Chiral separation analysis of urinary clenbuterol enantiomers in Chinese population by LC-MS-MS for doping control purpose

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Date: 2017.10

Overview

- ✓Introduction
- ✓Experimental
- ✓Results and discussion
- ✓Real cases
- ✓Conclusion
- ✓Future work
- ✓Acknowledgements

The number of clenbuterol-positive case increases rapidly in our routine analysis

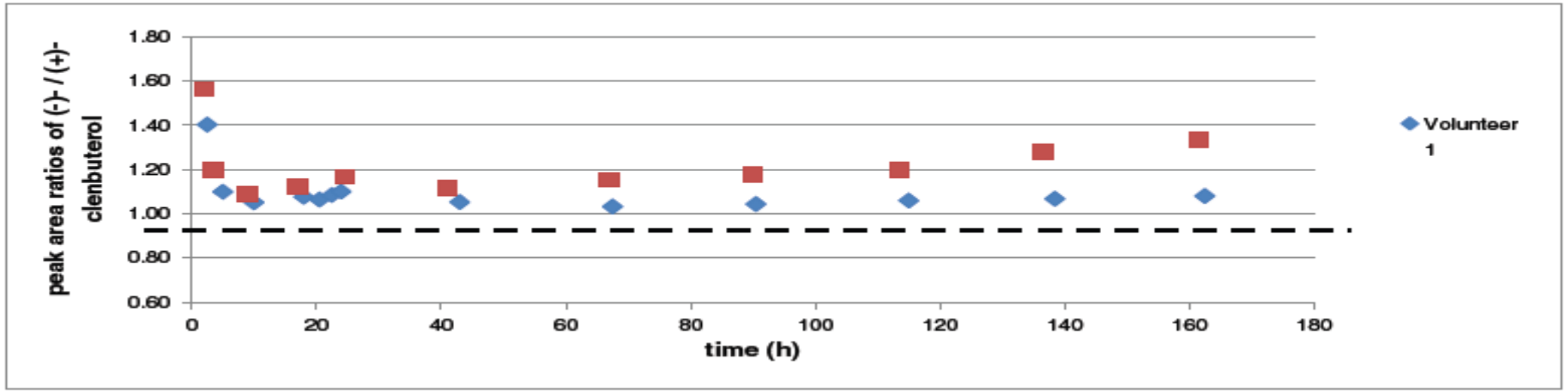
The concentration of most of clenbuterol-positive was less than 2ng/ml

the consumption of contaminated meat is a key factor

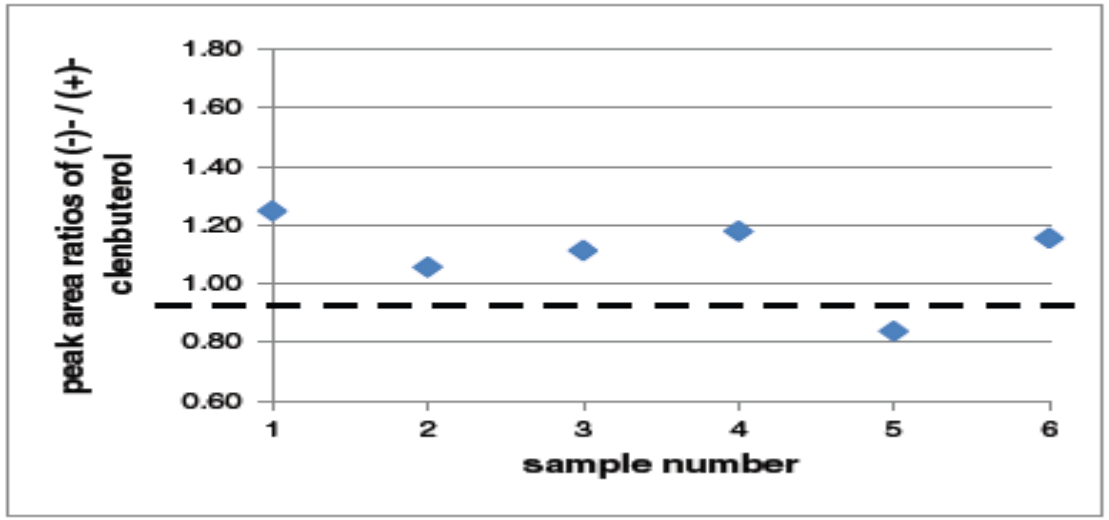
2013 RCM：

- LC-QTOF chiral separation of clenbuterol enantiomers calculation of R/S ratio
- the ratio lower than 1 is inconsistent with a drug administration, the ratio of oral intake of clenbuterol doesn’t fall below 1.0
- Enantiomer composition in animal tissue is time-dependent and relative to slaughtering time

a)



b)



- Other two papers have a similar opinion
- Hair analysis method by LC-MS-MS to differentiate therapeutic use from contamination

- No data in Chinese population was reported,
- the aim of this project was to investigate the R/S composition in Chinese population after ingestion of clenbuterol-contaminated pork and oral intake

- LC-MS-MS condition AB Sciex 5500Q LC-MS-MS, cloumn: CHIROBIOTIC^{M} T 150cmx2.1mm,5um, flow rate:0.3ml/min, isocratic condition with methanol(10um, ammonium formate), 10uL injection.

