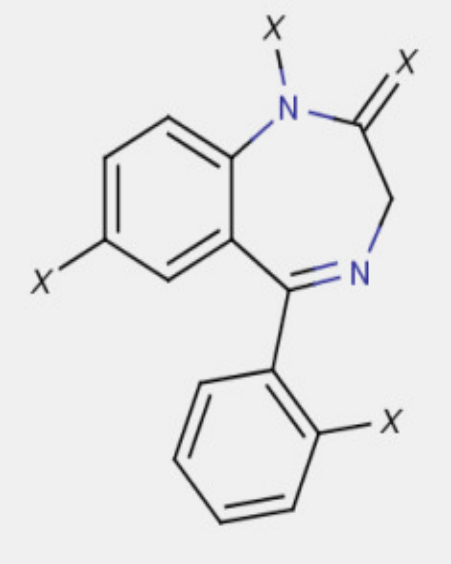
Determination of Benzodiazepines in serum by LC-on-line SPE-MS/MS using Spark Holland Symbiosis™ system and Recipe® ClinCal® calibrators.

Introduction:

A **benzodiazepine (benzo)** psychoactive drug whose core chemical structure is the fusion of a benzene ring and a diazepine ring. The first benzodiazepine, chlordiazepoxide (Librium)was discovered in 1955, and made available in 1960 by Hoffmann–La Roche, which has also marketed diazepam (Valium) since 1963.

Long-term use is controversial due to concerns about adverse psychological and physical effects, increased questioning of effectiveness and because benzodiazepines are prone to cause tolerance, physical dependence, and, upon cessation of use after long term use, a withdrawal syndrome. Withdrawal from benzodiazepines, in general, leads to improved physical and mental health. [1]

[1] text from Wikipedia



Recipe® introduced the ClinMass® Complete Kit for Benzodiazepines in October of 2011.

This kit provides a reliable quantification of 33 different benzodiazepines and metabolites. The chromatography is modified by using a Core shell column with an analysis runtime of 15 minutes including on-line SPE sample preparation. Manual sample preparation is no longer required.

Recipe® provided the quantifier and qualifier masses and MS settings for all 33 compounds and 20 Deuterated internal standards to be used on the AB Sciex API4000. For this application note the Clincal® Calibrators are used without the correction of the internal standards. This set contains one blank (level 0) and 3 calibration points (level 1-3). The concentrations in the three calibrators are different for the 33 benzodiazepines.

Method .

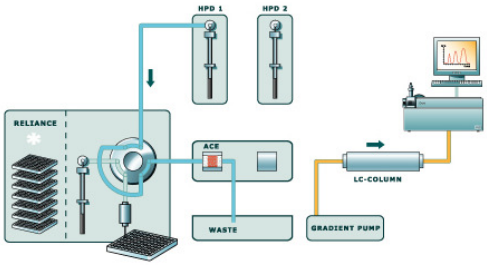
Because most benzodiazepines are neutral and non-polar at pH 7 and higher, water is used for the on-line SPE routine. For the development of the method a special standard containing 6 benzodiazepines in 1 mM Ammonium Carbonate (pH9) is used. The online SPE method is developed by testing the 8 different sorbents in the Spark HySphere™ method development tray (p/n: 90822.660).

The highest recoveries and best peak shapes were achieved with the HySphere™ C8EC SE (10x2 mm, 7µ) cartridge. After selecting the HySphere C8EC SE the wash was optimized by testing different percentages

Acetonitrile (ACN) in the cartridge wash solvent. The highest recoveries were achieved with 5% ACN in water wash.

The final on-line SPE (XLC)method is:

Cartridge:	HySphere C8EC SE (p/n:0822.611)
Solvation	1 mL ACN (5 mL/min)
Equilibration	1 mL water (5mL/min)
Sample application	1 mL water (1 mL/min)
Wash 1	1 mL water (5 mL/min)
Wash 2	1 mL 5% ACN in water (5 mL/min)
Elution time	4 minutes LC gradient



Autosampler:

The Recipe®MS6013 calibrator kit contains four small bottles with dried spiked serum. In each calibrator exactly 1 mL of water is added and mixed for 15 minutes. The calibrators are ready to use, no additional sample preparation steps are necessary.

