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The role of steroid sulphatase in regulating the oestrogenicity of oestrogen sulphamates

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Abstract

Oestrogen sulphamates have increased systemic, but reduced hepatic oestrogenicity which results from their sequestration and transport through the liver by red blood cells. Oestrogen sulphamates act as prodrugs for the release of natural oestrogens but, as yet, there is little information as to how the sulphamoyl moiety is cleaved from the steroid nucleus. In the present investigation we have used the potent steroid sulphatase (STS) inhibitor, 667 COUMATE, to explore the possibility that STS might be responsible for the hydrolysis of oestrogen sulphamates. Administration of oestrone sulphamate (10 μg/day, s.c., for 5 days) to ovariectomised rats resulted in a 3.5-fold increase in the uterine weights of treated animals. Co-administration of oestrone sulphamate and 667 COUMATE (2 mg/kg) completely blocked STS activity in treated animals and completely abrogated the ability of oestrone sulphamate to stimulate uterine growth. In vitro studies, using [^{3}H]oestrone sulphamate or [^{3}H]oestrone, revealed that the uptake of the sulphamate derivative (95.9 ± 2.4%) by red blood cells was considerably higher than that for the non-sulphamoylated oestrogen (25.1 ± 1.9%). Results from these studies demonstrate convincingly that STS is the enzyme responsible for the removal of the sulphamoyl group from oestrogen sulphamates. This enzyme therefore has a crucial role in regulating the oestrogenicity associated with this class of drug.

Keywords: Oestrogens; Oestrogen sulphamates; Steroid sulphatase; Carbonic anhydrase; Oral contraception; Hormone replacement therapy

Natural and synthetic oestrogens are in widespread use for oral contraceptive and hormone replacement therapy (HRT). A major disadvantage with the use of natural oestrogens is that they are rapidly inactivated during their passage through the liver after oral administration [1]. The synthetic oestrogen ethinyloestradiol (EE), which was first synthesised in 1938, is more resistant to metabolism due to the introduction of the 17α-ethinyl group which blocks oxidation of the 17β-hydroxyl group [2,3]. While natural and synthetic oestrogens are therapeutically effective the requirement to administer them at relatively high doses can give rise to a number of adverse side effects [4]. This results mainly from the oestrogenic effects that they exert during their transit and metabolism in the liver. Several factors, including some of the blood clotting system, sex hormone binding globulin, cortisol binding globulin, and angiotensinogen, are all regulated by oestrogens [5].

Recently, a number of sulphamate derivatives of natural oestrogens and EE were synthesised and found to be more active than EE on oral administration to rats [6]. Such compounds included oestradiol-3-*O*-sulphamate (E2MATE, Fig. 1, **1**). The oestrone analogue of this compound oestrone-3-*O*-sulphamate (EMATE,

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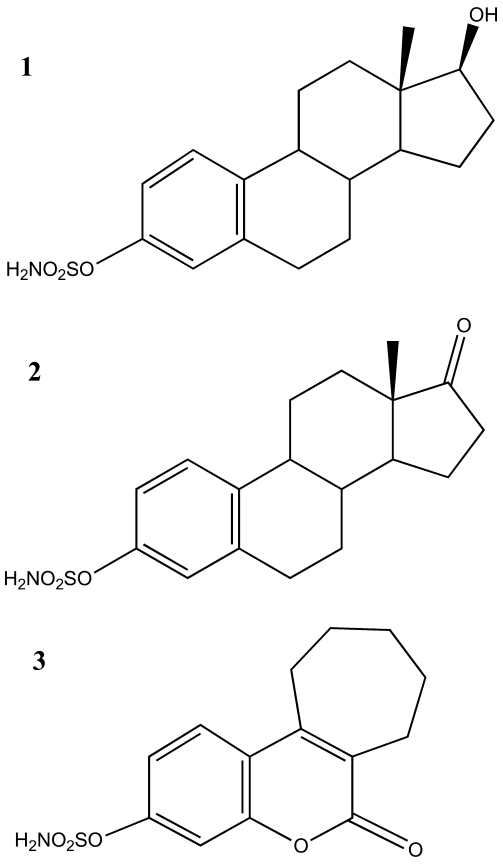

Fig. 1. Structures: **1**, oestradiol-3-*O*-sulphamate (E2MATE); **2**, oestrone-3-*O*-sulphamate (EMATE); and **3**, 6-oxo-8,9,10,11-tetrahydro-7*H*-cyclohepta-[*c*][1]benzopyran-3-*O*-sulphamate (667 COUMATE).

