

Synthesis and characterization of octopamine sulfate, norfenefrine sulfate and etilefrine sulfate reference materials for doping control

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Abstract

BACKGROUND: Doping is the use of prohibited substances by athletes to improve their performance. World Anti-Doping Agency (WADA)-accredited laboratories require various metabolite reference standards of the prohibited chemical substances or drugs for routine quality control. Therefore, it was proposed to develop efficient synthetic methodologies for highly pure reference materials of Phase II metabolites of octopamine, norfenefrine and etilefrine, which are prohibited in sports by WADA under the S6 stimulant category. The reference materials were characterized using various analytical techniques. New high-performance liquid chromatography with diode-array detection (HPLC-DAD) methods were developed for purity assessment.

RESULTS: The synthesized Phase II metabolite reference standards, i.e. octopamine sulfate, norfenefrine sulfate and etilefrine sulfate, were confirmed by ¹H NMR, ¹³C NMR, liquid chromatography–high-resolution mass spectrometry (LC-HRMS), attenuated total reflectance Fourier transform infrared and thermogravimetric analysis. In the LC-HRMS study, the mass error value of synthesized compounds was less than 10 ppm (error) which confirms the identity of the reference materials. New HPLC-DAD method were developed to ensure the purity of the reference materials. We used the HILIC column as metabolite reference standards are highly polar. The mobile phase was composed of water and acetonitrile in fixed composition. The HPLC-DAD purity of the developed reference materials was observed as 100%.

CONCLUSION: We have developed reproducible synthetic routes for octopamine sulfate, norfenefrine sulfate and etilefrine sulfate, which are prohibited in sports by WADA. The synthesized metabolites were characterized using different advanced analytical techniques. These reference standards will be helpful to all WADA-accredited laboratories in routine anti-doping testing. © 2023 Society of Chemical Industry (SCI).

Supporting information may be found in the online version of this article.

Keywords: synthesis; characterization; spectroscopy; process design

INTRODUCTION

The World Anti-Doping Agency (WADA) came into existence in 1999 to promote anti-doping practices.¹ WADA aims to provide a fair play environment and it is an independent international organization dealing with the illicit use of chemical substances or non-approved drugs.² The prohibited drug substances include anabolic androgenic steroids, 2-agonists, hormone antagonists and modulators, diuretics, various peptide hormones and growth factors that are subject to extensive drug testing for sports in doping control programs.³ Many prohibited substances have no threshold restrictions and identity confirmation because of the non-availability of reference materials.^{4,5} Various types of mushrooms, plants and mixtures of wine and herbs were used by ancient Greeks and Romans to enhance the performance of athletes in 776 BC.⁶ Since 2004, this agency has started generating

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lists of prohibited drugs and methods for doping-free sports under the world anti-doping code.⁷⁻¹⁰ As of now, athletes are allowed to take some substances with a corresponding urinary threshold as mentioned in the WADA Technical Document of 'Decision limits for the confirmatory quantification of exogenous threshold substances by chromatography-based analytical methods (TD DL)' to distinguish allowed use from prohibited systemic misuse. These drugs are permitted with a therapeutic use exemption up to a decision limit level in various situations, where alternatives of these threshold substances are not available.¹¹ The decision limit for urine samples is high, which also includes the combined measurement uncertainty, and urine samples of threshold substances containing more than the decision limit are considered an adverse analytical finding and will lead to a doping case. Currently, athletes use peptide molecules to improve their athletic performance.^{12,13} It is reported in the literature that a high oral dose of these substances has an anabolic or harmful impact.¹⁴ Octopamine, norfenefrine and etilefrine structure are closely related to norepinephrine and were restricted under the S6 stimulants category in 2005–2006 because of performance-amplifying properties.¹⁵ Clinically, octopamine and norfenefrine are being used as adrenergic drugs.¹⁶ Etilefrine works as an anti-hypotensive drug, also known as an analeptic and sympathomimetic agent.¹⁷ These drugs undergo Phase II metabolism and sulfate conjugation happens to excrete from the body as octopamine sulfate (OS), norfenefrine sulfate (NS) and etilefrine sulfate (ES).¹⁸⁻²⁰ These conjugated sulfate metabolites have better hydrophilicity and are excreted through urine. Liquid chromatography–mass spectrometry (LC/MS) is a widely used technique for quantifying different substances from biological samples because of its high sensitivity if standards are available.²¹ Drugs previously undetectable at particular sample concentrations are now determined using new methods and sophisticated advanced instruments.^{3,22} These conjugated standard metabolites are essential for monitoring the amount of doping agents in athletes' blood or urine samples.^{23,24} Previously, our laboratory has synthesized three different reference materials, carboxytoremifene, *para*-hydroxyprenylamine and norethylmorphine, and supplied them to NDTL for further supply to WADA-accredited laboratories for routine QAQC use.²⁵⁻²⁹ However, reference materials of the target metabolites are required for calibration of test results and confirmation of dope control methods, simplifying the metabolic assessment of prohibited drugs.³⁰ As such, it was proposed to develop efficient synthetic routes for highly purified reference materials of OS, NS and ES (as shown in Figure 1), which are prohibited in sports by WADA under the S6 stimulant category for anti-doping studies.³¹⁻³³

The synthesized material has already been delivered to NDTL, New Delhi for further supply to all the WADA-accredited laboratories for routine testing.³⁴ Consequently, synthesis of drug metabolites as reference materials has emerged as a new research area within synthetic organic chemistry, which bridges the gap between synthetic, medicinal and analytical chemistry.^{9,12,35-37}

