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The salmeterol anomaly and the need for a urine threshold

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Abstract (250 words)

Salmeterol is a long acting beta2-agonist (LABA) widely used for treatment of airways disease. There is evidence that beta2-agonists, including salmeterol, have the potential for performance enhancing effects when delivered at supratherapeutic doses. For this reason, all beta2-agonists are currently on the Prohibited List issued by the World Anti-Doping Agency (WADA) regardless of dosing route with some exemptions for inhaled salbutamol, formoterol, and salmeterol when used at therapeutic inhaled doses. For 2020, salmeterol use is permitted up to a therapeutic dosing threshold of 200 µg daily, but unlike salbutamol and formoterol, there is an anomaly; currently there is no urine threshold to control for supratherapeutic dosing beyond this dosing threshold. Salmeterol, however, is reportable as an Adverse Analytical Finding (AAF) at levels above 10 ng/ml. Complicating matters is that following inhalation, salmeterol parent drug is present at relatively low levels compared to other beta2-agonists due to rapid metabolism to the metabolite, alpha-hydroxysalmeterol, which is typically present at higher levels than parent drug. Moreover, peak parent drug levels following permitted therapeutic dosing are below the minimum required performance level (MRPL) of 10 ng/ml for salmeterol (50% of the MRPL that analytical laboratories are required to meet for non-threshold beta2-agonists), hence the presence of salmeterol may be unreported. For consistency, a urine threshold should be introduced for salmeterol as matter of priority, to balance the needs of athletes that use salmeterol therapeutically up to the agreed dosing threshold, with the need to control supratherapeutic dosing for doping intentions and athlete harm minimisation.

1. Introduction

Salmeterol is a long acting beta2-agonist (LABA) that is widely used for the treatment of airway disease, particularly asthma. Salmeterol is usually delivered via metered dose inhaler (MDI) or dry powder inhaler (DPI) twice daily and is used as a "symptom controller" with a duration of action around 12 hours compared to the short-acting beta2-agonist (SABA) salbutamol with a duration of action around 4 hours. Salmeterol is also a chiral compound and is usually administered as the racemate (50:50 mixture of (R)- and (S)-salmeterol), with (R)-salmeterol being the active enantiomer [1]. Enantiopure (R)-salmeterol is reportedly available for purchase online via chemical supply vendors.

There is solid evidence that members of the beta2-agonist drug class, including salmeterol, have the potential for performance enhancing effects when delivered at supratherapeutic doses. When administered at acute high doses, beta2-agonists can enhance muscle power and sprint performance [2-8], and if taken for longer periods, they possesses muscle anabolic properties [9-11], as recently reviewed[12]. For this reason, all beta2-agonists are on the 2020 Prohibited List issued by the World Anti-Doping Agency (WADA) [13] regardless of dosing route – albeit with three exemptions. Salbutamol, formoterol, and salmeterol are allowed by inhalation at therapeutic doses up to a dosing threshold. The therapeutic dosing threshold for salmeterol is 200 µg daily. However, unlike salbutamol and formoterol, there is no urine threshold for salmeterol. Despite being reportable at levels above 10 ng/ml (50% of the Minimum Required Performance Level (MRPL) [14], an unscrupulous athlete could inhale salmeterol at high doses with relative impunity for performance-enhancing purposes.

Here we outline the potential for supratherapeutic misuse of salmeterol and argue that a urine threshold needs to be introduced as a matter of priority.

2. Salmeterol as a performance enhancing drug

Few studies have investigated the potential performance-enhancing effects of salmeterol in humans. Early work in athletes suggested that used acutely at therapeutic low doses (50 µg), salmeterol did not enhance running performance, cycling anaerobic power or muscle strength [15,16]. However, more recent work has demonstrated that daily inhaled treatment with salmeterol (100 µg twice daily) enhanced 30 m sprint performance during a period of 5 weeks in combination with a power and strength program [17]. To our best knowledge, no study has been performed in humans that had muscle hypertrophy as an outcome measure, but studies in rodents show that salmeterol possesses anabolic properties. Thus, there appears to be beneficial effects in rat muscle [18] as well as rat dosing studies [19]. The work by Moore et al [19] was based on the theory that a long duration of action is required for beta2-agonists to evoke an anabolic response, and compared the potent anabolic relatively non-selective beta2-agonist clenbuterol with salmeterol by equimolar dosing of rats via different routes of administration. Given orally, salmeterol caused significant increases in body and carcass weight, and in the mass of mixed fibre gastrocnemius/plantaris and tibialis anterior muscles, but no increase in slow-twitch soleus muscles [19]. These effects were similar to that observed with clenbuterol, apart from an additional response seen in soleus muscle. However, when given by infusion, salmeterol demonstrated a dramatic increase in soleus muscle mass. These results indicate that the anabolic potency of salmeterol *in vivo* is dependent on the route of administration; this is highly relevant to dosing via the inhaled route where first-pass metabolism is limited to the swallowed fraction. The other findings from this work were that slow-twitch muscles are less sensitive than mixed-fibre muscles, and observance of ergogenic effects in humans may be