Fig. 1, **2**) was originally developed as the first potent irreversible inhibitor of steroid sulphatase (STS) activity [7]. While EMATE was being developed as an STS inhibitor, parallel studies were in progress to explore the use of oestrogen sulphamates for oral contraception and HRT. It was subsequently revealed that the reason for the enhanced oral activity of oestrogen sulphamates resulted from their ability to be sequestered into red blood cells (rbcs) and to transit the liver without undergoing first pass metabolism [7,8]. In addition to avoiding hepatic inactivation, their sequestration into rbcs also prevents them from exerting excessive oestrogenic effects on the liver. It has been suggested that the therapeutic use of oestrogen sulphamates would therefore combine the convenience of oral treatment with the metabolic advantages of transdermal administration [8].

While oestrogen sulphamates therefore have considerable potential for development for oral contraception and HRT a major question still to be resolved is how they exert their oestrogenic effects. Oestrogen sulphamates are unable to bind to the oestrogen receptor (ER) in rat uterine cytosol or to displace oestradiol from the ER, indicating that per se they are not oestrogenic [8]. It had previously been suggested that the mechanism by which compounds, such as EMATE, inhibit STS activity might involve the irreversible sulphamoylation of an amino acid at the active site of the STS enzyme, with the subsequent release of the oestrogen steroid nucleus [9]. Thus, it is possible that STS may have a crucial role in regulating the oestrogenicity associated with this new class of oestrogen. In the present study we have employed the non-steroidal, non-oestrogenic, STS inhibitor, 667 COUMATE (Fig. 1, **3**), to examine the role of STS in modulating the potent oestrogenicity of oestrogen sulphamates such as EMATE. Inhibition of STS activity by 667 COUMATE was found to totally abrogate the oestrogenicity associated with EMATE.

Materials and methods

Chemicals. Oestrone-3-*O*-sulphamate (EMATE) and 667 COUMATE were synthesised as described previously [7,10]. Both compounds exhibited spectroscopic and analytical data in accordance with their structures and were pure, as shown by high-performance liquid chromatography.

In vivo studies. Female ovariectomised Wistar rats (155–165 g) were purchased from Charles River (Margate, Kent, UK). Experiments were carried out under conditions that complied with institutional requirements. Groups of rats, with three rats in each group, each received EMATE (10 μg/day, s.c. for 5 days), 667 COUMATE (2 mg/kg/day for 5 days, p.o., in tetrahydrofuran: propylene glycol 1:9 v/v) or EMATE plus 667 COUMATE. Control animals received vehicle only. At the end of the 5-day treatment period rats were subjected to terminal anaesthesia, weighed and their uteri and samples of liver tissue, for STS analysis, obtained. Adhering fat tissue was trimmed from uteri prior to recording their weights. Results for uterine weights are expressed as uterine weight/body weight × 100.

Steroid sulphatase assay. STS activity in samples of liver from control and treated animals was measured as described previously [11]. Briefly, tissues were homogenised in phosphate-buffered saline (pH 7.4, 50 mM containing 250 mM sucrose) and supernatants were prepared by centrifugation (2000*g*, 4 °C for 20 min). Duplicate aliquots of tissue supernatants were incubated with [6,7-^{3}H]oestrone sulphate (4×10^5 dpm, Perkin–Elmer, LS, USA) adjusted to a final concentration of 20 μM with unlabelled oestrone sulphate (Sigma, Poole, Dorset, UK). [4-^{14}C]Oestrone (1×10^4 dpm, Perkin–Elmer) was included in the reaction mixture to monitor procedural loses. Samples were incubated for 60 min at 37 °C after which product oestrone was separated from oestrone sulphate by partition with toluene. An aliquot of toluene was removed and ^{3}H and ^{14}C radioactivity was measured by liquid scintillation spectrometry. The mass of oestrone sulphate hydrolysed was calculated from the ^{3}H counts detected corrected for procedural losses. The protein concentration was measured using the Bradford method. Results are expressed as nmol product formed/h/mg protein.

Red blood cell uptake of [^{3}H]EMATE and [^{3}H]oestrone. Whole blood (20 ml) was obtained from a male volunteer. [^{3}H]EMATE (5×10^5 dpm, 30 Ci/mmol, custom synthesised by Sibtech, CT, USA) or [^{3}H]oestrone (5×10^5 dpm, 30 Ci/mmol, Perkin–Elmer) was added to 2 ml aliquots of whole blood. After incubating samples, with mixing at 37 °C for 1 h, rbcs were separated from plasma by centrifugation. Radioactivity present in the rbc and plasma fractions was measured by liquid scintillation spectrometry.