MATERIALS AND METHODS

All chemicals, reagents and solvents were purchased from a commercial source and were used as received without further purification. High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were obtained from M/s Rankem Pvt and Limited, India. Potassium bromide (KBr) was purchased from M/s Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). HPLC-grade

water was used for the entire analysis from a Merck Milli-Q water purification unit installed at the Department of Biotechnology, NIPER Guwahati. ¹H NMR and ¹³C NMR spectra were recorded with Bruker 400 MHz, 500 MHz, 126 MHz and 100 MHz spectrometers, in D₂O, CDCl₃ or DMSO-*d*₆. The data are presented as chemical shift (δ) and coupling constant *J* in hertz (Hz). LC/MS/MS: Waters Xevo TQ-XS triple quadrupole mass analyzer. High-resolution MS (HRMS): Thermo Exactive Plus Orbitrap mass spectrometer, Thermo Scientific. HPLC-grade acetonitrile was obtained from M/S Fisher Scientific. Reversed-phase HPLC system: M/s Agilent Technologies series, 1260 infinity HPLC-II system. HPLC system was composed of a G7129A 1260 autosampler, column compartment and a 1260 diode-array detector (DAD). Agilent 1200 series LC pumps and auto-sampler (Agilent, CA, USA) system were used in conjunction with a triple quadrupole mass spectrometer with an electrospray ionization (ESI) source in the LC/MS/MS system. HRMS: Agilent G6545B LC-QTOF.

Chemistry

Conversion of **1** to **7**

To a stirred solution of octopamine hydrochloride (**1**; 1 equiv, 30 mmol, 5.68 g) and Boc₂O (1.1 equiv, 33 mmol, 7.19 g) in 40 mL of DCM at 0 °C, Et₃N (2 equiv, 60 mmol, 6.06 g) was added dropwise. After 30 min, the reaction was set at room temperature for an additional 3 h. The solvent was evaporated under reduced pressure upon completion of the reaction (indicated by TLC). The crude reaction mixture was purified by column chromatography on silica gel (EtOAc/hexane = 4:6) to afford **7** (98% yield, 7.50 g) as a white solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.24 (s, 1H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.65 (d, *J* = 8.5 Hz, 2H), 6.60 (s, 1H), 5.12 (d, *J* = 4.3 Hz, 1H), 4.42 (dt, *J* = 8.3, 4.5 Hz, 1H), 3.06–2.98 (m, 1H), 2.96–2.87 (m, 1H), 1.32 (s, 9H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 156.86, 156.13, 134.47, 127.66, 115.21, 78.06, 71.68, 48.72, 28.77. ATR-FTIR (solid/cm⁻¹) 3397, 3266, 2971, 2925, 2852, 1643, 1613, 1533, 1513, 1451, 1366, 1299, 1244, 1213, 1154, 1069, 958, 897, 850, 829, 794, 750, 704, 629. MS (ESI) *m/z* [M + Na]⁺ calculated for C₁₃H₁₉NNaO₄: 276.1212, found 275.96. HRMS (ESI) *m/z* [M + Na]⁺ calculated for C₁₃H₁₉NNaO₄: 276.1212, found 276.1208.

Conversion of **7** to **10**

TBSCl (4 equiv, 118.5 mmol, 17.77 g) and imidazole (6 equiv, 177.8 mmol, 12.09 g) were added to a solution of **7** (1 equiv, 29.6 mmol, 7.50 g) in 60 mL of DMF and was stirred at room temperature for 4 h. Upon completion of the reaction (indicated by TLC), 40 mL of water was slowly added to the reaction mixture. It was extracted with DCM (20 mL \times 4). Anhydrous Na₂SO₄ was used to dry the organic extract, and under reduced pressure, it was dried to obtain the crude product. Column chromatographic purification was done on silica gel (EtOAc/hexane = 1:10) to afford **10** (94% yield, 13.65 g) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.15 (d, *J* = 8.3 Hz, 2H), 6.77 (d, *J* = 8.5 Hz, 2H), 4.78 (s, 1H), 4.68 (dd, *J* = 7.9, 3.5 Hz, 1H), 3.40–3.29 (m, 1H), 3.02 (dt, *J* = 13.4, 6.5 Hz, 1H), 1.42 (s, 9H), 0.96 (s, 9H), 0.86 (s, 9H), 0.17 (s, 6H), 0.01 (s, 3H), –0.14 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 155.95, 155.01, 135.28, 127.21, 119.83, 79.21, 73.61, 49.12, 29.79, 28.51, 25.91, 25.75, 18.27, 1.11, –4.35, –4.64, –4.97. ATR-FTIR (solid/cm⁻¹) 3311, 2958, 2930, 2857, 1690, 1606, 1533, 1507, 1466, 1396, 1363, 1251, 1163, 1083, 1017, 944, 910, 783, 703, 666. MS (ESI) *m/z* [M + Na]⁺ calculated for C₂₅H₄₇NNaO₄Si₂: 504.2941, found 504.15. HRMS (ESI) *m/z* [M + Na]⁺ calculated for C₂₅H₄₇NNaO₄Si₂: 504.2941, found 504.2943.

Conversion of **10** to **13**

To a solution of **10** (1 equiv, 28.37 mmol, 13.65 g) in 40 mL of DMF, Cs_2CO_3 (1.5 equiv, 42.55 mmol, 13.86 g) was added in portions and was stirred at room temperature for 36 h. After completion of the reaction (indicated by TLC), 35 mL of water was slowly added to the reaction mixture and extracted with EtOAc (20 mL \times 4). Anhydrous Na_2SO_4 was used to dry the organic extract and evaporated under reduced pressure to obtain the crude product. Column chromatographic purification was done on silica gel (EtOAc/hexane = 3:7) to afford **13** (76% yield, 8.10 g) as a colorless sticky solid. ^1H NMR (400 MHz, CDCl_3) δ 7.09 (d, J = 8.5 Hz, 2H), 6.97 (s, 1H), 6.77 (d, J = 8.5 Hz, 2H), 4.99–4.87 (m, 1H), 4.66 (dd, J = 8.3, 3.2 Hz, 1H), 3.43–3.28 (m, 1H), 3.05–2.94 (m, 1H), 1.43 (s, 9H), 0.86 (s, 9H), 0.01 (s, 3H), –0.14 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 156.44, 155.79, 133.83, 127.31, 115.26, 79.85, 73.72, 49.31, 28.53, 25.92, 18.29, –4.58, –4.98. ATR-FTIR (solid/ cm^{-1}) 3350, 2932, 2858, 1686, 1607, 1509, 1455, 1364, 1250, 1164, 1082, 978, 942, 828, 775, 667. HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{19}\text{H}_{33}\text{NNaO}_4\text{Si}$: 390.2077, found 390.2074.