- MS parameters: MRM mode, 277.1> 168.0, 277.1>140.0

(clenbuterol enantiomers), 286.1 >169.0, 286.1 >141.0(D_9-clenbuterol)

Animal trial：

three swines were fed and dosed with clenbuterol

- administrated 30 days, slaughter time: 3, 5, 10 day after drug withdrawal,
- meat was cooked and distributed (5ug/ person,26 person: 13 males and 13 females),
- urine samples were collected prior to the administration and for 7 days post administration , each urine was collected at the beginning 3 days.

Human administration： the 26 persons were also administrated with 5ug clenbuterol, urine samples were also collected in the above method.

The studies were approved by the Research Ethics Committee of the Chinada.

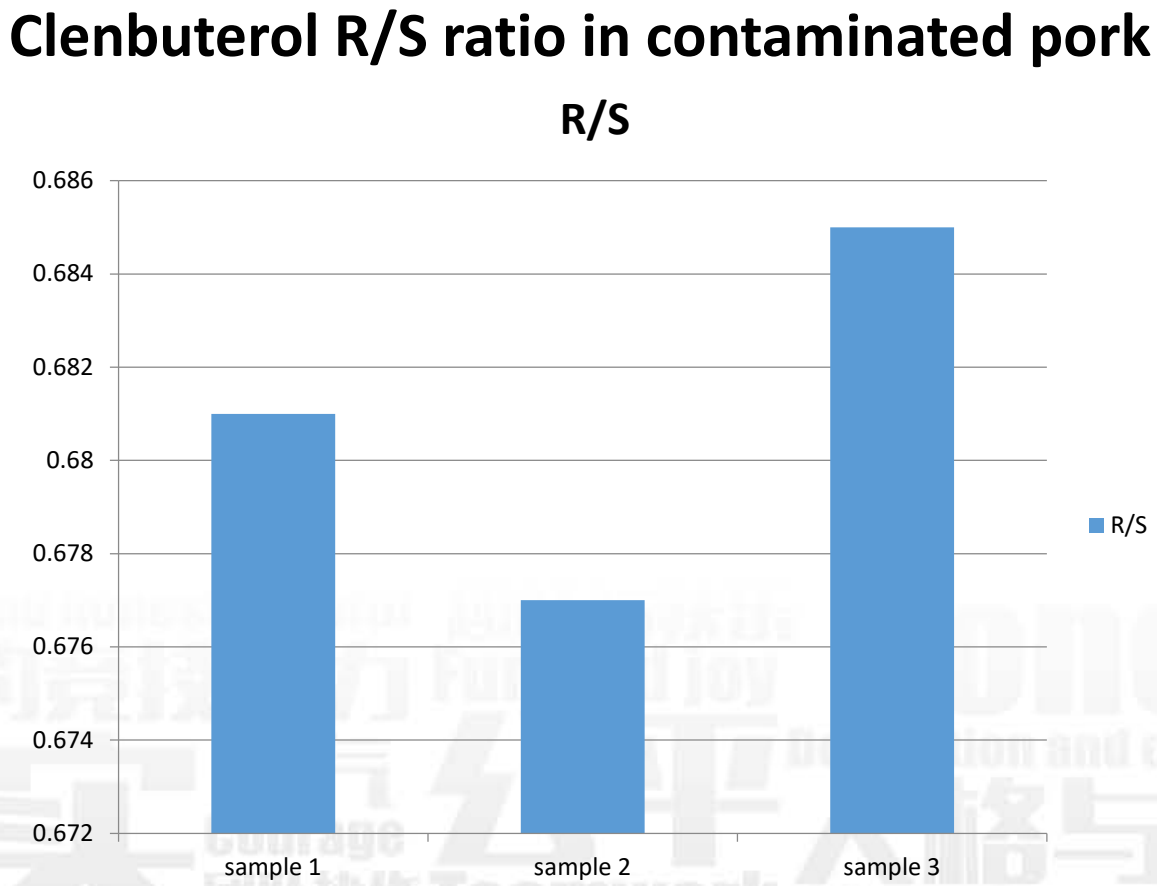
- Urine preparation 2mL urine, 0.5ng D_9-clenbuterol, 0.5mL 12M NaOH, 4mL TBME, shaking for 10min,dried and dissolved with 200uL MP, 10ul injection.

- 38 urine samples from routine analysis which were reported clenbuterol-positive were reanalyzed by R/S ratio.