Statistics. The significance of differences in uterine weights of treated and control animals was assessed using Student's *t* test.

Results and discussion

The increased oral potency of oestrogen sulphamates as oestrogens, compared with that of natural or synthetic oestrogens, prompted the present investigations into the role of STS in regulating the oestrogenicity of this new class of oestrogen. EMATE was the first irreversible inhibitor to be developed and proved to be active on oral application to rats [11]. This had not been expected due to the general lack of activity associated with oestrogens when administered by the oral route. While a postulated mechanism by which EMATE inhibits STS activity had previously suggested the possible release of the oestrogen nucleus, the high oestrogenic potency of EMATE and related compounds had not been anticipated. As subsequent studies revealed that oestrogen sulphamates per se were not able to bind to the ER, the role of STS in activating oestrogen sulphamates was examined [8].

Administration of a low dose of EMATE (10 μg/day for 5 days) resulted in a 3.5-fold increase in uterine weight compared with those in control ovariectomised animals ($p < 0.001$) (Fig. 2). The non-steroidal STS inhibitor 667 COUMATE has previously been shown to be non-oestrogenic in this uterine weight gain assay and this was confirmed in the present investigation [12]. The co-administration of EMATE plus 667 COUMATE, however, completely blocked the ability of EMATE to stimulate uterine growth, showing that EMATE per se is not oestrogenic. There was no significant difference in the uterine weights of control animals and those receiving EMATE plus 667 COUMATE. Measurement of STS activity in livers of animals receiving 667 COUMATE alone or EMATE plus 667 COUMATE revealed that it was almost completely inhibited (99.9%) (Fig. 3). These findings, therefore, convincingly demonstrate that STS is critically involved in regulating the oestrogenicity associated with oestrogen sulphamates and that it is the enzyme responsible for the cleavage of the sulphamoyl moiety from these compounds. For animals receiving the low dose of EMATE, which exerted a potent oestrogenic effect, STS activity was inhibited by 73% compared with that in control animals. For these animals, the remaining STS activity is presumably sufficient to cleave the suphamoyl moiety from EMATE allowing oestrone to exert its oestrogenic effects and stimulate uterine growth.

This finding has important implications for the therapeutic use of oestrogen sulphamates for oral contraception, HRT, and anti-oestrogenic therapy [13]. In a preliminary volunteer study a dose of 0.5 mg/kg EMATE inhibited STS activity in white blood cells (used to mon-

