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# Analysis of carbonic anhydrase inhibitors in doping control

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## Introduction

Diuretics are abused as a masking agent or for maintaining body weight particularly in weight category sports. Delbeke and Debackere reported that administration of carbonic anhydrase inhibitors, such as acetazolamide, can reduce urinary excretion of basic doping substances, e.g. mephentermine, phentermine etc. due to alteration of metabolic clearance of these drugs through an increase of urinary pH. In this paper, we report the following studies of six carbonic anhydrase inhibitors: acetazolamide(ACZ), methazolamide(MTZ), ethoxzolamide(ETZ), dorzolamide(DRZ), brinzolamide(BRZ) and dichlorphenamide(DCP).

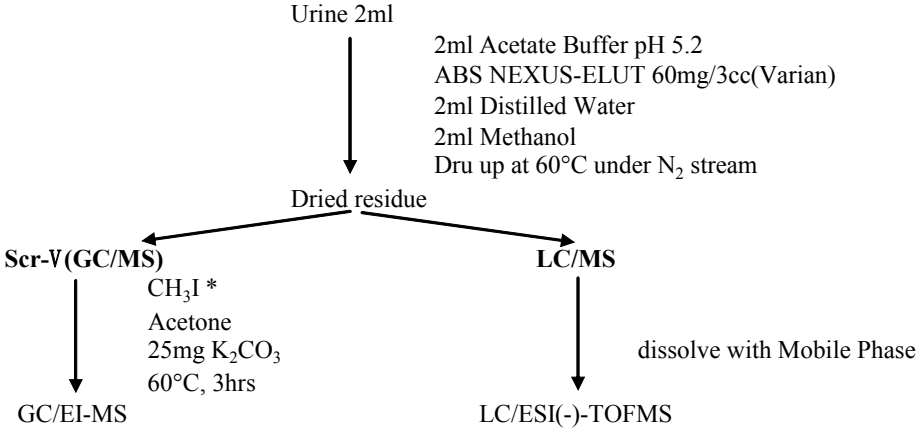
- -pH profiles of solid phase extraction(SPE)
- -Screening and confirmation of ACZ and MTZ by GC/MS
- -LC/MS analysis in negative ESI mode

Our report on LC/MS procedure also refers to Ritalinic acid(RA) and Carboxy-finasteride(CF), which are not suitable for GC /MS screening.

## Experimental

Sample preparation by solid phase extraction was performed using ABS ELUT-NEXUS(60mg, Varian, CA,USA) with acetate buffer at pH 5.2 followed by methanol elution. Sample extracts were analyzed directly for LC/MS, after derivatization with $CH_3I$ for GC/MS screening, and $C_2H_5I$ for GC/MS confirmation(Fig. 1).

All of other reagents were analytical grade. LC/Q-TOF MS instrument was QSTAR XL MS/MS System from Applied Biosystems (Foster City, CA, U.S.A.). GC/MS instrument was Agilent 6890N/5973 GC/MS from Agilent Technologies (Hachioji, Tokyo, Japan). GC/MS and LC/MS parameters are described in Table 1.




*)$CH_3CH_2I$ in case of confirmation of actazolamide and methazolamide

Fig.1 Sample preparation

Table 1 GC/MS and LC/MS conditions

| GC/MS parameters | |
|---|---|
| Instrument | Agilent 6890N/5973 inert GC/MS |
| Column | Ultra-II (Agilent) 0.25mm I.D. X 12.5m 0.33μm |
| Oven | Initial 150°C(hold 1.0 min), 300°C(19.5°C/min) hold 7.0min |
| Injector | 280°C, Split 11:1, 2μl injection |
| Transfer line | 300°C |
| Electron impact | 70eV |
| Acquisition | Scan mode: m/z 50 to 400 |

| LC/MS parameters | |
|---|---|
| Instrument | QSTAR XL MS/MS System (Applied Biosystems) |
| Column | Discovery C18 4.0mm x 50mm (SUPELCO) |
| Mobile Phase | A: 5mM $CH_3COONH_4$(pH5.2 with 1% $CH_3COOH$) |
| | B: $CH_3CN$ |
| Gradient | 0-1.5min: A 90% hold |
| | 1.5min-6.5min: A 90% - A 20% |
| | 6.5-7.5min: A 20% hold |
| | 7.5-8.5min: A 20% - A90% |
| Run time | 11min |
| Flow rate | 250 μl/min |
| Oven temp. | 25°C |
| Ionization condition | Negative ESI |
| Neblizer Gas | 2.85L/min |
| Aux. Gas | 4.80L/min |
| Ion spray Temp. | 450°C |
| Ion spray Voltage | -4,200V |
| TOF MS range | m/z140 to 500 |

## Results and Discussion

*-pH profiles of solid phase extraction*

Recovery of ACZ and MTZ from alkaline urine is relatively low(Fig.2).

This phenomenon was also observed in cases of XAD-2, OASIS HLB and Bond Elut C18

(No data shown). It is necessary to adjust the urinary pH to <7.0 for a successful solid phase extraction of ACZ and MTZ.

-*Screening by GC/MS*

It is not possible to separate ACZ and MTZ after methylation(Table 2).

As recovery of RA with liquid-liquid extraction is less than 1%, MRPL sample of RA can not be detected by our screening procedure-II. CF cannot be detected by the traditional GC/MS screening procedure(Table 2).