Method evaluation

- specifiecity:50 urine samples (35 from routine and 15 from the lab staff) no endogenous interfering substance
- LOD 50pg/ml
- Extraction Recovery: 86.2%
- matrix effect: R 91.5%, S 89.8%

- sample1:salughter after 3-day
- sample2:salughter after 5-day
- sample3:salughter after 10-day



Stable enantiomer composition (around 0.7)after 3-day drug withdrawal until slaughtering

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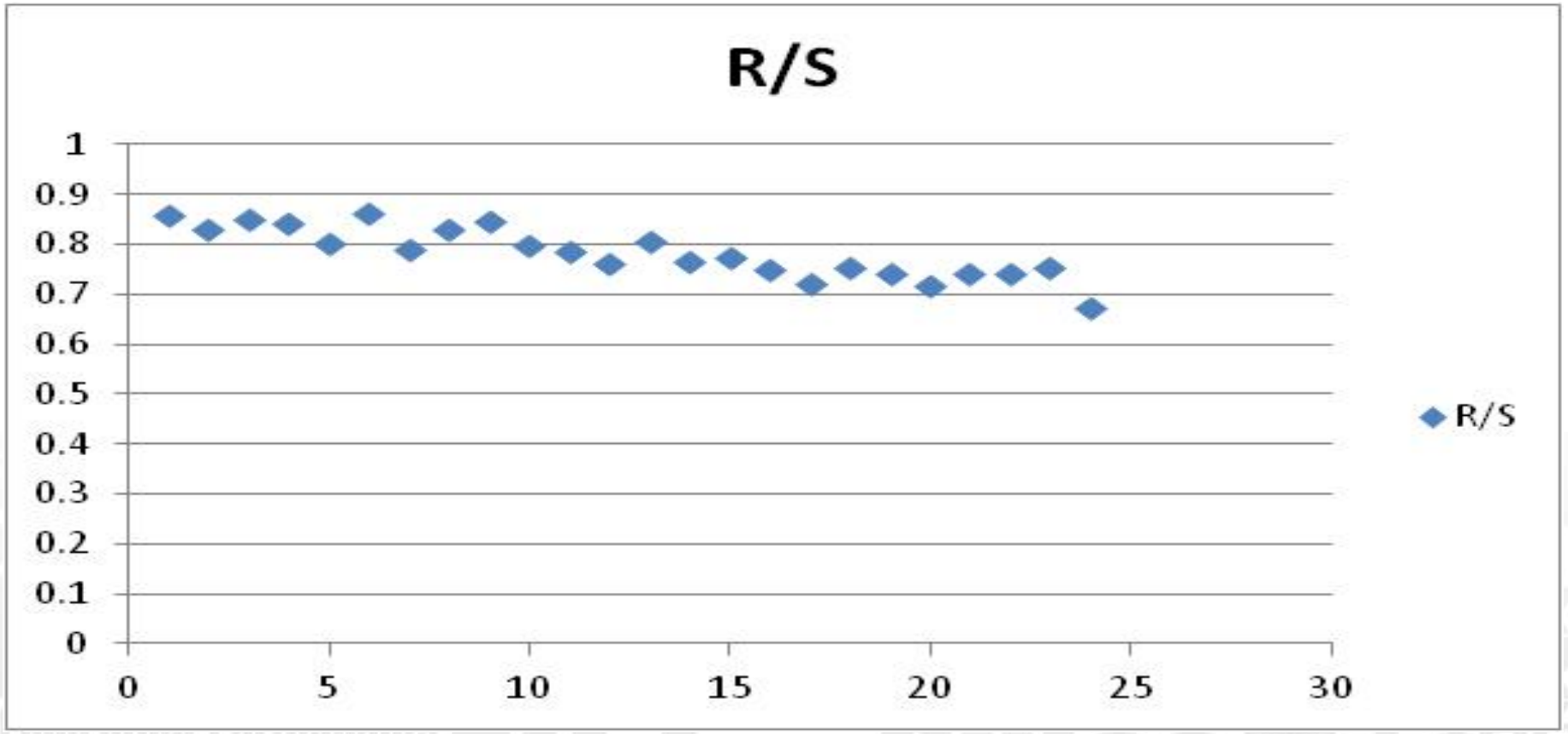
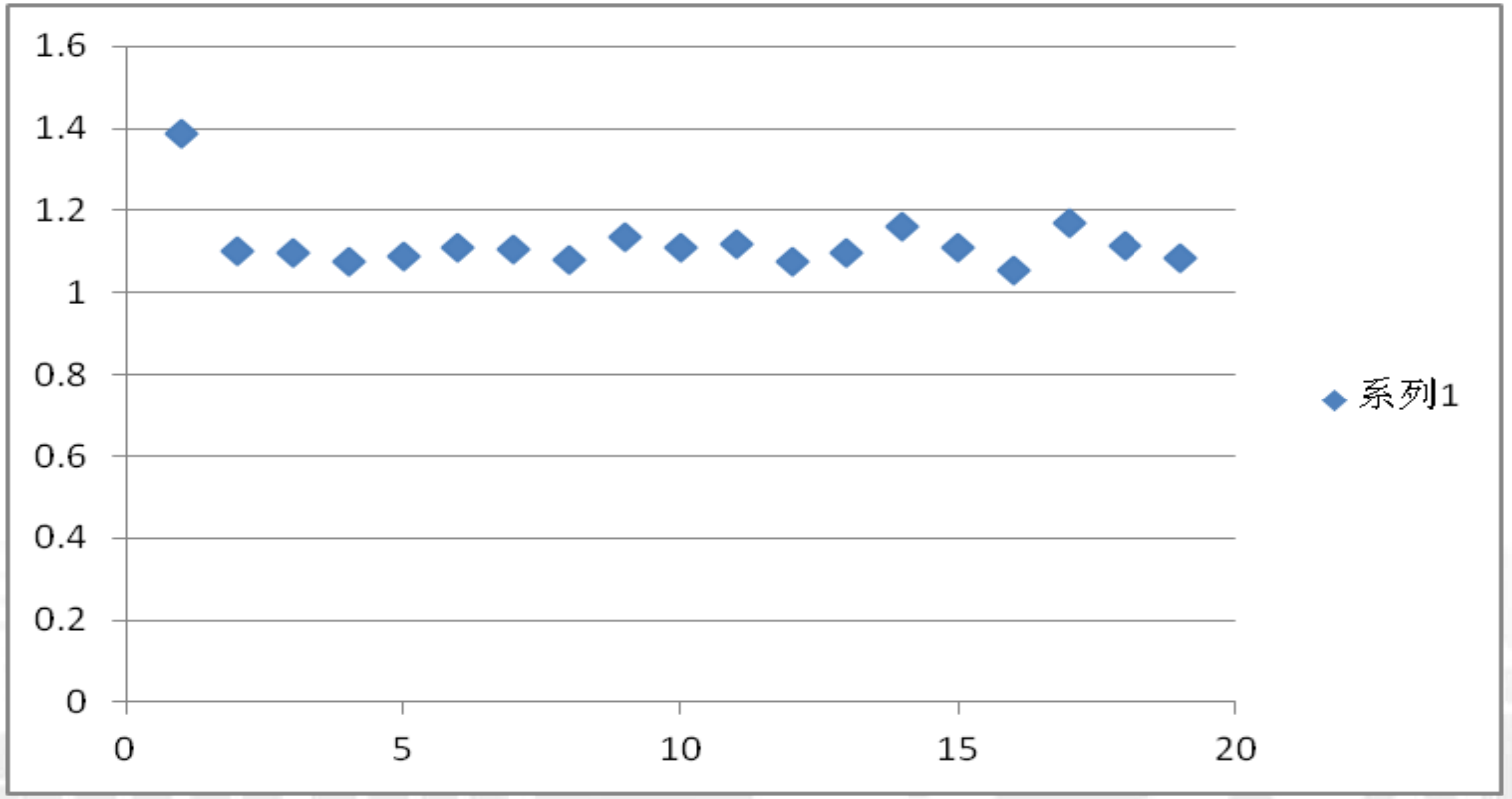
Result from the ingestion of contaminated pork by a volunteer



