Research Article

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Bioanalysis

Advancing the analysis of terbutaline in urine samples using novel enzyme hydrolysis

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Aim: To investigate the efficiency of two new fast-acting enzymes, recombinant arylsulfatase (IMCS-PSF) and mutant β-glucuronidase from *Escherichia coli* (IMCSzyme), in hydrolyzing specific terbutaline metabolites. **Materials & methods:** Two purified novel enzymes are used to precisely determine the amount of each metabolite in urine at different time points after oral administration. After systematically evaluating the hydrolysis efficiency of the novel enzymes compared with commercially available enzymes, these recently developed enzymes were applied to establish the separate urine concentration profiles of terbutaline and each metabolite. **Results & discussion:** The results highlight the highly efficient arylsulfatase enzyme expressed from *E. coli* for urine analysis of terbutaline while suggesting sulfoconjugates as the main terbutaline metabolites. **Conclusion:** This study demonstrated the high efficiency of the IMCS-PSF enzyme in hydrolyzing terbutaline conjugates in comparison with other enzyme reagents typically used for the analysis of terbutaline and sulfoconjugates are the main terbutaline metabolites in urine.

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Terbutaline is a typical short-acting β-agonist that has marketing authorization for use as a treatment for pulmonary diseases, a tocolytic agent (a drug that hinders premature labor) and as a short-term asthma treatment [1–3]. Although it is banned from usage in food production, farmers and manufacturers continue to illegally administer this drug as a growth promoter to their livestock [4]. In addition, terbutaline is most often misused by athletes as a performance enhancing substance, where it has been shown to increase muscle strength and peak power [5], increase the rate of glycolysis [6] and induce muscle hypertrophy [7,8]. Therefore, terbutaline appears on the prohibited substances list of the World Anti-Doping Agency (WADA) both in and out of competition [9]. The issue then occurs with the government regulation of the illegal usage of terbutaline. As a result of its clinical use for the treatment of bronchial diseases, the government cannot completely ban terbutaline as a legal prescription drug. Thus, the only viable solution is for the government to monitor the misusage of terbutaline in athletes effectively.

Despite different administration methods, the major elimination pathway of terbutaline is through urine [10,11]. However, similar to other β-agonists, only trace amounts of the parent drug are present in urine due to extensive metabolism and conjugation [12,13]. Conjugated metabolites in the form of glucuronides and sulfoconjugates are major urinary species (Figure 1) [11,14,15].

In drug testing, hydrolysis of the glucuronide and sulfate linkage prior to analysis is necessary for improved detection [16]. Although attempts have been made to monitor intact glucuronides, such methods are limited by the availability of glucuronide reference standards [11]. Enzyme hydrolysis of β-agonists by commercially available β-glucuronidase(BGus)/arylsulfatase(PAS) enzymes from *Helix pomatia* (crude enzyme) is the standard method used to treat urine samples before instrument detection, other than chemical hydrolysis with hydrochloric acid [10,11,17–19]. More importantly, it has been reported that the ineffective, nonselective and time-consuming nature of this