muscle/exercise test specific, possibly accounting for some of the negative findings in older athlete studies. Ryall et al [20] examined the effect of intra-peritoneal injections of formoterol and salmeterol in rats for therapeutic applications in muscle wasting disease. It was found that while formoterol was more potent than salmeterol, salmeterol demonstrated greater muscle hypertrophy (~12%) in the extensor digitorum longus (fast twitch) and soleus (slow twitch) muscle compared to formoterol, and that both drugs used at around 25 microgram/kg/day are capable of eliciting skeletal muscle hypertrophy with minimal effects on heart muscle. A summary of muscle studies is shown in Table 1.

3. Salmeterol on the Prohibited List

Salmeterol, like formoterol and salbutamol, is permitted to be used via inhalation under certain dosing thresholds to allow athletes with asthma to compete. These thresholds are designed to distinguish between therapeutic dosing (need to treat asthma symptoms) and supratherapeutic dosing (where a performance benefit may be obtained).

Thresholds for salbutamol and formoterol consist of dosing via the inhalation route (metered dose inhaler) with a maximum permitted dose in a 12 and 24 hour period, respectively [13], importantly with corresponding urine thresholds [22] to limit the potential for supratherapeutic dosing for doping purposes. For salmeterol, there is only a dosing threshold with no corresponding urine threshold. Despite the drug being detectable in urine, this effectively means there is no practical way to limit supratherapeutic dosing other than relying on an athlete's honesty to report their dose in the previous 24 hours. This is a similar situation to the SABA terbutaline when administered under a Therapeutic Use Exemption (TUE) [23].

A review of anti-doping figures for the three permitted beta2-agonists, salbutamol, formoterol, and salmeterol, from publicly available sources indicate that there are only a relatively small number of Adverse Analytical Findings (AAFs) attributable to salmeterol as shown in Table 2. Salmeterol, present as the un-metabolised parent drug, is reportable as an AAF at levels above 10 ng/ml (50% of the Minimum Required Performance Level (MRPL)) [14]. From 2010, there has been a significant change in formoterol AAFs per year, a result of formoterol being permitted to be used with corresponding dosing and urine threshold from 2012 onwards. While there appears to be low numbers of salmeterol AAFs, this is most likely due to differences in the pharmacokinetics of salmeterol compared to salbutamol and formoterol, which results in very low urine concentrations as discussed below.

4. Urine levels following dosing

Only a few studies have reported urinary levels of salmeterol or the alpha-hydroxy metabolite, with reports in horses [25,26] and humans [27-29]. Data on salmeterol urinary levels from human studies are summarised in Table 3.

Part of the reason for the lack of data is the relatively low concentrations observed in urine meaning the assay is technically demanding, particularly for routine screening. As reported by Hostrup et al [27], the median urine concentration of the alpha-hydroxysalmeterol metabolite after 4 hours was 2.9 ng/ml, and the median salmeterol concentration was around 8-fold lower at 0.38 ng/ml in non-athletes male subjects who inhaled a 100 microgram dose via a DPI device in resting conditions.