Autosampler method

Injection Mode: ul Pickup (Zero loss)
Injection volume: 10 µL
Speed: Normal
HeadSpace pressure: Off
Air segment: Off
Cooling: 10°C

Wash method:

Wash volume (ul)	Wash solvent
700	5% ACN 0.2% FA
1000	40% ACN 0.2% FA
700	5% ACN 0.2% FA

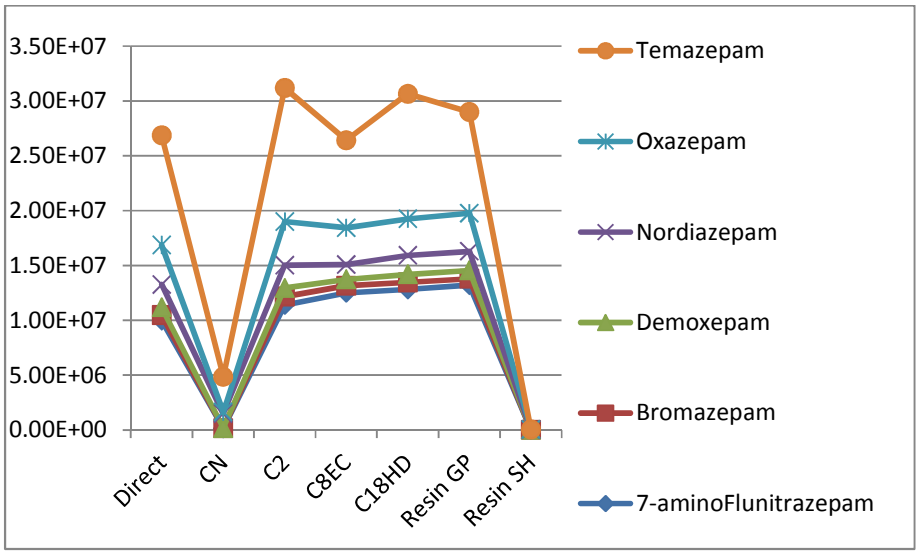
LC method

For the chromatography a Kinetex™ XB-C18 (50*2.1 mm 2.6µ) Core Shell column from Phenomenex® is selected. Mobile phase A is 0.2% formic acid in water and mobile phase B is acetonitrile

Pump time	Pump flow (ml/min)	Pump Fraction A %	Pump Fraction B %
00:00:01	0.30	90	10
00:00:30	0.30	90	10
00:09:00	0.30	5	95
00:10:00	0.30	5	95
00:10:15	0.30	90	10
00:15:00	0.30	90	10

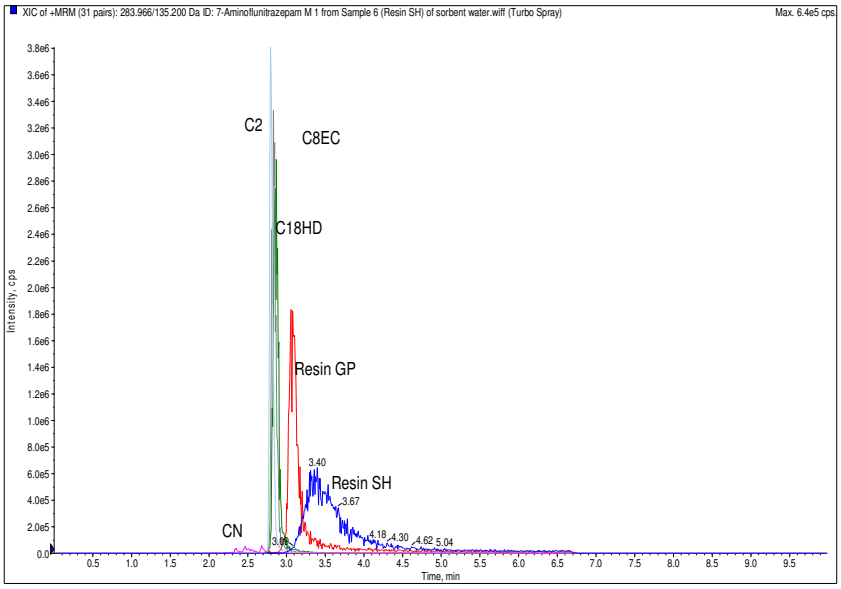
Results

To develop the method a mixture of 6 Benzodiazepines (1 mm Ammonium Carbonate pH9) is used, this allows to check for recoveries of the on-line SPE method compared to the direct LC injection.