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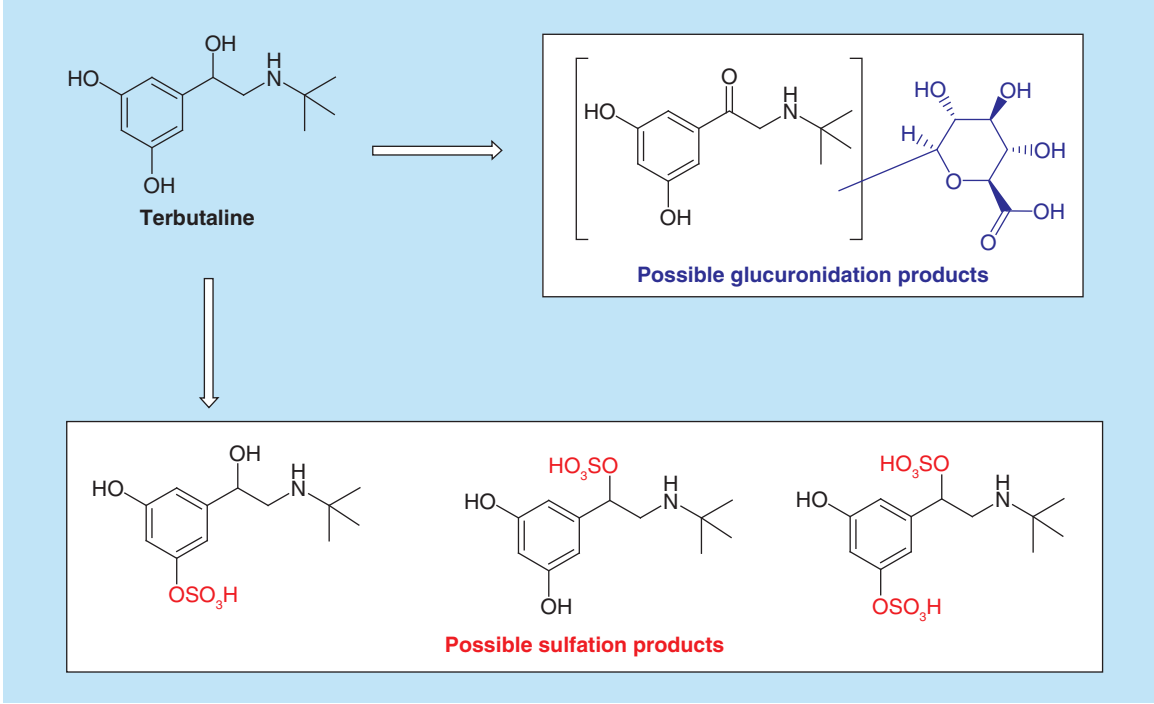

Figure 1. Major metabolic pathways of terbutaline. Terbutaline undergoes sulfation and transforms into terbutaline sulfoconjugates. Terbutaline undergoes glucuronidation and transforms into terbutaline glucuronides.

enzyme results in the inefficient hydrolysis of terbutaline metabolites [11], leading to the inaccurate quantification of terbutaline in urine. Additionally, since most commercial BGus/PAS enzymes are either purified from *H. pomatia* or from abalone (which continually come with unquantified sulfatase in the form of impurities), it is impossible to profile the composition of terbutaline metabolites accurately. From the limited research existing on the metabolism of terbutaline, a cohesive conclusion on the exact metabolic pathways of terbutaline has yet to be solidified. Due to the lack of pure and efficient enzymes in the hydrolysis process of terbutaline metabolites, studies that have attempted to develop a screening process for terbutaline in urine have been unsuccessful.

The purpose of this study was to investigate the efficiency of two new fast-acting enzymes, recombinant arylsulfatase (IMCS-PSF) and mutant β-glucuronidase from *Escherichia coli* (IMCSzyme), in hydrolyzing specific terbutaline metabolites [20–23].This application of enzyme hydrolysis allows for the accurate detection of the unmodified terbutaline concentration in urine using LC–MS/MS, leading to its significant role in the accurate quantification of terbutaline in urine analysis.

Materials & methods

Subjects & ethical approval

Two healthy volunteers (44–48 years; weight: 60–68 kg; height: 163–173 cm; one male, one female) were included in this study. Subjects were without any chronic diseases and without a regular user of other medicines. All subjects gave their written consent to participate in the study. The study was performed in accordance with the Helsinki-II-Declaration and ethical approval was obtained from the local ethical committee. The study was conducted according to the rules of good clinical practice.