Conversion of **13** to **16**

Compound **13** (1 equiv, 22.07 mmol, 8.10 g) was dissolved in 33 mL of THF and 22 mL of *N,N'*-dimethylpropylene urea (DMPU), and the resulting solution was cooled to –75 °C. Sodium bis(trimethylsilyl)amide (1 mol L^{-1} in THF, 1.1 equiv, 24.27 mmol, 4.44 g, 23.76 mL) was added dropwise, and for 15 min, it was stirred. After that, neopentyl chlorosulfate (1.4 equiv, 30.89 mmol, 5.74 g) was added to the reaction mixture, and the stirring was continued for 3 h. After 3 h, the reaction mixture was warmed to room temperature. After the reaction was complete (indicated by TLC), ethyl acetate (20 mL) and saturated aqueous NaHCO_3 solution were subsequently added, and then it was extracted with EtOAc (20 mL \times 4). Over anhydrous Na_2SO_4 the organic extract was dried and evaporated under reduced pressure to obtain the crude product. The crude product was purified by column chromatography on silica gel (EtOAc/hexane = 1:10) to afford **16** (88% yield, 10.04 g) as a pale-yellow solid. ^1H NMR (400 MHz, CDCl_3) δ 7.36 (d, J = 8.5 Hz, 2H), 7.26–7.23 (m, 2H), 4.82–4.75 (m, 2H), 4.04 (s, 2H), 3.39–3.30 (m, 1H), 3.05–2.97 (m, 1H), 1.41 (s, 9H), 0.96 (s, 9H), 0.87 (s, 9H), 0.03 (s, 3H), –0.12 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 155.91, 149.53, 141.84, 127.52, 120.86, 83.51, 79.50, 73.14, 49.13, 32.00, 28.47, 26.00, 25.86, 18.25, –4.69, –4.95. ATR-FTIR (solid/ cm^{-1}) 2958, 2927, 2857, 1699, 1509, 1467, 1402, 1367, 1255, 1156, 1085, 948, 887, 827, 779, 743, 671, 629. MS (ESI) m/z $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{24}\text{H}_{44}\text{NO}_7\text{Si}$: 518.2608, found 518.39. HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{24}\text{H}_{43}\text{NNaO}_7\text{Si}$: 540.2427, found 540.2427.

Conversion of **16** to **17**

To a solution of **16** (1 equiv, 19.42 mmol, 10.04 g) in THF at 0 °C, *tetra-n*-butylammonium fluoride (70% in water, 1.5 equiv, 29.13 mmol, 7.60 g, 10.8 mL) was added dropwise and the reaction mixture was stirred for 1 h. Then, it was warmed to room temperature, and the stirring continued for 3 h. After the reaction was complete (indicated by TLC), under reduced pressure, THF was evaporated, and 20 mL of ACN was added. It was then extracted with EtOAc (20 mL \times 4), and over anhydrous Na_2SO_4 the organic extract was dried. Under reduced pressure, it was evaporated to obtain the crude product, which was purified by column chromatographic on silica gel (EtOAc/hexane = 3:7). Compound **17** (96% yield, 7.45 g) was afforded as a white solid. ^1H NMR

(400 MHz, CDCl_3) δ 7.40 (d, J = 8.6 Hz, 2H), 7.27 (d, J = 8.7 Hz, 2H), 4.95 (s, 1H), 4.87–4.78 (m, 1H), 4.07 (s, 2H), 3.54 (s, 1H), 3.44 (dd, J = 12.0, 6.7 Hz, 1H), 3.20 (dt, J = 13.8, 6.5 Hz, 1H), 1.43 (s, 9H), 0.99 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3) δ 157.34, 149.66, 141.22, 127.49, 121.15, 83.60, 80.29, 73.55, 48.53, 32.03, 28.36, 25.98. ATR-FTIR (solid/ cm^{-1}) 3362, 2961, 2922, 2858, 1673, 1595, 1515, 1463, 1397, 1379, 1315, 1264, 1145, 1064, 1024, 950, 866, 799, 749, 699, 645. MS (ESI) m/z $[\text{M} - \text{H}]^-$ calculated for $\text{C}_{18}\text{H}_{28}\text{NO}_7\text{S}$: 402.1586, found 402.90. HRMS (ESI) m/z $[\text{M} - \text{COOH}]^-$ calculated for $\text{C}_{19}\text{H}_{30}\text{NO}_9\text{S}$: 448.1641, found 448.1651.

Conversion of **17** to **20**

In a round-bottom flask fitted with a reflux condenser, compound **17** (1 equiv, 18.50 mmol, 7.45 g) was dissolved in 40 mL of DMF, and then sodium azide (1.5 equiv, 27.75 mmol, 1.80 g) was added. In an oil bath, the resulting reaction mixture was stirred at 75 °C for 24 h. After the reaction was complete (indicated by TLC), under reduced pressure, the DMF was evaporated, and the crude reaction mixture was directly subjected to column chromatographic purification on silica gel (EtOH/DCM = 2:8) to afford **20** (97% yield, 6.39 g) as a white solid. ^1H NMR (400 MHz, D_2O) δ 7.28 (d, J = 8.6 Hz, 2H), 7.17 (d, J = 8.7 Hz, 2H), 3.23 (dp, J = 14.0, 8.0, 7.2 Hz, 2H), 1.77 (s, 1H), 1.22 (s, 9H). ^{13}C NMR (100 MHz, D_2O) δ 158.09, 150.70, 138.95, 127.68, 121.55, 100.00, 80.99, 71.91, 46.45, 27.60, 23.27. ATR-FTIR (solid/ cm^{-1}) 3566, 3505, 3385, 2968, 2923, 2857, 1672, 1531, 1426, 1242, 1167, 1046, 867, 805, 735, 714. MS (ESI) m/z $[\text{M} - \text{Na}]^-$ calculated for $\text{C}_{13}\text{H}_{18}\text{NO}_7\text{S}$: 332.0804, found 331.90. HRMS (ESI) m/z $[\text{M} - \text{Na}]^-$ calculated for $\text{C}_{13}\text{H}_{18}\text{NO}_7\text{S}$: 332.0804, found 332.0808.