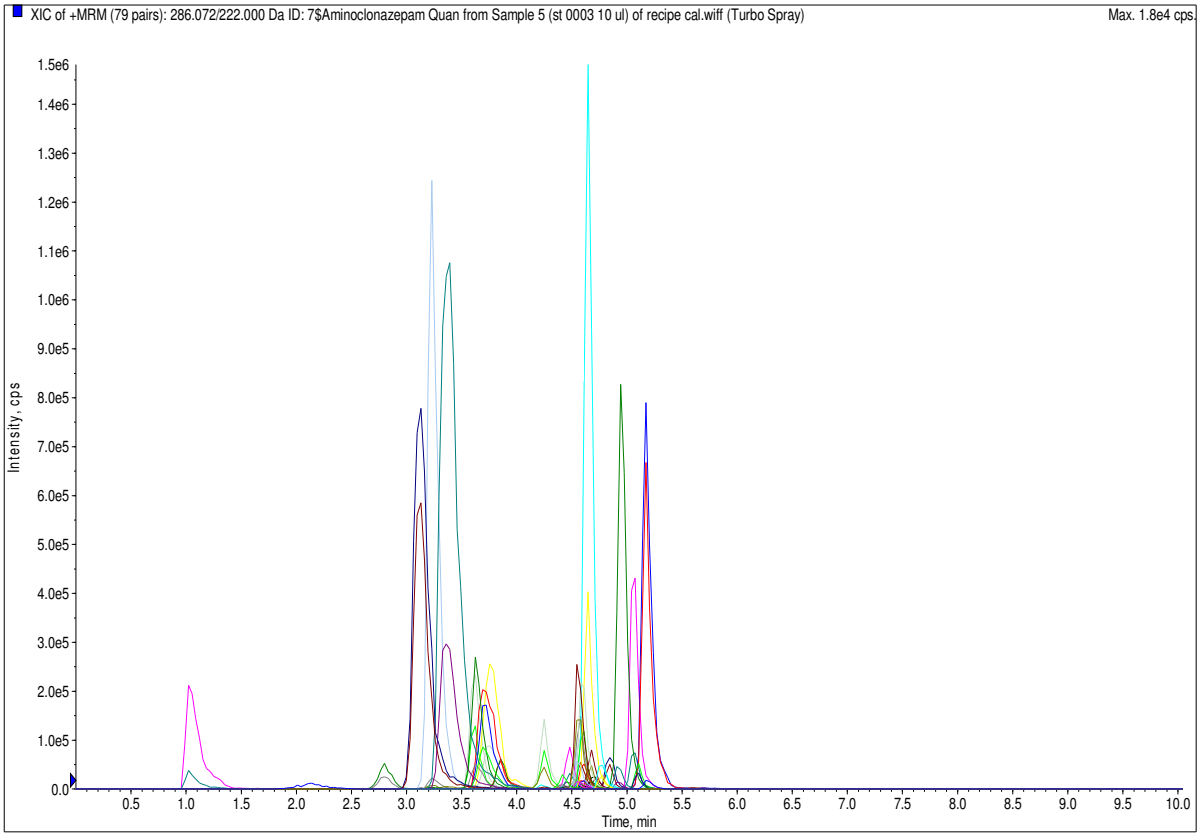
Comparison of the peak area of the direct LC injection and the 6 reversed phase sorbents of the HySphere ™ method development tray.

The HySphere™ C2, C8EC, C18HD and Resin GP achieve recoveries around 100% for the 6 compounds, the Resin GP is excluded from the wash optimization steps because of the poor peak shape for all compounds.



Overlay of 6 reversed phase sorbents peaks of 7-amino-flunitazepam.

The 33 Recipe® benzodiazepine compounds have a very wide polarity range.
The C2 has a limited polarity range for trapping all the compounds, C18HD has too much retention of the stronger non-polar benzodiazepines and generate peaks with a large peak width. Therefore the C8EC SE is selected for running the Recipe® standards as it handles a wide polarity range and the compounds elute with a better peakshape from the C8EC SE than from the C18HD.



On-line SPE: 10 µL level 2 serum standard on a C8EC SE cartridge

The Recipe® Clin Cal MS6013 Calibrator kit contains 4 levels. One level is a blank serum and the other three levels are spiked serum samples with different concentrations for the 33 benzodiazepines. Each level is injected three times and from the spiked levels a calibration curve is created. The list below shows the three levels per compound and the calibration curve correlation coefficient(R).
For the calibration curve the quantifier MS/MS setting results of the 33 Benzodiazepines are used. The qualifier MS/MS chromatogram is only used to confirm the retention time of the benzodiazepines.