Chemicals & reagents

All solvents were purchased from Merck (Millipore, MA, USA). Ultrapure water was made using a Milli-Q Ultrapure system (Millipore, MA, USA). Terbutaline was purchased from Abcam (catalog number: ab142554, MA, USA). Internal standards Terbutaline-D_9 (ISTD) was purchased from WITEGA Laboratorien (catalog number: BA048, Berlin, Germany). All standard solutions were prepared by dissolving preweighed 1 mg of solid analyte from the manufacturer in 1 ml methanol (MeOH) to make 1 mg/ml stock before diluting to desired concentrations in MeOH and stored at -20°C until use. Recombinant glucuronidase (IMCSzyme™, catalog number: 04-ESF3S-050) and

Table 1. Composition of solutions mixture tested in the experiments.

Test condition	Urine	Water	Buffer	Enzyme
Blank urine sample	100 µl	75 µl	25 µl (tris buffer)	None
IMCS sulfatase (IMCS-PSF)	100 µl	50 µl	25 µl (tris buffer)	25 µl
IMCS glucuronidase (IMCSzyme)	100 µl	50 µl	25 µl (tris buffer)	25 µl
H. Pomatia BGus by Sigma (crude enzyme)	100 µl	50 µl	25 µl (NaOAC buffer)	25 µl

BGus: β-glucuronidase; IMCS: Integrated Micro-Chromatography System; PSF: Recombinant arylsulfatase.

arylsulfatase (IMCS-PSF™, catalog number: PAS 17–1106), were obtained from Integrated Micro-Chromatography Systems (SC, USA). BGus from *H. pomatia* (catalog number: G7017), sodium acetate and glacial acetic acid were purchased from Sigma-Aldrich (MO, USA). Tris buffer (1 M, pH = 8.0, catalog number: T1150) was purchased from Solarbio Life Science (Beijing, China).

Instrumental analysis

Analyses were performed using a Waters XEVO Triple Quadrupole Mass Spectrometer (Waters Corporation, MA, USA) coupled to a Waters Acquity UPLC Class I equipped with Waters Acquity UPLC BEH-C_{18} column (2.1 × 50 mm, 1.7 µm, Waters Corporation). The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Our previous study has shown this elution system can effectively separate terbutaline and analogs [24]. The gradient started at 4% B, ramped to 95% B at 3.0 min, where it remained until 5 min and was re-equilibrated to 4% B. The total run time was 8 min. The column flow rate was 0.2 ml/min. Mass spectrometer parameters were: capillary voltage, 3000 V; desolvation temperature, 350°C; desolvation gas flow, 650 l/h; and nebulizer pressure, 7 bar. To quantify free terbutaline in urine samples, the analyte as well as the ISTD were detected by multiple reaction monitoring mode (terbutaline parent: m/z 226.3–152.2 [quan], 226.3–107.2 [qual] and terbutaline-D_9: 235.3–153.0 [quan]). For quantification of the unconjugated terbutaline, calibrant concentrations between 0.1 ng/ml and 20 ng/ml were run. The LOD for terbutaline was 0.1 ng/ml and the LOQ was 0.3 ng/ml.

Urine sample preparation

Urine sample collection

Three tablets of terbutaline (2.5 mg for each tablet as sulfate salt) were administered to two healthy volunteers (44–48 years; weight: 60–68 kg; one male, one female). Urine was collected before drug administration and continuously collected after for 24 h for the male volunteer and 3 days followed by morning urine specimens taken on the two subsequent days for the female volunteer.