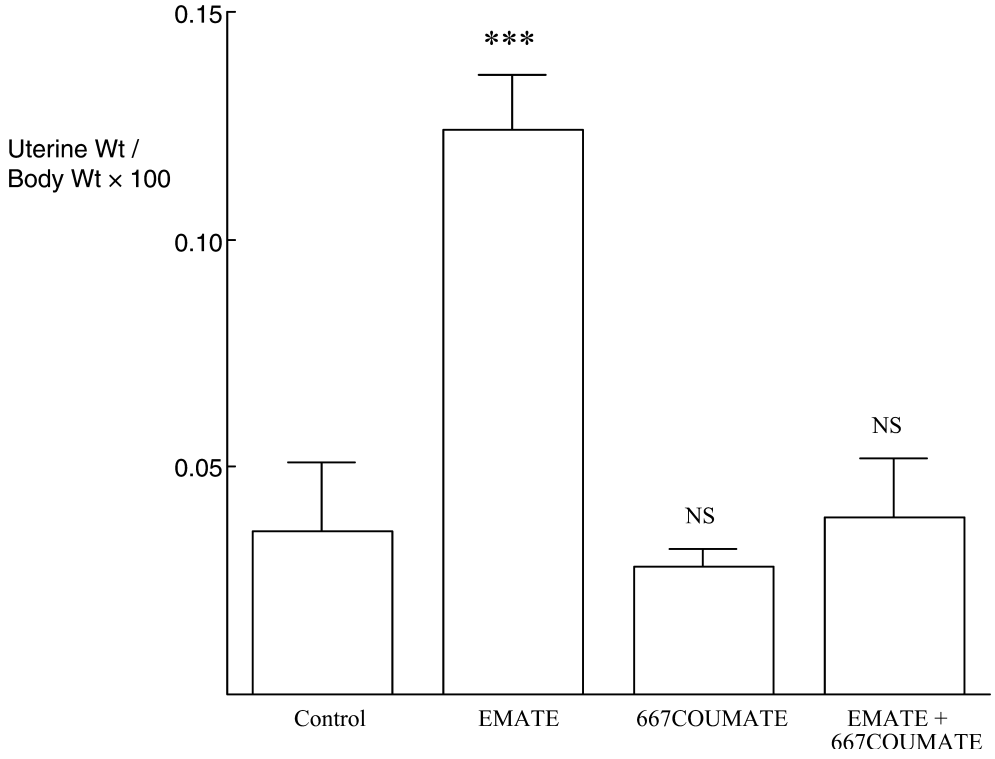

Fig. 2. Inhibition of oestrone-3-*O*-sulphamate (EMATE) stimulated uterine growth by co-administration of the steroid sulphatase inhibitor, 667 COUMATE. Adult female ovariectomised rats were treated with EMATE (10 μg/day, s.c., for 5 days), 667 COUMATE (2 mg/kg/d, p.o.) or with a combination of EMATE and 667 COUMATE. EMATE significantly stimulated uterine growth but its ability to do so was completely abrogated by co-administration of 667 COUMATE (means ± SD, $n = 3$, ***$p < 0.001$).

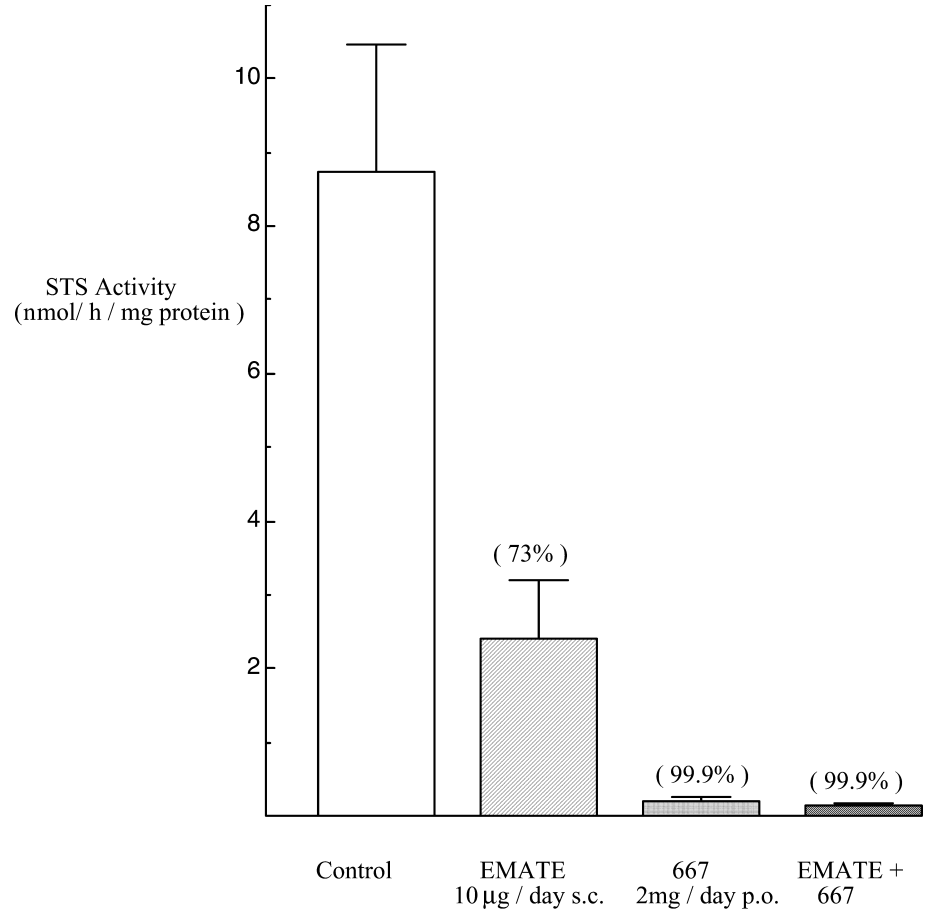

Fig. 3. Inhibition of steroid sulphatase (STS) activity by oestrone-3-*O*-sulphamate (EMATE) or 667 COUMATE. EMATE (10 µg/day, s.c., for 5 days) inhibited STS activity by 73%. 667 COUMATE alone, or in combination with EMATE, almost completely blocked STS activity (99.9%) (means ± SD, $n = 3$. STS activity was significantly inhibited by all treatments, $p < 0.001$, compared with controls).

itor the extent and duration of STS inhibition) within 4 h with complete inhibition being maintained for a prolonged period of time [14]. At this dose, therefore, it would appear unlikely that sufficient residual STS activity would remain to render EMATE oestrogenic. Thus, it may be necessary to use relatively low doses of oestrogen sulphamates to ensure some STS activity remains if this form of compound is to be used therapeutically.