Calibration curve: Recipe® MS6013 Calibrators				
	Concentration MS6013			
Analyt name	Level 1	Level 2	Level 3	R>=
7-Aminoclonazepam	5.17	26.5	78.3	0.996
7-Aminoflunitrazepam	5.24	27.1	80.2	0.998
7-Aminonitrazepam	22.1	111	332	0.996
alpha-Hydroxyalprazolam	5.34	29.1	83.8	0.999
alpha-Hydroxymidazolam	21.5	105	310	0.995
alpha-Hydroxytriazolam	4.62	22.5	65.5	0.997
Alprazolam	5.25	26.3	82.7	0.995
Bromazepam	31.5	153	456	0.996
Chlordiazepoxlde	223	1060	2970	0.998
Clobazam	47.7	238	716	0.994
Clonazepam	5.06	25.5	73.4	0.999
Demoxepam	216	1090	3290	0.992
Desalkylflurazepam (Norfludiazepam)	9.67	50.8	151	0.996
Desmethylflunitrazepam (Norflunitrazepam)	4.54	24.9	74.2	0.991
Diazepam	102	450	1370	0.993
Estazolam	42.8	214	643	0.996
Flunitrazepam	5.23	27.1	79.0	0.996
Flurazepam	8.54	43.1	128	0.995
Lorazepam	20.6	103	297	0.997
Lormetazepam	1.82	9.11	27.3	0.991
Medazepam	42.8	206	604	0.999
Midazolam	31.0	151	459	0.993
Nitrazepam	21.0	103	311	0.999
Norclobazam	266	1360	4040	0.990
Nordiazepam	83.3	397	1170	0.993
Oxazepam	125	627	1860	0.999
Prazepam	83.3	433	1220	0.990
Temazepam	43.3	220	636	0.995
Tetrazepam	40.9	204	613	0.999
Trazodone	165	828	2520	0.990
Triazolam	4.02	20.1	60.3	0.993
Zaleplon	8.37	43.3	131	0.999

Conclusion:

From this study it can be concluded that within a time frame of only a few days it is possible to develop a XLC-MS method for the 33 Benzodiazepines with a recovery of more than 90% for the 6 test Benzodiazepines.
For the 33 Benzodiazepines in serum the calibration curve correlation coefficient is more than 0.99% for all compounds.
The accuracy is between 90-110% for all 33 compounds and the three levels.
The precision of all compounds is below 15% for the level 1 (LOQ) and less than 10% for level 2 and 3.
All measurement are performed without the use of an internal standard and each sample is injected 3 times (n=3)

Acknowledgment

Spark Holland b.v. would like to thank Recipe ® for the supply of the Benzodiazepine ClinCal 6013 kit and the MS settings for the AB Sciex API 4000.

Appendix:

Recipe ® Mass settings with quantifier, qualifier and internal standards mass settings for AB Sciex API 4000 and 4000qtrap.

Analyt name	Q1	Q3	dwell	DP	EP	CE	CXP
7$Aminoclonazepam Quan	286.072	222	20	71	10	37	16
7$Aminoclonazepam$D4	290.031	226.1	20	76	10	35	14
7$Aminoflunitrazepam Quan	284.197	135	20	81	10	41	12
7$Aminoflunitrazepam Qual	284.197	227.2	20	81	10	37	14
7$Aminoflunitrazepam$D7	291.222	138.1	20	61	10	41	10
7$Aminonitrazepam Quan	252.175	121	20	76	10	37	10
7$Aminonitrazepam Qual	252.175	94.2	20	76	10	59	12
alphaOHAlprazolam Quan	325.122	297.2	20	46	10	37	12
alphaOHAlprazolam Qual	325.122	216.1	20	46	10	55	14
alphaOHAlprazolam$D5	330.228	302.2	20	36	10	41	12
alphaOHMidazolam Quan	342.124	324	20	76	10	31	22
alphaOHMidazolam Qual	342.124	203	20	76	10	37	18
alphaOHMidazolam$D4	346.073	168.1	20	76	10	59	10
alphaOHTriazolam Quan	359.3	176	20	41	10	37	20
alphaOHTriazolam$D4	362.954	243	20	56	10	65	24
Alprazolam Quan	309.177	205.2	20	51	10	61	14
Alprazolam$D5	314.182	286.1	20	71	10	41	20
Bromazepam Quan	316.091	182.1	20	71	10	49	18
Bromazepam Qual	316.091	209.1	20	71	10	37	12
Chlordiazepoxid$D5	305.193	286.1	20	56	10	33	22
Chlordiazepoxide Quan	300.056	227	20	61	10	35	18
Clobazam Quan	300.948	258.9	20	56	10	29	16
Clobazam Qual	300.948	224	20	56	10	47	14
Clonazepam Quan	316.053	269.9	20	56	10	39	24