A survey of 7045 routine doping urine samples collected over 1 year, demonstrated that only a small number (0.6%, 45/7045 samples) contained salmeterol, and only eight had levels higher than 0.5 ng/ml, with the two highest at 1.81 ng/ml and 1.29 ng/ml [28]. This was similar to the maximum observed concentration in an accompanying excretion volunteer study (1.27 ng/ml) [28]. The most remarkable result was that only 8 samples had a concentration higher than 0.5 ng/ml, meaning that 87% of the samples had a concentration below the lower limit of quantification of the method. Interestingly from the same manuscript in a volunteer study; in 6 subjects who inhaled an acute dose of 100 microgram salmeterol (MDI device), nine samples (50%, 9/18 samples) were above 0.5 ng/ml compared to 18% (8/45) in the doping control figures where salmeterol was detected. This difference should be largely attributed to sampling outside the time of peak excretion, highlighting that doping control figures cannot be meaningfully compared with clinical pharmacokinetics studies.

In terms of published clinical studies, there are only two other studies [27,30]. Our salmeterol study [30] demonstrated a median (range) of 0.08(3) ng/ml for a 50 µg dose, and 2.1(5) ng/ml for

a 200 µg dose both with urine at 2 h. Both of these doses have not been previously reported so any direct comparison is challenging. Studies at a 100 µg dose have been reported [27,28] but unlike salbutamol, there is no information on the linearity of the dose-urine concentration relationship [31] so concentrations cannot be extrapolated either up or down. Based on a comparison of the three different dosages now reported (50, 100, 200 µg), it appears that this relationship may not be linear at least for peak levels (Figure 1) and appears also highly variable – this is a different situation to salbutamol which is metabolised via a different pathway and is linear [31]. Observation of our previous data [30] shows that at the 50 µg dose, one subject had noticeably high levels compared to the other subjects, but the remainder were less than 0.16 ng/ml which is less than half the median observed by Hostrup et al [27] and less than a quarter of that for Deventer et al [28] . At the 200 µg dose, there was another high outlier (5.7 ng/ml), but the remainder of samples were below 3.5 ng/ml, with a median of 2.1 which was higher than both the Hostrup et al [27] (median 0.38) and Deventer et al [28] (median of 0.64 ng/ml) studies. Both the Hostrup et al [27] and Deventer et al [28] studies were half the dose. In addition, the Hostrup et al [27] samples were corrected for SG whereas salmeterol samples from our study [30] were not corrected for SG. In-house data from our laboratory reveal an inter-subject variability upwards of 50% for urine salmeterol levels, whereas the intra-subject variability is around 40% for urine levels, but only 20% for 0-12-h excretion.

Given the extensive CYP3A4 metabolism of salmeterol, the large inter-subject variability may also be explained by inter-subject pharmacogenetic differences for this enzyme [32], as well as significant potential for dietary inhibitors (well known CYP3A4 dietary inhibitors such as grapefruit juice etc.), which was not controlled in any of these urine levels studies. Like the other beta2-agonists, salbutamol and terbutaline, exercise and dehydration are likely to have a significant effect on urine levels and increase the chance of exceeding any threshold limits [33,34].

To account for this, the urine decision limits for threshold substances, such as salbutamol and formoterol, are adjusted to a USG of 1.020 if the urine samples exceed a USG of 1.018 + 0.002 (the latter represent the maximum uncertainty for the USG measurement). Hence, if a threshold is to be introduced for salmeterol, the corresponding decision limit would be adjusted upwards for concentrated urine samples.

5. The salmeterol anomaly

Based on the observations that salmeterol possesses muscle anabolic effects in rodents, and that other beta2-agonists within the same drug class can enhance muscle power and sprint performance, abductive reasoning suggests that salmeterol's place on WADAs 2020 Prohibited List is warranted. Unlike salbutamol and formoterol that are permitted to be used below a dosing threshold, along with a corresponding urine threshold to limit supratherapeutic dosing, salmeterol only has a dosing threshold with no published urine threshold.

The minimum required performance level (MRPL), is the minimum concentration of a prohibited substance, metabolite or marker that can be reliably detected and identified in routine daily operations, and is used for non-threshold substances.[14] The basic premise of the MRPL is that it harmonises the analytical methods of performance for non-threshold substances across the world. The MRPL for the S3 beta2-agonist class is 20 ng/ml, and salmeterol is reportable as an AAF at levels above 10 ng/ml (50% of the Minimum Required Performance Level (MRPL).[14] This level is around 5-10-fold higher than maximum levels typically observed following salmeterol inhalation therapy. The fact that salmeterol has a dosing threshold but no urine threshold [22] appears an anomaly in itself, aside from the fact that even if salmeterol has a reporting limit (50% MRPL), unlike salbutamol and formoterol, laboratories may not necessarily be capable of measuring salmeterol down to the required level where the active drug is likely to be found (Table 3). Similarly, while the active metabolite appears to be a suitable candidate to measure salmeterol via inhalation and thus act as a urine threshold analyte [27], there is no provision to measure the alpha-hydroxysalmeterol metabolite, either as a threshold or non-threshold substance, and for this to be reported as an AAF.