Enzyme hydrolysis condition optimization

Urine collected from the male volunteer at 10 h after terbutaline administration was used for the metabolite profiling with enzyme hydrolysis and the sample collected at 5 h was used for the evaluation of the enzyme hydrolysis efficiency. As shown in Table 1, four conditions were employed for the sample preparation. The total volume of each sample was 200 µl. For each condition, six-time points of incubation were used and each condition was performed in triplicates. The sample mixtures were then incubated at 45°C for 0, 15, 30, 60 and 120 min, and overnight, respectively, before being stored at -20°C until further diluted 1000-folds with mobile phase, spiked with ISTD (final concentration is 20 ng/ml) and injected to LC–MS/MS. The temperature selected for this study was acquired from company-recommended optimum conditions for enzyme hydrolysis [25]. For the enzyme concentration optimization study, 100 µl of urine and 25 µl of 1 M Tris buffer pH 8 was incubated with 0, 5, 10, 15, 20, 25 and 50 µl of PSF4 enzyme, respectively, and nanopure water was added to make 200 µl total volume before completion of hydrolysis at 45°C for 1 h. Multi-point calibration curves were prepared in mobile phase with the following calibrators in established linear range for each analyte (0.1–20 ng/ml): 0.1, 0.5, 1, 5, 10 and 20 ng/ml.

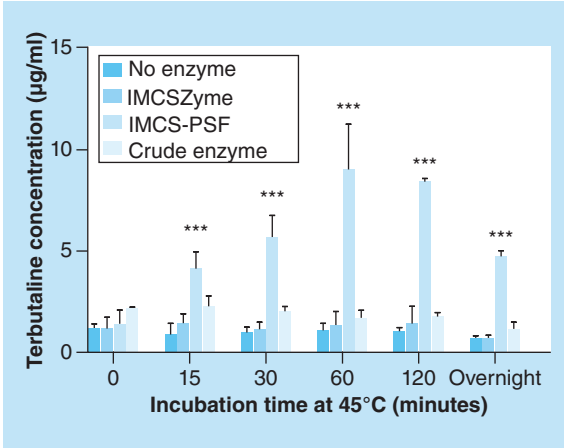

Figure 2. Comparison of the temporal hydrolysis profile of terbutaline metabolites upon hydrolysis with no enzyme, IMCSZyme, IMCS-PSF and crude enzyme.
The data are expressed as mean ± SD (n = 3).
***p ≤ 0.001 is based on ANOVA.
ANOVA: Analysis of variance; SD: Standard deviation.

Urine concentration study

Urine samples collected from the female volunteer were used for the urine concentration study. 100 µl of urine collected at each time point was added with 50 µl water, 25 µl 1 M Tris HCl buffer pH 8 and 25 µl water, PSF4 or BGus enzyme. Then the mixture was incubated at 45°C for 1 h prior to further being diluted 1000-folds with mobile phase, spiked with ISTD (final concentration is 20 ng/ml) and stored at -20°C until injected to LC–MS/MS.

Quantification & statistical analysis

The chromatograms were analyzed and terbutaline concentrations were quantified using the Waters MassLynx software system. Statistical analyses were completed using GraphPad Prism 5 software.

Results & discussion

As shown in Figure 2, at 0 min (no incubation time), the four different sample conditions depicted approximately the same amount of terbutaline concentration. The higher amount of terbutaline detected in samples incubated with crude enzyme could be the result of a matrix effect of the enzyme, which enhances terbutaline signal. Since the current method only used the dilute-and-shoot technique, incorporation of a better sample preparation may correct this condition. Significantly higher hydrolysis efficiency of the PSF4 enzyme on the terbutaline metabolites was observed after just 15 min of incubation at 45°C. After 60 min of incubation, the hydrolysis of the terbutaline metabolites reached plateau, inferring the complete hydrolysis of the metabolites in this particular sample. As shown in Figure 2, the average terbutaline concentration upon PSF4 hydrolysis was 8.5 ppm, which is about four-times higher than the results from crude enzyme hydrolysis (~2.4 ppm), BGus hydrolysis (~1.5 ppm) and no enzyme control (~0.9 ppm). Incubation time from 60 to 120 min did not alter the hydrolysis efficiency of any enzyme. Surprisingly, we observed the decrease of terbutaline in all conditions after overnight incubation. This could be the result from terbutaline degradation after a long period of exposure to relatively high temperatures in complex media such as urine. However, further studies on the stability of terbutaline in different conditions need to be completed to further confirm this phenomenon.