In addition to the role that STS has in regulating the oestrogenicity of these compounds another intriguing finding to emerge from studies with the oestrogen sulphamates is their ability to be transported in rbcs. This not only prevents them from being inactivated during transit through the liver but also protects the liver from excessive oestrogenic stimulation. In the present study the ability of rbcs to specifically take up and retain oestrogen sulphamates was confirmed (Fig. 4). For EMATE, almost all of the labelled compound added to whole blood (95.9 ± 2.4%) was taken up into rbcs in contrast to a much lower value for [^{3}H]oestrone (25.1 ± 1.9%). These findings are in agreement with previous experiments carried out using rat blood [15].

It was not initially apparent as to what compounds such as EMATE might bind to in rbcs. However, as it is known that sulphonamides, which are structurally related to sulphamates, bind to carbonic anhydrase II (CAII) in rbcs, the ability of a number of steroidal and non-steroidal sulphamates to dock into the CAII active site or to inhibit its activity was tested [16,17]. Using an assay for hCAII the sulphonamide acetazolamide, which is used therapeutically, had an IC_{50} value of 25 nM. Using the same assay, IC_{50} values for 667 COUMATE and EMATE were 25 and 42 nM, respectively. It was also revealed that EMATE binds reversibly to hCAII in contrast to its irreversible inhibition of STS activity [17]. Therefore, oestrogen sulphamates have the ability to be transported in rbcs by binding to CAII in a reversible manner. Once transported through the liver the oestrogen sulphamates must be released from rbcs with subsequent removal of the sulphamoyl group being required before they can exert their oestrogenic effects. Although STS is widely distributed throughout the body, little is known about the sites at which such cleavage occurs. As liver is a major site of STS activity, inhibition by oestrogen sul-