Conversion of **20** to **4**

To a stirring solution of compound **20** (1 equiv, 18.00 mmol, 6.39 g) in 40 mL of DCM, CF_3COOH (10 equiv, 180 mmol, 20.52 g, 13.8 mL) was added dropwise, and the resulting reaction mixture was stirred for 4 h at room temperature. After the reaction was complete (indicated by TLC), the white precipitate formed was isolated by simple filtration. It was then washed with DCM (15 mL \times 4) to afford pure product **4** (98% yield, 4.10 g) as a white solid. ^1H NMR (400 MHz, D_2O) δ 7.36–7.31 (m, 2H), 7.21 (dd, J = 8.4, 1.5 Hz, 2H), 4.89 (dd, J = 8.0, 3.6 Hz, 1H), 3.21–3.14 (m, 1H), 3.13–3.04 (m, 1H). ^{13}C NMR (100 MHz, D_2O) δ 151.19, 137.35, 127.46, 121.95, 69.10, 45.21. ATR-FTIR (solid/ cm^{-1}) 3417, 3225, 3157, 2966, 2881, 1682, 1604, 1496, 1213, 1130, 1044, 1004, 938, 873, 832, 728, 653. MS (ESI) m/z $[\text{M} - \text{H}]^-$ calculated for $\text{C}_8\text{H}_{10}\text{NO}_5\text{S}$: 232.0280, found 231.91. HRMS (ESI) m/z $[\text{M} - \text{H}]^-$ calculated for $\text{C}_8\text{H}_{10}\text{NO}_5\text{S}$: 232.0280, found 232.0282.

Conversion of **2** to **8**

To a stirred solution of norfenefrine hydrochloride (**2**; 1 equiv, 25 mmol, 4.740 g) and Boc_2O (1.1 equiv, 27.5 mmol, 5.995 g) in 35 mL of DCM, Et_3N (2 equiv, 50 mmol, 5.05 g) was added dropwise at 0 °C. After 30 min, the reaction was set at room temperature and continued to stir for an additional 3 h. After the reaction was complete (indicated by TLC), under reduced pressure, the solvent was evaporated. The crude reaction mixture was then purified by column chromatography on silica gel (EtOAc/hexane = 4:6) to afford **8** (99% yield, 6.29 g) as a colorless sticky solid. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.25 (s, 1H), 7.05 (t, J = 7.8 Hz, 1H), 6.67 (dd, J = 17.2, 9.5 Hz, 3H), 6.57 (s, 1H), 5.24 (d, J = 4.3 Hz, 1H), 4.45 (dt, J = 8.4, 4.5 Hz, 1H), 3.05 (ddd, J = 13.3, 6.3, 4.8 Hz, 1H), 2.91 (ddd, J = 13.3, 7.9, 5.2 Hz, 1H), 1.33 (s, 9H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 157.66, 156.16, 145.81,

129.41, 117.13, 114.37, 113.38, 78.11, 71.99, 48.80, 28.77. ATR-FTIR (solid/cm⁻¹) 3466, 3328, 3149, 3096, 2975, 2925, 1657, 1586, 1546, 1479, 1445, 1371, 1259, 1159, 1088, 1050, 989, 882, 786, 686. MS (ESI) *m/z* [M + Na]⁺ calculated for C₁₃H₁₉NNaO₄: 276.1212; found 276.01. HRMS (ESI) *m/z* [M - H]⁻ calculated for C₁₃H₁₈NO₄: 252.1236, found 252.1239.

Conversion of **8** to **11**

TBSCl (4 equiv, 99.2 mmol, 14.88 g) and imidazole (6 equiv, 148.8 mmol, 10.11 g) were added to a solution of **8** (1 equiv, 24.8 mmol, 6.29 g) in 55 mL of DMF. The reaction mixture was stirred at room temperature for 4 h. After completion of the reaction (indicated by TLC), 40 mL of water was slowly added to the reaction mixture and was extracted with DCM (20 mL × 4). The organic extract was dried over anhydrous Na₂SO₄, and the solvent was evaporated under reduced pressure to obtain the crude product, which was further purified by column chromatography on silica gel (EtOAc/hexane = 1:10) to afford **11** (98% yield, 11.68 g) as a colorless oil. ¹H NMR (500 MHz, CDCl₃) δ 7.15 (t, *J* = 7.8 Hz, 1H), 6.89 (d, *J* = 7.6 Hz, 1H), 6.81 (s, 1H), 6.71 (dd, *J* = 7.9, 1.7 Hz, 1H), 4.81–4.72 (m, 1H), 4.69 (dd, *J* = 7.8, 3.8 Hz, 1H), 3.43–3.28 (m, 1H), 3.02 (dt, *J* = 13.4, 6.5 Hz, 1H), 1.42 (s, 9H), 0.96 (s, 9H), 0.88 (s, 9H), 0.17 (s, 6H), 0.03 (s, 3H), -0.11 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 155.94, 155.65, 144.19, 129.27, 119.31, 119.17, 117.83, 79.29, 73.58, 49.04, 28.52, 25.92, 25.77, 18.31, 18.28, -4.30, -4.62, -4.98. ATR-FTIR (solid/cm⁻¹) 3461, 3376, 2940, 2893, 2859, 1712, 1593, 1489, 1362, 1259, 1163, 1093, 971, 951, 832, 776, 700, 668. HRMS (ESI) *m/z* [M + Na]⁺ calculated for C₂₅H₄₇NNaO₄Si₂: 504.2941, found 504.2932.