To confirm the complete hydrolysis of terbutaline metabolites in urine samples as well as determine the optimal enzyme concentration needed for general terbutaline analysis in urine, different concentrations of IMCS-PSF enzyme were tested. Urine collected from the male volunteer at 5 h after terbutaline administration was used for this study because prior sample screening as well as previous reports have shown maximum terbutaline sulfoconjugate excretion at this time point [11]. As shown in Figure 3, there is a clear positive relationship between the enzyme volume and the detected free terbutaline response. However, there is no significant difference between 25 and 50 µl of IMCS-PSF enzyme. Therefore, 25 µl of enzyme was selected to be used in the subsequent urine concentration study.

To further validate the application of the novel enzymes in the analysis of terbutaline and its metabolites in urine samples, the time pattern of terbutaline concentration in urine was evaluated. In this study, terbutaline, glucuronidated terbutaline and sulfoconjugated terbutaline were analyzed and calculated from urine samples at

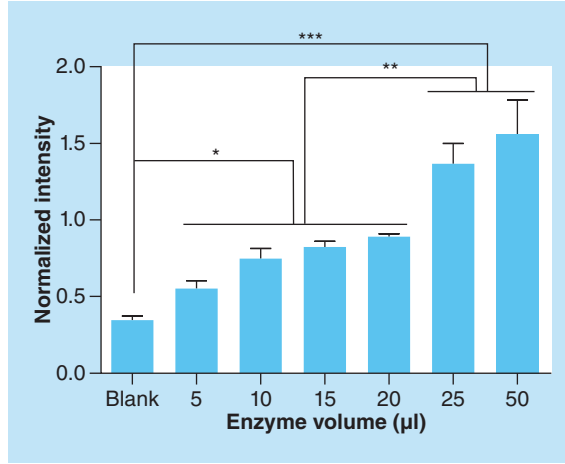

Figure 3. Effect of enzyme concentration on terbutaline metabolite hydrolysis. The 100 µl of urine sample was added with 25 µl 1 M Tris HCl buffer pH 8, varied volume of IMCS-PSF enzyme and water to make 200 µl total sample volume. The hydrolysis temperature and time was controlled at 45°C and 1 h, respectively. The data are expressed as mean ± standard deviation (n = 3, p values based on analysis of variance [ANOVA]).
*$p \leq 0.05$.
**$p \leq 0.01$.
***$p \leq 0.001$.