Table 2 Summary of screening analysis of acidic compounds by GC/MS

| Substance | Screening | Derivatives | $t_R$(min.) | $M^+$ | Fragment Ions | | MRPL (ng/ml) | LOD (ng/ml) |
|---|---|---|---|---|---|---|---|---|
| Acetazolamide | V | tri-$CH_3$ | **5.3** | **264** | **249** | **108** | 250 | 15 |
| Methazolamide | V | di-$CH_3$ | **5.3** | **264** | **249** | **108** | 250 | 10 |
| Ethoxzolamide | V | di-$CH_3$ | 6.7 | 286 | 179 | 151 | 250 | 25 |
| Dorzolamide | V | tri-$CH_3$ | 8.6 | 366 | 152 | 108 | 250 | 20 |
| Brinzolamide | V | tri-$CH_3$ | 9.7 | 425 | 152 | 260 | 250 | 10 |
| Dichlorphenamide | V | tetra-$CH_3$ | 7.5 | 360 | 253 | 108 | 250 | 30 |
| Mefruside (IS) | V | di-$CH_3$ | 9.1 | 410 | 85 | 325 | 250 | 10 |
| Ritalinic acid | II | N-TFA,O-TMS | 7.9 | 387 | 180 | 372 | 500 | **4,000** |
| Carboxy-finasteride | - | – | – | – | – | – | 250 | - |

-*Confirmation of ACZ and MTZ by GC/MS*

ACZ and MTZ can be distinguished using $C_2H_5I$ instead of $CH_3I$(Table 3 and Fig.3).

-*LC/MS analysis in negative ESI mode*

All carbonic anhydrase inhibitors could be detected at MRPL of 250ng/ml directly.

RA and CF could be also detected by same procedure as carbonic anhydrase inhibitors.

As can see Table 4, no matrix influence is observed for spiked sample of these compounds in water matrix, however, it can be observed for that of these compounds in urine matrix. It may be suggested that ion suppression was induced by any suppressants in urine matrix.

## Conclusion

Thus, use of LC/MS in negative ion mode for screening of acidic compounds has been found to have some advantages, however, we need to modify a sample clean-up or LC conditions in order to perform more high sensitivity analysis without matrix influences.

## Reference

Delbeke FT, Debackere M(1985). J Pharm Biomed Anal 3: 141-148

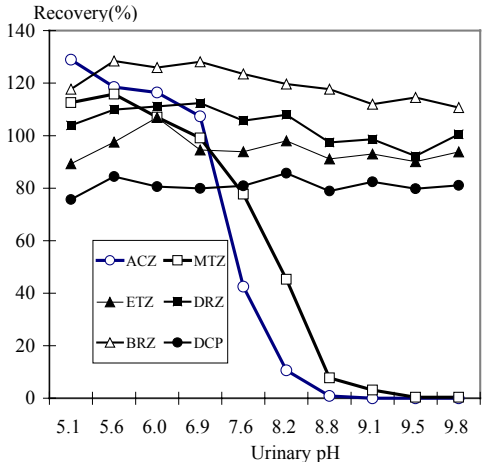



Fig. 2 pH profile of SPE

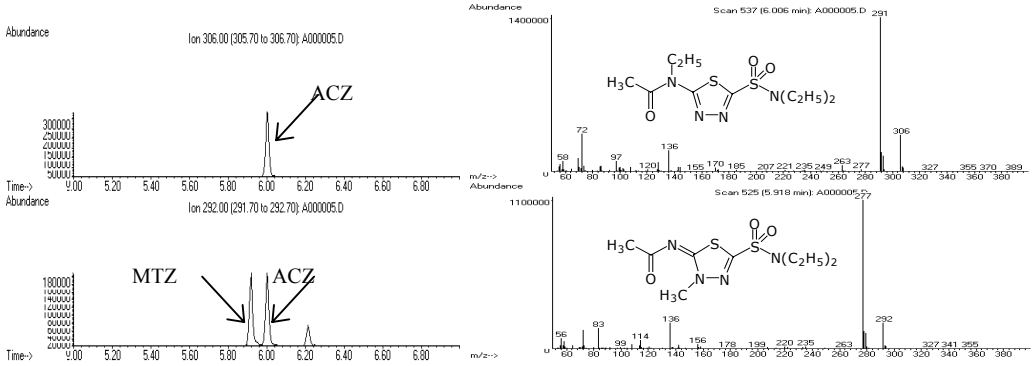



Fig. 3 GC/MS chromatogram and mass spectrum of ACZ and MTZ after ethylation

Table 3 Confirmation analysis of ACZ and MTZ by GC/MS

| Substance | Derivatives | $t_R$(min.) | $M^+$ | Fragment Ions | |
|---|---|---|---|---|---|
| Acetazolamide | tri-$CH_2CH_3$ | 6.0 | 306 | 291 | 136 |
| Methazolamide | di-$CH_2CH_3$ | 5.9 | 292 | 277 | 136 |
| Mefruside (IS) | di-$CH_2CH_3$ | 9.6 | 438 | 85 | 231 |

Table 4 Analysis of acidic compounds by LCESI(-)-TOFMS

| Substance | $[M-H]^-$ | $t_R$(min.) | Recovery(%) at 250ng/ml | |
|---|---|---|---|---|
| | | | Water matrix | Urine matrix |
| Acetazolamide | 221 | 5.6 | **93.2** | **72.6** |
| Methazolamide | 235 | 7.1 | **90.2** | **6.0** |
| Ethoxzolamide | 257 | 9.1 | 79.2 | 25.6 |
| Dorzolamide | 323 | 7.1 | 93.2 | 8.2 |
| Brinzolamide | 382 | 8.5 | 75.3 | 6.1 |
| Dichlorphenamide | 303 | 8.2 | 107.3 | 17.6 |
| Mefruside (IS) | 381 | 9.3 | 73.7 | 29.4 |
| Ritalinic acid | 218 | 6.9 | 67.7 | 5.7 |
| Carboxy-finasteride | 401 | 7.8 | - | - |

-: no data (certified standard missing)