Conversion of **11** to **14**

To a solution of **11** (1 equiv, 24.2 mmol, 11.68 g) in 40 mL of DMF, Cs₂CO₃ (2.5 equiv, 60.50 mmol, 19.60 g) was added in portions and was stirred at room temperature for 48 h. After completion of the reaction (indicated by TLC), 40 mL of water was slowly added to the reaction mixture and was extracted with EtOAc (20 mL × 4). Anhydrous Na₂SO₄ was used to dry the organic extract, evaporating under reduced pressure to obtain the crude product. This was purified by column chromatography on silica gel (EtOAc/hexane = 3:7) to afford pure product **14** (98% yield, 8.70 g) as a colorless sticky solid. ¹H NMR (400 MHz, CDCl₃) δ 7.24 (t, *J* = 7.8 Hz, 1H), 6.97–6.79 (m, 3H), 6.38 (s, 1H), 4.96 (s, 1H), 4.79 (d, *J* = 7.4 Hz, 1H), 3.47 (dd, *J* = 13.7, 6.4 Hz, 1H), 3.13 (dt, *J* = 13.0, 6.2 Hz, 1H), 1.53 (s, 9H), 0.98 (s, 9H), 0.12 (s, 3H), 0.00 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 156.27, 156.10, 144.21, 129.47, 118.05, 114.62, 113.11, 79.69, 73.73, 49.06, 28.51, 25.92, 18.30, 1.10, -4.63, -5.00. ATR-FTIR (solid/cm⁻¹) 3351, 3240, 3066, 2962, 2892, 2858, 1675, 1587, 1538, 1471, 1372, 1332, 1259, 1161, 1091, 1020, 973, 934, 867, 818, 777, 698, 662, 633. MS (ESI) *m/z* [M - H]⁻ calculated for C₁₉H₃₂NO₄Si: 366.2101, found 366.09. HRMS (ESI) *m/z* [M - H]⁻ calculated for C₁₉H₃₂NO₄Si: 366.2101, found 366.2095.

Conversion of **14** to **18**

Compound **14** (1 equiv, 23.7 mmol, 8.70 g) was dissolved in 33 mL of THF and 2 mL of DMPU, and the resulting solution was cooled to -75 °C. Sodium bis(trimethylsilyl)amide (1 mol L⁻¹ in THF, 1.1 equiv, 26.07 mmol, 4.77 g, 26.01 mL) was added dropwise, and the resulting reaction mixture was stirred for 15 min. After that, neopentyl chlorosulfate (1.4 equiv, 33.18 mmol, 6.17 g) was added to the reaction mixture, and the stirring was continued for 3 h. Then the reaction mixture was warmed to

-20 °C and stirred for 2 h. After completion of the reaction (indicated by TLC), ethyl acetate (20 mL) and saturated aqueous NaHCO₃ solution were added (at -20 °C), and then the reaction system was allowed to reach room temperature. After that, it was extracted with EtOAc (20 mL × 4). The organic extract was dried over anhydrous Na₂SO₄, and the solvent was evaporated under reduced pressure to obtain the crude product, which was further purified by column chromatography on silica gel (EtOAc/hexane = 3:7) to afford **18** (45% yield, 4.30 g) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.38 (t, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 1.9 Hz, 1H), 7.30 (d, *J* = 7.7 Hz, 1H), 7.22 (ddd, *J* = 8.1, 2.5, 1.0 Hz, 1H), 4.93 (s, 1H), 4.89–4.82 (m, 1H), 4.08 (s, 2H), 3.58–3.40 (m, 2H), 3.22 (dt, *J* = 13.8, 7.1 Hz, 1H), 1.44 (s, 9H), 1.00 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 150.48, 144.65, 130.04, 124.82, 120.27, 118.70, 100.00, 83.56, 80.36, 73.65, 48.55, 32.03, 28.40, 26.03. ATR-FTIR (solid/cm⁻¹) 3422, 2970, 2933, 2876, 1691, 1612, 1584, 1513, 1484, 1447, 1396, 1370, 1247, 1189, 1166, 1128, 1100, 1047, 957, 860, 793, 694, 636. MS (ESI) *m/z* [M + Na]⁺ calculated for C₁₈H₂₉NNaO₇S: 426.1562, found 426.36. MS (ESI) *m/z* [M + Na]⁺ calculated for C₁₈H₂₉NNaO₇S: 426.1562, found 426.1551.

Conversion of **18** to **21**

In a round-bottom flask fitted with a reflux condenser, compound **18** (1 equiv, 10.6 mmol, 4.3 g) was dissolved in 40 mL of DMF, and then sodium azide (1.5 equiv, 15.75 mmol, 1.02 g) was added. The reaction mixture was stirred at 75 °C in an oil bath for 24 h. After completion of the reaction (indicated by TLC), the DMF was evaporated under reduced pressure, and the crude reaction mixture was directly purified by column chromatography on silica gel (EtOH/DCM = 2:8) to afford **21** (69% yield, 2.60 g) as a white solid. ¹H NMR (500 MHz, D₂O) δ 7.25 (t, *J* = 8.0 Hz, 1H), 7.12–7.04 (m, 3H), 4.61 (t, *J* = 6.1 Hz, 1H), 3.16 (qd, *J* = 13.9, 6.2 Hz, 2H), 1.17 (s, 9H). ¹³C NMR (100 MHz, D₂O) δ 158.11, 151.21, 143.23, 129.94, 123.99, 120.90, 119.33, 81.02, 71.96, 46.47, 27.58. ATR-FTIR (solid/cm⁻¹) 3399, 2968, 2924, 2852, 2119, 1691, 1519, 1448, 1389, 1364, 1237, 1176, 1141, 1064, 950, 917, 873, 806, 740, 719, 669, 641. MS (ESI) *m/z* [M - Na]⁻ calculated for C₁₃H₁₈NO₇S: 332.0804, found 331.98. MS (ESI) *m/z* [M - H]⁻ calculated for C₁₃H₁₇NNaO₇S: 354.0623, found 354.2967.