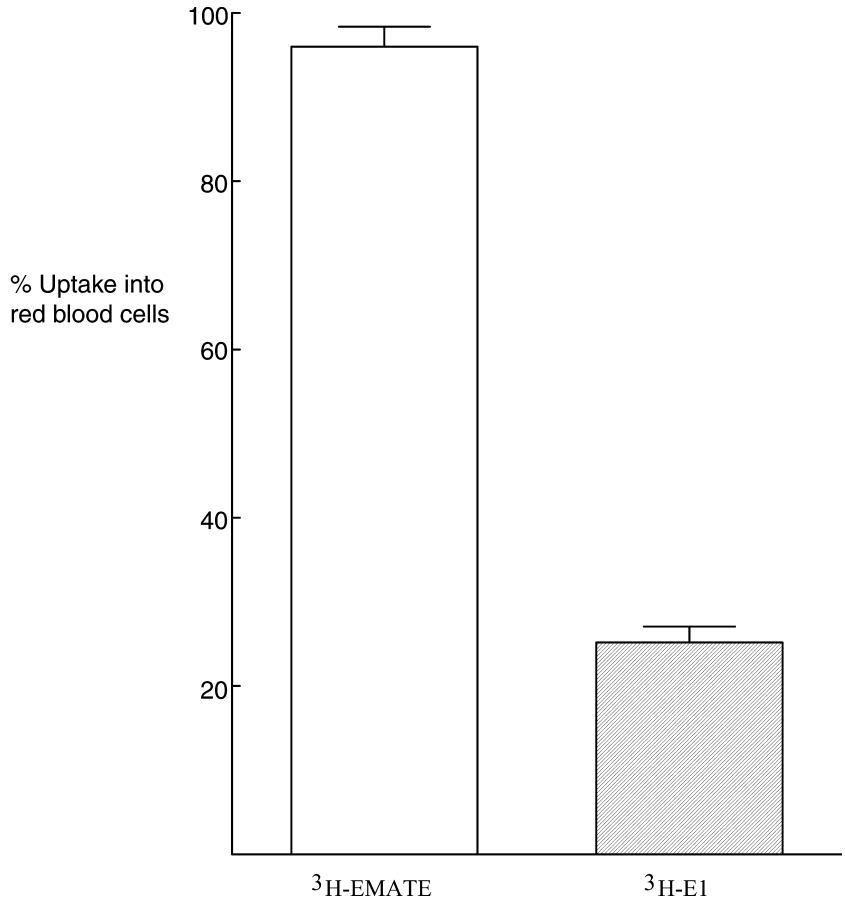

Fig. 4. Uptake of [^{3}H]oestrone-3-*O*-sulphamate ^{3}H-EMATE or [^{3}H]oestrone (^{3}H E1) into red blood cells. Significantly less ^{3}H E1 was taken up into red blood cells than [^{3}H]EMATE ($p < 0.001$) (means ± SD, $n = 3$).

phamates after their oral administration probably contributes to the lack of oestrogenic effects that these compounds have on the liver. In preliminary experiments in rats after a single 10 mg/kg oral dose of an oestrogen sulphamate liver STS activity was found to be completely inactivated within 5–10 min (data not shown).

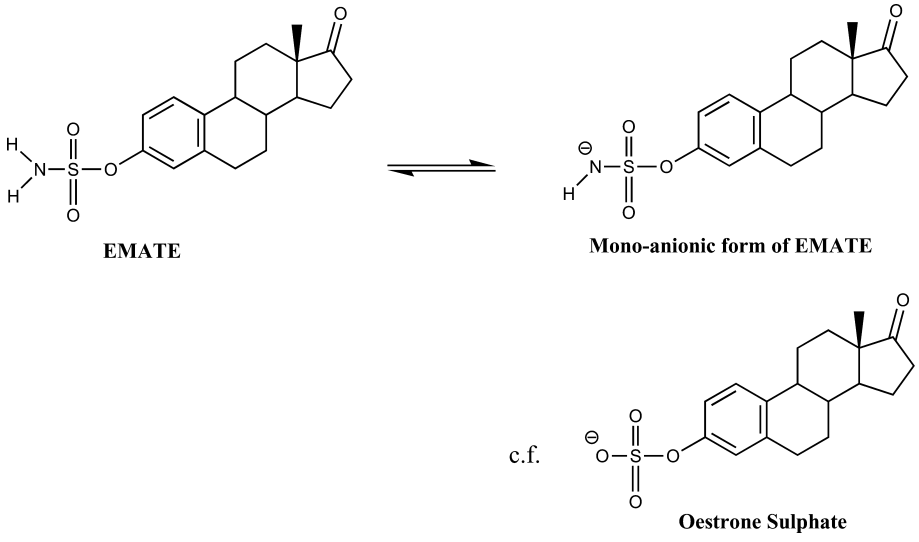

Fig. 5. The ionisation of the sulphamate group of oestrone-3-*O*-sulphamate (EMATE) to its mono-anionic conjugate base form. Oestrone sulphate in its anion form is included for comparison.

Biochemically it is relatively straightforward to understand why an apparently neutral sulfamate should be cleaved by a steroid sulphatase, an enzyme that normally turns over a charged sulphate ester. It has been shown crystallographically that EMATE binds to the crystal structure of carbonic anhydrase II, as expected, using a coordination of the sulphamate *anion* to the active site zinc atom [18]. Moreover, previous studies on various aryl sulphamates by our group and others have shown that the N-proton is fairly acidic with a pK_a value of ca. 9.5 for EMATE (in 70% aqueous MeOH) [19] and ca. 9.1 for 667 COUMATE (in 50% aqueous MeOH) [10]. This implies that at physiological pH a proportion of the weakly acidic EMATE and 667 COUMATE would be in their conjugate base form. Since such a mono-ionised sulphamate will be essentially isoelectronic and isosteric with the ionised ester (Fig. 5) it is anticipated that this species will be well recognised by the sulphatase. What limits the "pro-drug" effect, however, in this case is that enzymatic cleavage is accompanied by irreversible inhibition.

In summary, oestrogen sulphamates represent the first new class of oestrogen to be developed since the identification of EE in 1938. However, it is apparent from the present study that while this new class of oestrogen has some novel properties, i.e., ability to be sequestered by rbcs, its ability to act as an oestrogen is controlled by STS. If this form of oestrogen therapy is to be successfully used therapeutically it will be important to use doses of the compounds which do not completely inhibit STS activity. It is predicted that complete inhibition of STS activity would suppress the oestrogenicity of this class of compound.

Acknowledgment

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