R/S ratio after ingesting 5 ug clenbuterol by the same volunteer



The above result is in agreement with RCM 2013, R/S ratio is below 1.0 after ingesting contaminated pork, and higher than 1.0 by oral intake.

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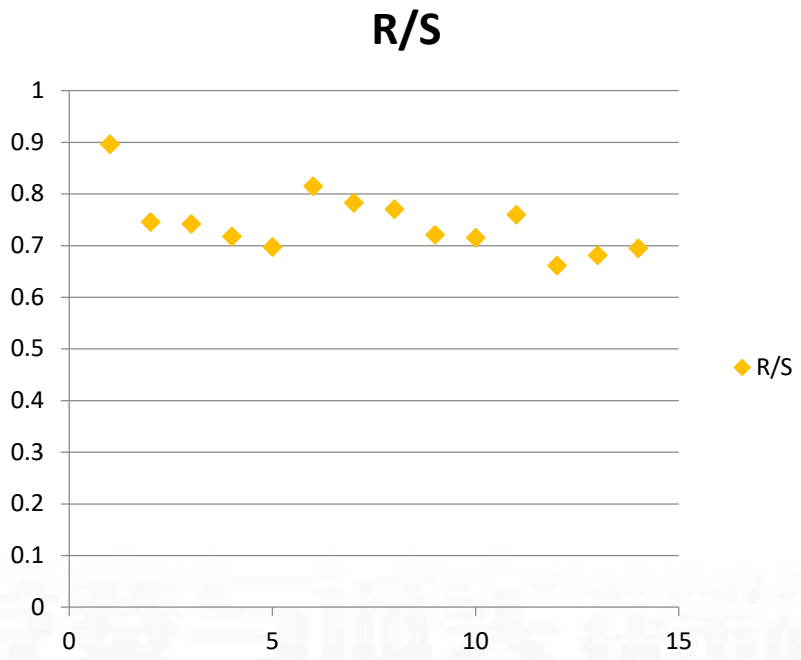
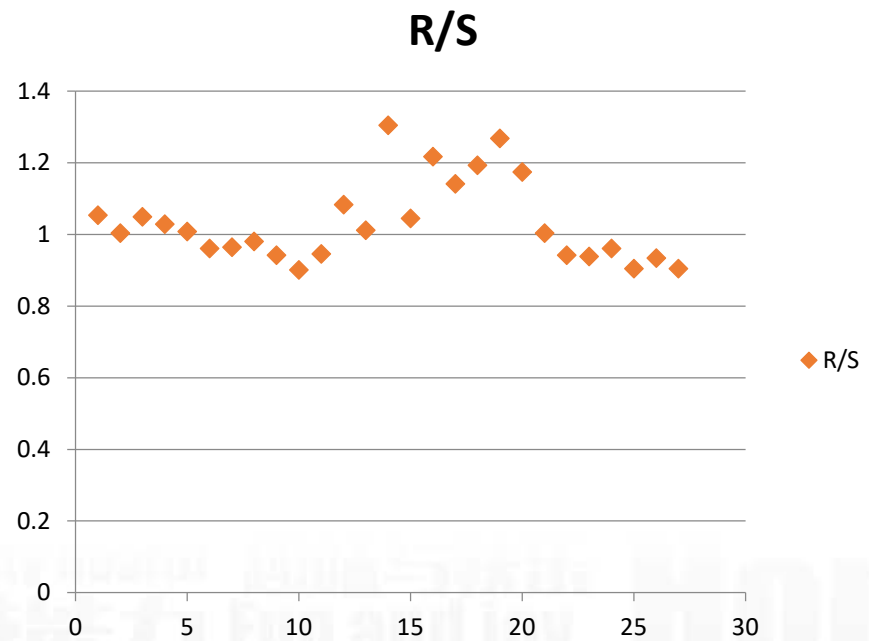
Abnormal case studies： case1