Conversion of **21** to **5**

To a stirring solution of compound **17** (1 equiv, 7.3 mmol, 2.60 g) in 30 mL of DCM, CF₃COOH (6 equiv, 43.80 mmol, 4.99 g, 3.35 mL) was added dropwise, and the resulting reaction mixture was stirred at room temperature for 8 h. After completion of the reaction (indicated by TLC), DCM was evaporated under reduced pressure. Then to the crude reaction mixture, EtOH (20 mL) was added, resulting in the formation of a white precipitate, which was purified by simple filtration and washed with EtOH (10 mL × 4) to afford the pure product **5** (90% yield, 1.53 g) as a white solid. ¹H NMR (400 MHz, D₂O) δ 7.36 (t, *J* = 8.0 Hz, 1H), 7.25–7.13 (m, 3H), 4.91 (dd, *J* = 8.4, 3.7 Hz, 1H), 3.20 (dd, *J* = 13.1, 3.8 Hz, 1H), 3.09 (dd, *J* = 13.1, 8.6 Hz, 1H). ¹³C NMR (100 MHz, D₂O) δ 151.42, 141.57, 130.40, 123.72, 121.72, 119.13, 69.11, 45.22. ATR-FTIR (solid/cm⁻¹) 3533, 3154, 3091, 3053, 2971, 1681, 1605, 1517, 1481, 1447, 1313, 1223, 1120, 1042, 1001, 951, 922, 880, 846, 812, 792, 727, 696, 667, 635. MS (ESI) *m/z* [M - H]⁻ calculated for C₈H₁₀NO₅S: 232.0280, found 231.92. HRMS (ESI) *m/z* [M - H]⁻ calculated for C₈H₁₀NO₅S: 232.0280, found 232.0290.

Conversion of **3** to **9**

To a stirred solution of etilefrine hydrochloride (**3**; 1 equiv, 25 mmol, 5.24 g) and Boc_2O (1.1 equiv, 27.5 mmol, 5.99 g) in 40 mL of DCM, Et_3N (2 equiv, 50 mmol, 5.05 g) was added dropwise at 0 °C. After 30 min, the reaction was set at room temperature and continued to stir for an additional 3 h. After completion of the reaction (indicated by TLC), the solvent was evaporated under reduced pressure, and the crude reaction mixture was

purified by column chromatography on silica gel ($\text{EtOAc}/\text{hexane} = 4:6$) to afford **9** (90% yield, 6.29 g) as colorless sticky solid. ^1H NMR (500 MHz, CDCl_3) δ 7.35 (s, 1H), 7.13 (t, $J = 7.7$ Hz, 1H), 6.89 (s, 1H), 6.80 (d, $J = 7.6$ Hz, 1H), 6.72 (d, $J = 7.3$ Hz, 1H), 4.92–4.83 (m, 1H), 3.56–3.09 (m, 4H), 2.30 (s, 1H), 1.46 (s, 9H), 1.02 (t, $J = 7.0$ Hz, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 156.58, 143.73, 129.66, 117.70, 114.98, 113.01, 80.85, 74.26, 55.64, 44.25, 28.40, 13.55. ATR-FTIR (solid/ cm^{-1}) 3327, 2975, 2932, 2875, 1659,

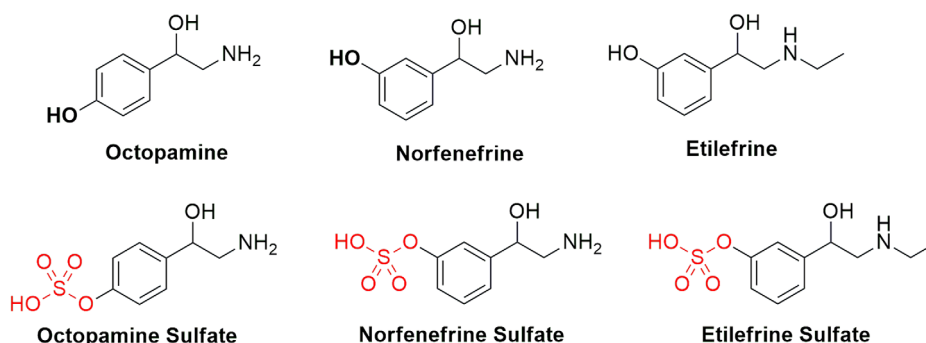
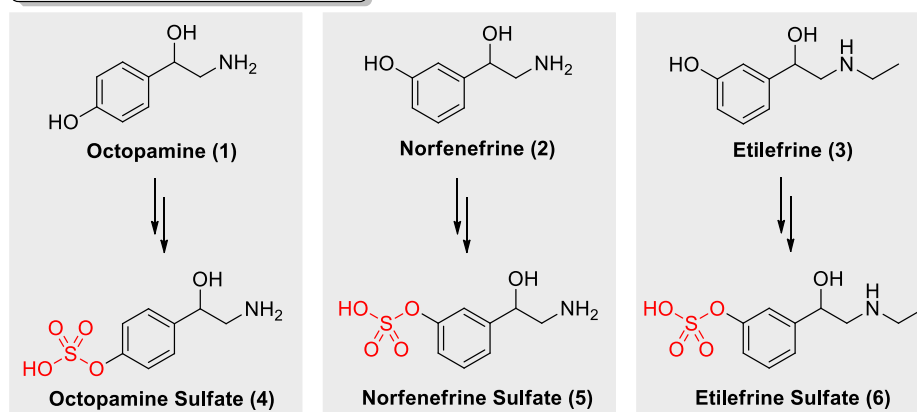


Figure 1. Structures of octopamine, octopamine sulfate, norfenefrine, norfenefrine sulfate, etilefrine and etilefrine sulfate.