To our knowledge there is no published research linking the seemingly arbitrary urine concentration of 10 ng/ml (50% MRPL) to the maximum dose threshold of 200 μg. Moreover,

this 10 ng/ml level could be considered generous when considering peak levels from a maximal dose (200 μg) from two studies have been 5.7 ng/ml (Table 3). This could potentially lead to a situation where an athlete could essentially use as much inhaled salmeterol as they wish, limited only by adverse effects. If an AAF did arise, it could be claimed that the result was attributable to maximum threshold dosing levels. The administration route could also be parenteral or oral if so desired. With no way of checking for dosing compliance using a urine threshold, supratherapeutic use could result in performance enhancements and athlete harm.

Conclusion

Salmeterol is likely to be performance enhancing in supratherapeutic doses and is allowed in- and out of competition but since there is no urine threshold, there is currently no way of ensuring use is restricted to dosing thresholds apart from the seemingly arbitrary requirement for reporting at 10 ng/ml. Despite some limitations, the urine thresholds introduced by WADA for salbutamol and formoterol to restrict supratherapeutic dosing works well for the vast majority of athletes who use beta2-agonists therapeutically under permitted regimens. For consistency, a urine threshold should also be introduced for salmeterol as matter of priority, to balance the needs of athletes that use salmeterol therapeutically up to the agreed dosing threshold, with the need to control supratherapeutic dosing for doping purposes and athlete harm minimisation. Further work would be required to ascertain whether the metabolite or parent drug would be the best candidate, and the apparent non-linear dose-urine concentration needs greater exploration.

Conflict of interest

GJ and MH have provided independent scientific reports in anti-doping investigations involving beta2-agonists. Both authors are funded by independent research grants from WADA that pertains to beta2-agonist pharmacology and physiology of the related beta2-agonists salbutamol, terbutaline and formoterol.

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Table 1. In vivo studies of salmeterol effects on muscle mass.

Study	Dose	Species	Duration	Effect				
				Tibialis anterior (increased mass, mg (%) versus control)	Gastrocnemius/ plantaris (increased mass, mg (%) versus control)	Soleus (increased mass, mg (%) versus control)	Extensor digitorum longus (increased mass, mg (%) versus control)	Heart (increased mass, mg (%) versus control)
Ryall et al. 2006[20]	Once daily intraperitoneal (ip) injection µg/kg/d 1 10 25 250 500 1000 2500	Rats (Fisher 344) 270 g	4 weeks	-	-	No effect 5 (5%) 9 (9%) 20 (21%) 27 (28%) 26 (27%) 27 (28%)	No effect 3 (3%) 10 (9%) 31 (28%) 35 (32%) 43 (39%) 43 (39%)	No effect No effect 43 (6%) 109 (16%) 132 (19%) 174 (25%) 112 (16%)
Carbo et al (1997) [21]	Route of administration not specified 3.5 µmol/kg/d (1455 µg/kg/d)	Rats (Wistar) 100-150 g	7 days	-	75 mg/100mg (11%)	No effect	-	No effect
Moore et al [19]	Oral 120 µg/day 2.4 mg/day	Rats (Wistar) 140-190 g	10 days	No effect 212 (24%)*	No effect 655 (25%)*	No effect No effect	No effect No effect	No effect 110 (15%)*

	(631-857 µg/kg/d and 12.6-17.1 mg/kg/d)							
	Osmotic mini-pump 133 µg/day (578-782 µg/kg/d)	170-230 g		212 (24%)*	695 (26%)*	54 (19%)*	54 (28%)*	259 (31%)*

* Estimated from manuscript figures using ImageJ (https://imagej.nih.gov/ij/)

Table 2. Currently permitted beta2-agonists identified as Adverse Analytical Findings (AAFs) in the S3 Beta2-agonist drug class (all sports) from 2010-2017 [24], reflecting the addition of a dosing and urine threshold for formoterol in 2012. *AAFs and ATFs (atypical finding)