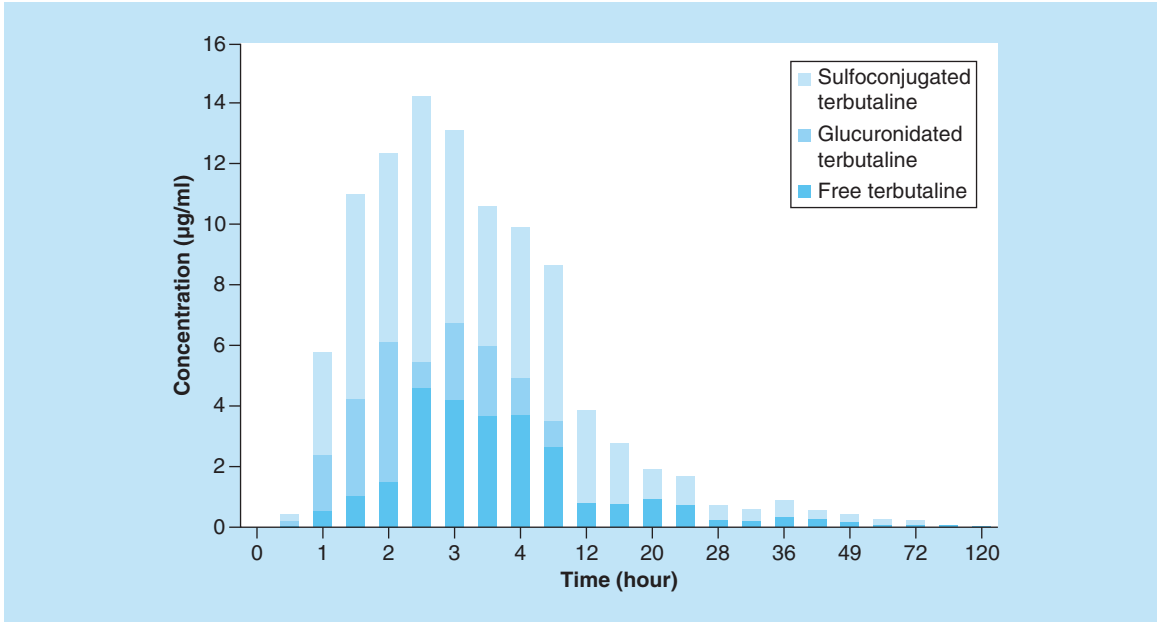

Figure 4. Urine concentration profile of sulfoconjugated terbutaline, glucuronidated terbutaline and free terbutaline. 100 µl of urine collected at each time point was added with 50 µl water, 25 µl 1 M Tris HCl buffer pH 8 and 25 µl water, IMCS-PSF or IMCSzyme. The mixture was incubated at 45°C for 1 h.

different time points. Unmodified terbutaline concentrations at each time point were obtained from urine samples without incubation with any enzyme (control). The glucuronidated terbutaline concentration and sulfoconjugated terbutaline concentration in urine can be calculated by subtracting terbutaline concentration in the samples that were incubated with IMCSzyme enzyme and IMCS-PSF enzyme with terbutaline concentration in the control sample without enzyme, respectively. As shown in Figure 4, terbutaline and its metabolites were detected in urine specimens for approximately 4–5 days. In this study, terbutaline metabolites were detected in urine specimens as early as 30 min after administration while unmodified terbutaline observed was lower than the LOD (0.1 ng/ml). No detection of terbutaline and its metabolites in the blank urine were collected prior to terbutaline administration. The concentration of terbutaline and its metabolites in urine continued to increase within the first 2 h. The highest total terbutaline concentration was observed at 2.5–3 h after administration before it started to decrease. The urine concentration profile of free terbutaline is consistent with literature data obtained in plasma or serum where the peak serum level is obtained around 3–4 h after oral administration [26,27]. Although the maximum concentration of free terbutaline previously reported in urine appeared at lower concentration after 4 h [11,26,27], this could be due

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to the variation between each individual. Additionally, studies have shown that urine concentrations of β-agonists are partially related to the specific gravity and concentrated urine samples which can lead to a higher concentration of the drug in urine [28,29]. This could be another possible cause of the higher urine concentration observed in this study. But more importantly, the curve progression in all studies are similar. In addition, these data were obtained from one volunteer, which is considered too small of a sample size to make robust inferences concerning the accurate time-pattern of urine concentration of terbutaline and its metabolites. For further investigation of the accurate quantification of terbutaline and its metabolites in urine, a higher number of volunteers need to be included.

Although a cohesive conclusion on the exact metabolic pathways of terbutaline is yet to be solidified since contradictory results were previously reported by several groups [11,14,15,30] in our study, terbutaline sulfoconjugates are unarguably the major metabolites of terbutaline, while glucuronide conjugates appear as minor metabolic products (Figure 4). In the 1970s, Nilsson *et.al.* and Davies *et al.* first described sulfoconjugates as the primary metabolites and glucuronides as the minor metabolites of terbutaline in urine samples after BGus and crude enzyme hydrolysis. Contradictorily, in 2009, a study conducted by Orlovius *et al.* identified terbutaline sulfates as the sole metabolites in urine, without prior hydrolysis of the urine samples. They directly detected each terbutaline metabolite using in-house synthesized terbutaline sulfoconjugates as the reference standards for LC–MS/MS analysis. Orlovius *et al.* argued that the detection of the glucuronidated terbutaline in the previous literature reports may have been due to a cross-reactivity of the enzymes used and the less sensitive detection method. Our study has introduced the use of the efficient and selective BGus and PAS enzymes combined with the highly sensitive LC–MS/MS instrument to endorse terbutaline sulfoconjugates as the major metabolites and terbutaline glucuronides as the minor metabolites of terbutaline in urine after oral administration. It is possible that the lack of terbutaline glucuronide reference material in the previous study may have contributed to the misdetection of the low concentration of glucuronidated terbutaline in a complex matrix-like urine specimen.