(a) Sulfate conjugate metabolites:



CHALLENGES AND OBJECTIVES

- multiple reaction sites in the parent drug molecules?
- selective sulfation of phenolic OH group?
- identification of suitable sulfating agent?
- overall yield?
- HPLC purity?
- analytical methods and analytical standards for anti-doping?

(b) Proposed synthetic route:

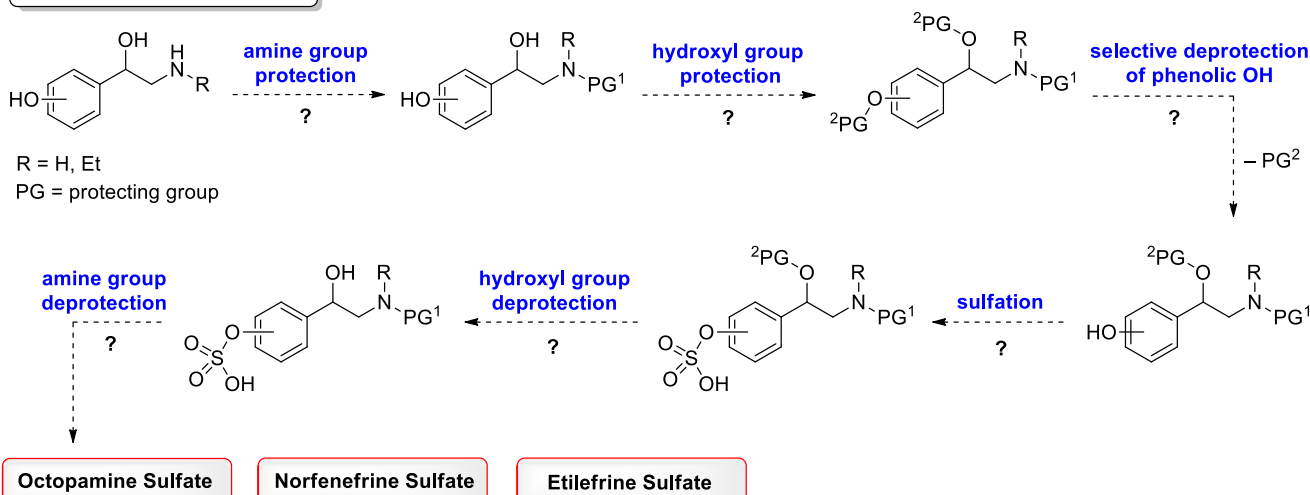


Figure 2. Hypothesis for synthesizing OS, NS and ES.

1610, 1590, 1479, 1455, 1416, 1366, 1276, 1249, 1221, 1151, 1074, 961, 870, 776, 700. MS (ESI) m/z $[M - H]^-$ calculated for $C_{15}H_{22}NO_4$: 280.1549, found 279.98. HRMS (ESI) m/z $[M - H]^-$ calculated for $C_{15}H_{22}NO_4$: 280.1549, found 280.1556.