	2010*	2011*	2012*	2013*	2014	2015	2016	2017
salbutamol	9	6	6	11	8	16	15	12
formoterol	78	84	0	0	0	0	0	0
salmeterol	6	1	3	9	7	1	1	0

Table 3. Data on urine concentrations of salmeterol after inhaled administration in humans

				Individuals' peak salmeterol urine concentration (ng/ml)			Individuals' peak alpha-hydroxysalmeterol urine concentration (ng/ml)			
Study	**Type**	**Dose**	**Subjects**	**max**	**mean**	**median**	**max**	**mean**	**median**	
Deventer et al. 2011 [26]*	PK-study	100 µg (MDI)	Healthy (23-39 yrs), n=6	1.3	0.6	0.6	-	-	-	2 h post dose
	Biobank analysis of 45 anonymized samples containing salmeterol	Unknown	Athletes	1.8	-	-	-	-	-	n/a
Hostrup et al. 2012 [25]*	PK-study	100 µg (DPI)	Asthmatics, n=10	0.6	0.3	0.4	7.6	3.9	3.8	0-4 h post dose
			Non-asthmatics, n=10	0.7	0.4	0.4	12.3	4.1	3.7	0-4 h post dose
Jacobson et al. 2016 [28]	PK-study	50 µg (MDI)	Healthy, n=6	3	0.6	0.1	-	-	-	2 h post dose
		200 µg (DPI)	Healthy, n=4	5.7	2.6	2.1	-	-	-	2 h post dose
Hostrup et al. (unpublished)*	In-house trials	100 µg (DPI)	Healthy, n=7	0.9	0.4	0.3	15.1	4.6	3.1	0-8 h post dose
		200 µg (DPI)	Athletes, n=14	5.7	2.5	2.0	30.9	6.0	3.3	0-6 h post dose

DPI: dry-powder inhalation; MDI: metered dose inhaler; PK: pharmacokinetics. *analysis by WADA accredited laboratory

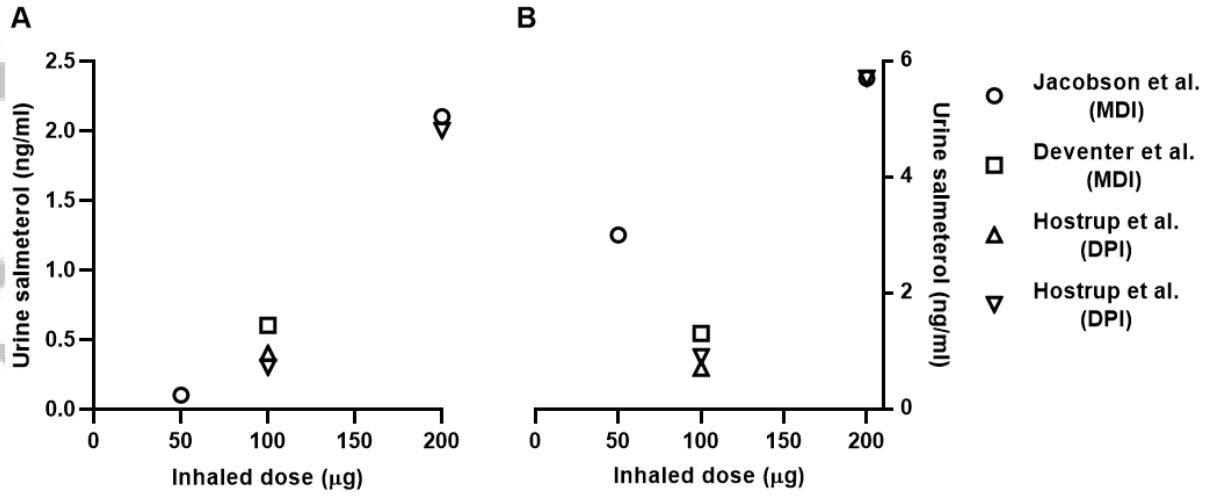

Figure 1. Comparison of median (panel A) and maximum (panel B) free salmeterol urine concentrations from studies reported in Table 3 demonstrating a linear dose-urine relationship for median levels and a non-linear dose-urine relationship for peak concentrations.