Ingestion of contaminated pork



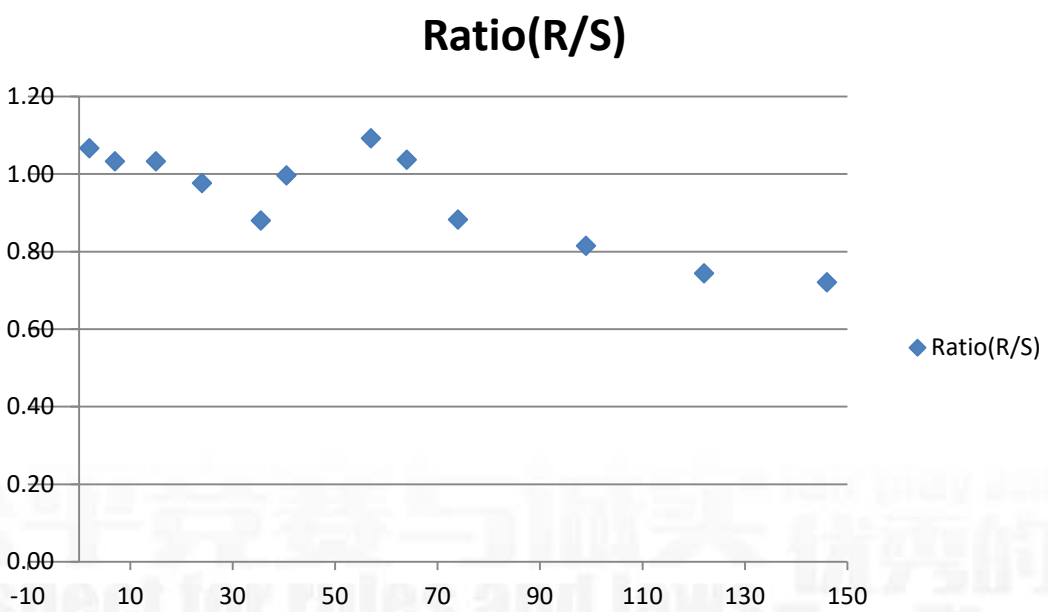
oral intake

some points were between 0.9 and 1.0 after oral intake of 5 ug clenbuterol which may be misjudged as food contamination.

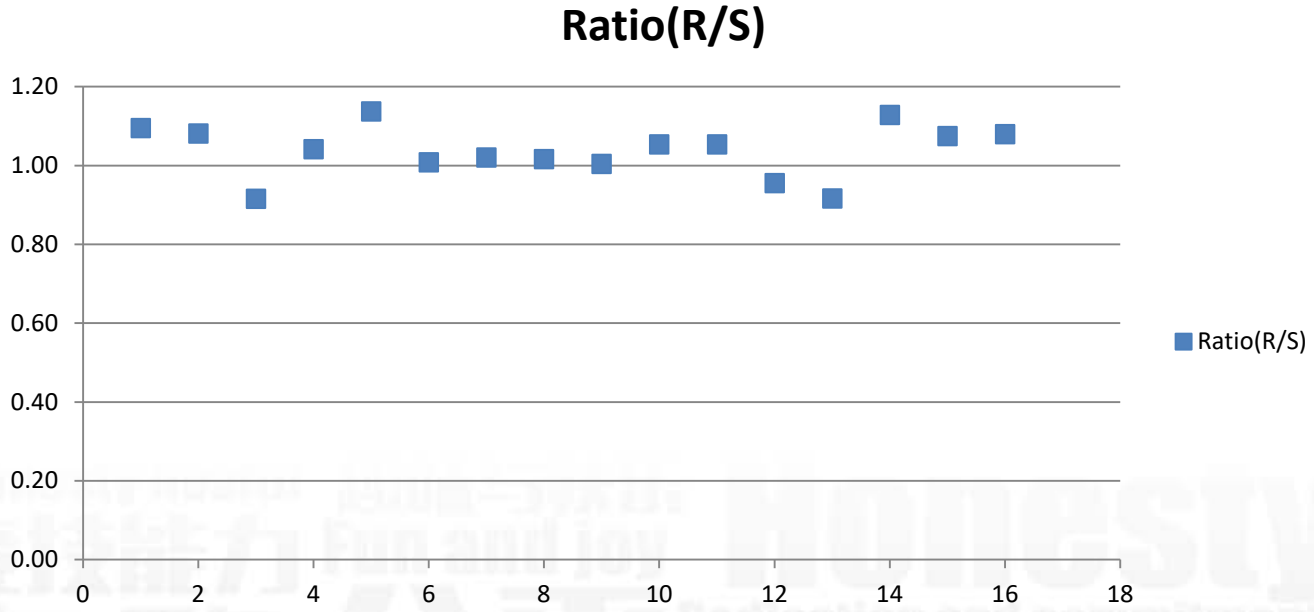
Of all 26 volunteers, 6 volunteers show R/S ratio less than 1 and more than 0.9 after ingesting 5 ug clenbuterol，