Conclusion

This study demonstrated the high efficiency of the recombinant arylsulfatase enzyme in hydrolyzing terbutaline conjugates in comparison with other enzyme reagents typically used for the analysis of terbutaline. This illustrates the possibility of the accurate quantification of terbutaline and its major metabolites in urine. In addition, the amount of terbutaline detected in the urine samples began to degrade after overnight incubation. This degradation highlights the advantage of using fast-action enzymes in the hydrolysis and quantification of terbutaline metabolites in urine samples. While other existing protocols are unable to accurately quantify both sulfoconjugates and glucuronic conjugates, this method has confirmed the existence and quantified both kinds of metabolites while establishing sulfoconjugates as the main terbutaline metabolites in urine. However, further studies with a greater number of volunteers need to be conducted to correctly establish the metabolic pathway and/or the excretion profile of terbutaline. This new method of analyzing terbutaline metabolites in urine can lead to the development of more efficient screening protocols. It potentially allows regulatory agencies to enforce accurate antidoping control screening for terbutaline. In addition to its application in antidoping screening, this method can be applied to the regulation of terbutaline and potentially other β-agonists in livestock since they are also widely misused in the food industry.

Future perspective

With the advancement of advanced tandem and hybrid LC–MS instruments, the sensitivity as well as efficiency of the instruments will continuously increase. This requires the parallel improvement of sample preparation to match the ultralow detection limit and shorter analysis time. Enzyme hydrolysis of conjugated analysts before analysis will become a necessary step to improve the detection limit and reduce total analysis time. In addition, there has been a shift from parent compound analysis to nontarget analysis using high-resolution MS and analysis of metabolites and transformation products in the past decade. It is becoming evident that more research is needed to determine the breakdown pathways and to evaluate the fate of transformation products formed. With the development of purified enzyme that can specifically hydrolyze a type of conjugated metabolite, the metabolic pathway studies for many drugs/compounds can be conducted as never before.

Acknowledgements

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Summary points

- Terbutaline sulfoconjugates are the major metabolite of terbutaline, while glucuronide conjugates appear as minor metabolic products.
- The IMCS-PSF enzyme shows higher hydrolysis efficacy in comparison with other enzyme reagents typically used for the analysis of terbutaline.
- The IMCS-PSF enzyme alone is sufficient to hydrolyze terbutaline conjugates in urine for the analysis of terbutaline.
- One-hour incubation of IMCS-PSF enzyme with urine sample at 45°C is sufficient to complete the hydrolysis of terbutaline sulfoconjugates.
- A total of 25 μl IMCS-PSF enzyme per 100 μl of urine sample is sufficient for the analysis of terbutaline in urine samples.
- Long incubation time of hydrolysis enzyme and urine sample should be avoided to prevent terbutaline degradation.
- This new method of analyzing terbutaline metabolites in urine can lead to the development of a more efficient screening protocol to enforce accurate antidoping control screening and the regulation of terbutaline and potentially other β-agonists in livestock.
- This method of analyzing compound metabolites may apply to other studies aiming to identify metabolic pathway of a certain drug and/or routine analysis for therapeutic drug monitoring.

Financial & competing interests disclosure

Q Wang is a shareholder of Integrated Micro-Chromatography Systems (IMCS), LLC. The terms of this arrangement have been reviewed and approved by IMCS, LLC. in accordance with its policy on objectivity in research. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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