Clonazepam Qual	316.053	214	20	56	10	57	16
Clonazepam$D4	320.118	274	20	56	10	37	24
Demoxepam Quan	287.4	180.2	20	60	10	34	15
Demoxepam Qual	287.4	269.1	20	60	10	43	15
Desalkylflurazepam Quan	289.143	140	20	81	10	41	12
Desalkylflurazepam Qual	289.143	226.1	20	81	10	41	18
Desmethylflunitrazepam Quan	300.1	254.3	20	66	10	37	14
Desmethylflunitrazepam Qual	300.1	198.1	20	66	10	51	18
Diazepam Quan	285.171	153.9	20	71	10	39	14
Diazepam Qual	285.171	193	20	71	10	45	14
Diazepam$D5	290.163	154	20	51	10	39	10
Estazolam Quan	295.082	267.1	20	36	10	39	12
Estazolam Qual	295.082	205.1	20	36	10	61	18
Estazolam$D5	300.204	271.9	20	56	10	35	12
Flunitrazepam Quan	314.108	268.1	20	81	10	37	28
Flunitrazepam Qual	314.108	239	20	81	10	49	16
Flunitrazepam$D7	321.158	246	20	66	10	51	16
Flurazepam Quan	388.16	315.1	20	71	10	35	18
Flurazepam Qual	390.15	317.1	20	66	10	33	20
Lormetazepam Quan	335.077	289	20	56	10	29	12
Lormetazepam Qual	337.07	291	20	61	10	33	18
Lorazepam Quan	322.989	276.9	20	51	10	29	18
Lorazepam Qual	320.996	229	20	71	10	43	22
Lorazepam$D4	325.046	278.9	20	51	10	31	20
Medazepam Quan	271.226	206.9	20	36	10	39	16
Medazepam Qual	271.226	91	20	36	10	45	12
Midazolam Quan	326.054	291.1	20	81	10	39	20
Midazolam Qual	326.054	249	20	81	10	55	16
Midazolam$D4	330.158	295	20	76	10	39	12
Nitrazepam Quan	282.113	236	20	66	10	37	12
Nitrazepam Qual	282.113	180	20	66	10	55	16
Nitrazepam-D5	287.041	185	20	31	10	53	18
Norclobazam Quan	287.068	245	20	41	10	29	14
Norclobazam Qual	287.068	210	20	41	10	45	12
Nordiazepam Quan	271.206	140	20	41	10	39	12
Nordiazepam Qual	271.206	165	20	41	10	43	14
Nordiazepam-D5	276.02	140	20	76	10	41	12
Oxazepam Quan	287.106	241.1	20	41	10	19	14
Oxazepam Qual	287.106	268.8	20	41	10	31	14
Oxazepam-D5	292.145	245.9	20	41	10	35	22
Prazepam Quan	325.23	271	20	71	10	33	26
Prazepam-D5	330.132	276.1	20	61	10	35	16
Temazepam Quan	301.082	255	20	46	10	35	22
Temazepam-D5	306.302	260.1	20	46	10	33	16
Tetrazepam Quan	289.116	197	20	71	10	53	16
Tetrazepam Qual	289.116	167	20	71	10	83	14
Trazodone Quan	372.1	148	20	60	10	52	12
Trazodone Qual	372.1	176.1	20	60	10	52	12

Triazolam Quan	**343.025**	**308.1**	**20**	**71**	**10**	**39**	**18**
Triazolam Qual	**343.025**	**239.1**	**20**	**71**	**10**	**59**	**14**
Triazolam-D4	**346.901**	**312.1**	**20**	**91**	**10**	**39**	**10**
Zaleplon Quan	**306.17**	**236**	**20**	**76**	**10**	**43**	**14**
Zolpidem Quan	**308.094**	**234.9**	**20**	**46**	**10**	**51**	**16**
Zolpidem Qual	**308.094**	**236**	**20**	**46**	**10**	**39**	**14**
Zolpidem-D6	**314.275**	**234.8**	**20**	**60**	**10**	**52**	**12**