all volunteers after intake of 5 ug clenbuterol are more than 0.9

- Case 2



a bad cold



recovery

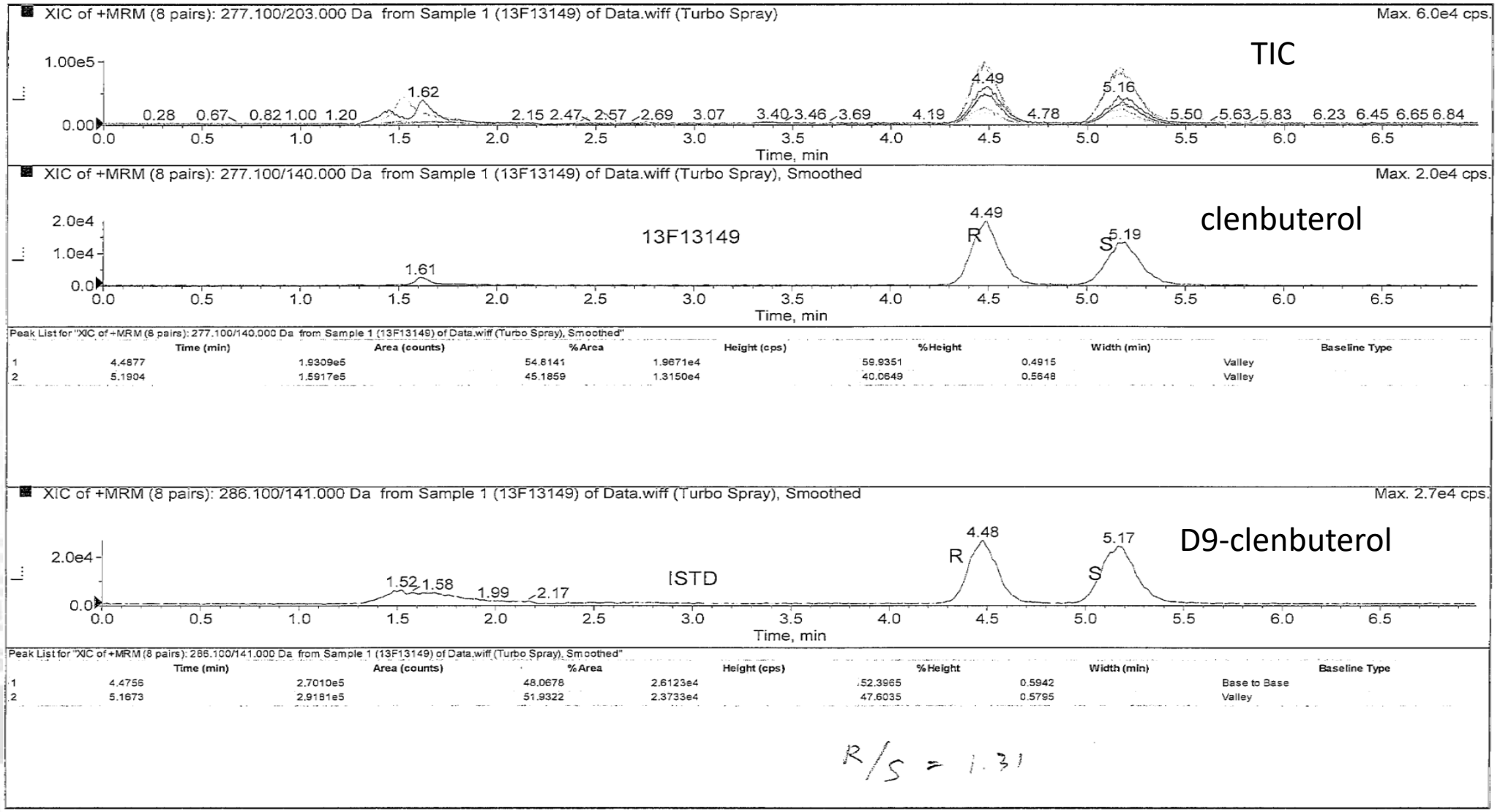
A volunteer had a bad cold during the period of ingesting 5 ug clenbuterol, R/S ratio show less than 0.9 in 3 days . after his recovery, all time points were higher than 0.9. Do the cold medication, endocrine or bacteria influence R or S depletion?

- 20 cases were in agreement with RCM 2013 result , (contaminated pork < 1, oral intake >1)

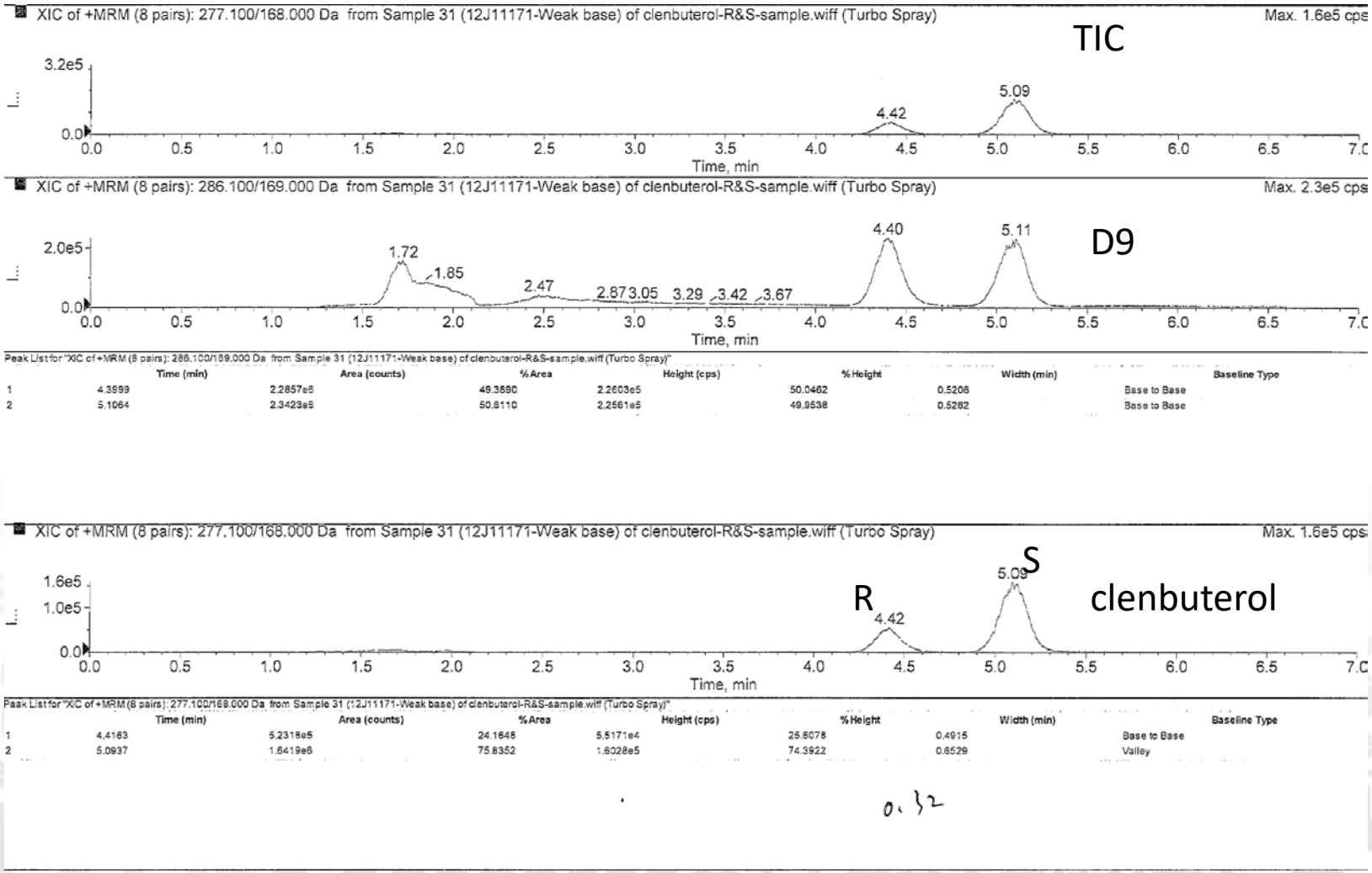
- 6 cases, some time point samples were more than 0.9 and less than 1.

- the criteria is suggested as 0.9 in Chinese population

- **Case 1**


XIC of +MRM (8 pairs): 277.100/203.000 Da from Sample 1 (13F13149) of Data.wiff (Turbo Spray)
Max. 6.0e4 cps.
TIC
1.62
4.49
5.16
Time, min
XIC of +MRM (8 pairs): 277.100/140.000 Da from Sample 1 (13F13149) of Data.wiff (Turbo Spray), Smoothed
Max. 2.0e4 cps.
13F13149
clenbuterol
1.61
4.49
R
5.19
S
Time, min
Peak List for "XIC of +MRM (8 pairs): 277.100/140.000 Da from Sample 1 (13F13149) of Data.wiff (Turbo Spray), Smoothed"
Time (min) Area (counts) %Area Height (cps) %Height Width (min) Baseline Type
1 4.4877 1.9309e5 54.8141 1.9671e4 59.9351 0.4915 Valley
2 5.1904 1.5917e5 45.1859 1.3150e4 40.0649 0.5648 Valley
XIC of +MRM (8 pairs): 286.100/141.000 Da from Sample 1 (13F13149) of Data.wiff (Turbo Spray), Smoothed
Max. 2.7e4 cps.
ISTD
D9-clenbuterol
1.52
1.58
1.99
2.17
4.48
R
5.17
S
Time, min
Peak List for "XIC of +MRM (8 pairs): 286.100/141.000 Da from Sample 1 (13F13149) of Data.wiff (Turbo Spray), Smoothed"
Time (min) Area (counts) %Area Height (cps) %Height Width (min) Baseline Type
1 4.4756 2.7010e5 48.0678 2.6123e4 52.3965 0.5942 Base to Base
2 5.1673 2.9181e5 51.9322 2.3733e4 47.6035 0.5795 Valley
R/S = 1.31

•Case 2


XIC of +MRM (8 pairs): 277.100/168.000 Da from Sample 31 (12J11171-Weak base) of clenbuterol-R&S-sample.wiff (Turbo Spray)
Max. 1.6e5 cps
TIC
3.2e5
0.0
4.42
5.09
Time, min
XIC of +MRM (8 pairs): 286.100/169.000 Da from Sample 31 (12J11171-Weak base) of clenbuterol-R&S-sample.wiff (Turbo Spray)
Max. 2.3e5 cps
D9
2.0e5
1.72
1.85
2.47
2.87
3.05
3.29
3.42
3.67
4.40
5.11
Time, min
Peak List for "XIC of +MRM (8 pairs): 286.100/169.000 Da from Sample 31 (12J11171-Weak base) of clenbuterol-R&S-sample.wiff (Turbo Spray)"
Time (min) | Area (counts) | % Area | Height (cps) | % Height | Width (min) | Baseline Type
1 4.3699 2.2857e6 49.3890 2.2603e5 50.0462 0.5208 Base to Base
2 5.1064 2.3423e6 50.6110 2.2561e5 49.9538 0.5282 Base to Base
XIC of +MRM (8 pairs): 277.100/168.000 Da from Sample 31 (12J11171-Weak base) of clenbuterol-R&S-sample.wiff (Turbo Spray)
Max. 1.6e5 cps
S
R
clenbuterol
1.6e5
1.0e5
0.0
4.42
5.09
Time, min
Peak List for "XIC of +MRM (8 pairs): 277.100/169.000 Da from Sample 31 (12J11171-Weak base) of clenbuterol-R&S-sample.wiff (Turbo Spray)"
Time (min) | Area (counts) | % Area | Height (cps) | % Height | Width (min) | Baseline Type
1 4.4163 5.2318e5 24.1648 5.5171e4 25.6078 0.4915 Base to Base
2 5.0937 1.6419e6 75.8352 1.6028e5 74.3922 0.6529 Valley
0.32

- 38 clenbuterol-positive urine samples obtained from routine analysis were reanalyzed by R/S ratio method, 6 urine samples were less than 0.9 (0.32, 0.55, 0.37 0.39, 0.42, 0.3),32 urine samples were higher than 1.0(the highest: 1.82), these 6 sample could be determined as food contamination.

Conclusion

- A method for resolving clenbuterol enantiomers and measuring R/S ratio was developed and validated.

- Three swines were fed and dosed by clenbuterol, the R/S ratio of pork tissue was close to 0.7 and stable after at least 3-day drug withdrawal until slaughter.

- 26 volunteers participated in two experiments(oral intake of 5ug clenbuterol and ingestion of contaminated pork containing the same amount of clenbuterol

Conclusion

- More than 1,000 urine samples were collected and analyzed by LC-MS-MS with the R/S method
- The R/S ratio was below 0.9 to all volunteers after ingestion of contaminated pork containing 5 ug clenbuterol.
- The R/S ratio for oral intake of 5 ug clenbuterol was between 1.0 and 1.3 in 20 cases and indicated between 0.9 and 1.0 in some time points for 6 cases .
- Clenbuterol could be detected at least one week after a single 5ug-doseage intake
- The criteria is revised as 0.90 in Chinese population and different from the literature result (1.0)
- Other medications may influence clenbuterol enantiomers depletion?

Future work

- Can a bad cold influence clenbuterol enantiomers depletion?
- The R/S ratios after intake of clenbuterol-contaminated beef and mutton
- Metabolic difference between ingestion of contaminated meat and oral intake

Acknowledgements

- Many thanks for financial support from China Anti-Doping Agency and General Administration of Sport of China

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THE END

THANK YOU