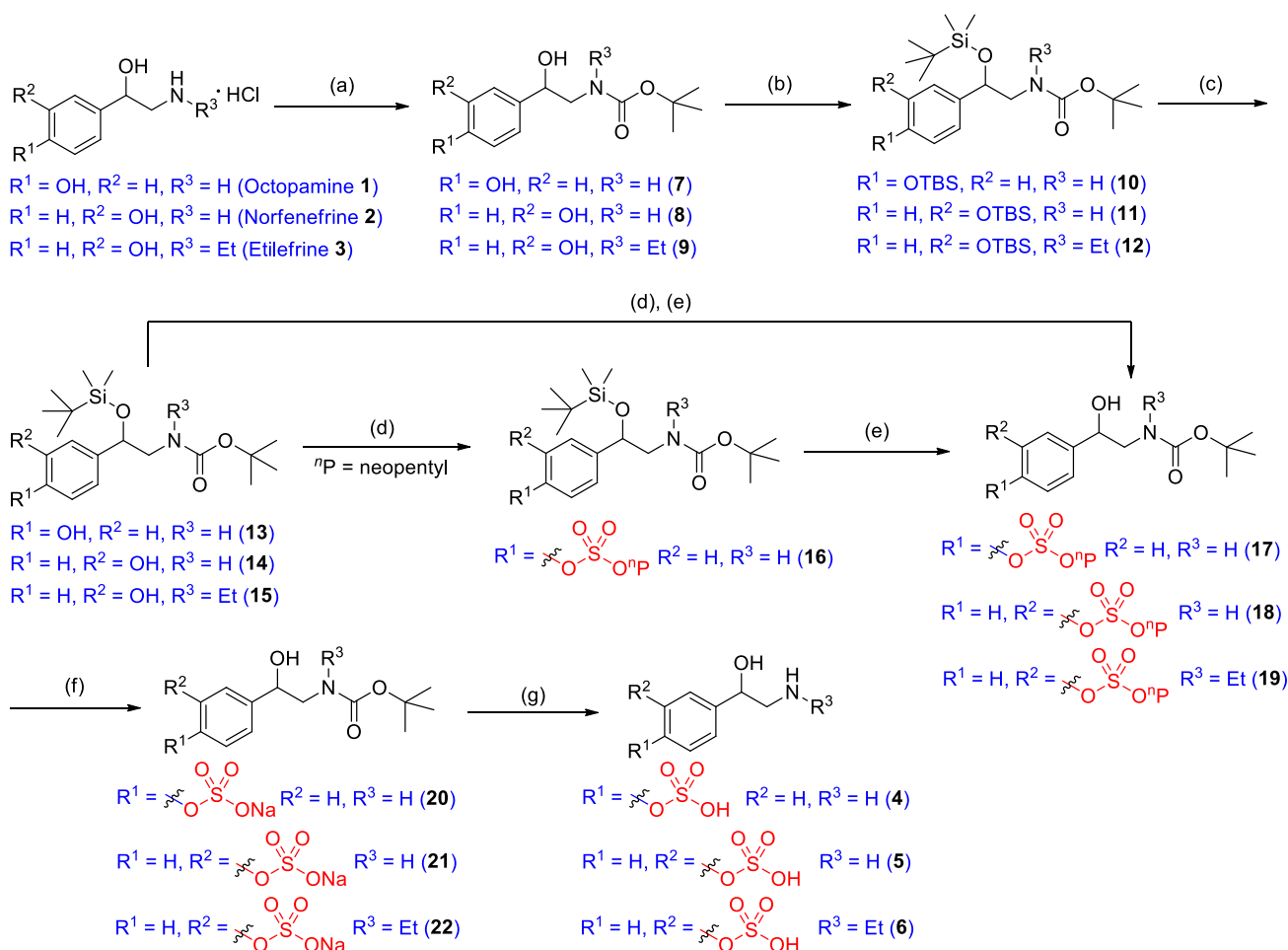
Conversion of **9** to **12**

TBSCl (4 equiv, 98.48 mmol, 14.84 g) and imidazole (6 equiv, 147.72 mmol, 10.04 g) were added to a solution of **9** (1 equiv, 24.62 mmol, 6.29 g) in 55 mL of DMF. The reaction mixture was stirred at room temperature for 4 h. After completion of the reaction (indicated by TLC), 45 mL of water was slowly added to the reaction mixture and was extracted with DCM (20 mL \times 4). Anhydrous Na_2SO_4 was used to dry the organic extract. Under reduced pressure, the solvent was evaporated to obtain the crude product, which was purified by column chromatography on silica gel (EtOAc/hexane = 1:10) to afford **12** (96% yield, 11.07 g) as a colorless oil. 1H NMR (500 MHz, $CDCl_3$) δ 7.14 (q, J = 7.5 Hz, 1H), 6.95–6.77 (m, 2H), 6.70 (t, J = 9.6 Hz, 1H), 4.89 (ddd, J = 61.4, 8.5, 4.0 Hz, 1H), 3.39–3.22 (m, 2H), 3.17–2.92 (m, 2H), 1.47 (d, J = 10.3 Hz, 9H), 1.01–0.97 (m, 3H), 0.96 (s, 9H), 0.86 (s, 9H), 0.16 (s, 6H), 0.00 (s, 3H), –0.14 (d, J = 4.3 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 155.69, 155.65, 155.55, 155.19, 145.02, 144.86, 129.21,

129.04, 119.32, 119.25, 119.16, 118.96, 117.87, 117.77, 79.35, 79.11, 74.02, 73.09, 56.08, 55.60, 44.63, 43.59, 28.71, 28.63, 25.98, 25.94, 25.78, 25.76, 18.29, 18.27, 18.20, 18.17, 13.50, 13.02, –4.32, –4.73, –4.84, –4.89, –4.96. ATR-FTIR (solid/ cm^{-1}) 2939, 2893, 2860, 1695, 1593, 1472, 1405, 1366, 1262 1154, 1084, 950, 832, 778, 699. MS (ESI) m/z $[M + H]^+$ calculated for $C_{27}H_{52}NO_4Si_2$: 510.3435, found 510.3. HRMS (ESI) m/z $[M + H]^+$ calculated for $C_{27}H_{52}NO_4Si_2$: 510.3435, found 510.3405.

Conversion of **12** to **15**

To a solution of **12** (1 equiv, 21.74 mmol, 11.07 g) in 40 mL of DMF was added Cs_2CO_3 (2 equiv, 43.49 mmol, 14.13 g), and the reaction mixture was stirred at room temperature for 48 h. After completion of the reaction (indicated by TLC), 40 mL of water was slowly added to the reaction mixture and was extracted with EtOAc (20 mL \times 4). Anhydrous Na_2SO_4 was used to dry the solvent and evaporated under reduced pressure to obtain the crude product. Column chromatographic purification of the crude product was done on silica gel (EtOAc/hexane = 3:7) to afford pure product **15** (98% yield, 8.18 g) as a colorless sticky solid. 1H NMR (400 MHz, $CDCl_3$) δ 7.14 (q, J = 8.0 Hz, 1H), 6.91–6.68 (m, 3H), 4.89 (ddd, J = 49.5, 8.0, 4.1 Hz, 1H), 3.40 (ddd, J = 16.3, 11.6,



Reagents and conditions: (a) $(Boc)_2O$, Et_3N , DCM, 0 °C to rt; (b) TBSCl, 1*H*-Imidazole, DMF, rt; (c) Cs_2CO_3 , DMF, rt; (d) NaHMDS THF, –78 °C, neopentyl chlorosulfate; (e) 70% TBAF in H_2O , THF, 0 °C to rt; (f) NaN_3 , DMF, 70 °C; (g) TFA, DCM, rt

Scheme 1. Synthesis of OS, NS and ES.

5.1 Hz, 1H), 3.23 (td, $J = 13.7, 6.1$ Hz, 1H), 3.18–2.89 (m, 2H), 1.46 (d, $J = 5.2$ Hz, 9H), 1.02–0.96 (m, 3H), 0.86 (s, 9H), 0.01 to –0.01 (m, 3H), –0.13 (d, $J = 11.1$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ 156.03, 155.84, 155.50, 145.00, 129.45, 129.33, 118.11, 114.52, 114.30, 113.05, 79.69, 79.48, 73.71, 73.31, 56.01, 55.37, 44.62, 43.53, 28.63, 25.95, 25.92, 25.73, 18.20, 13.48, 12.95, –3.51, –4.74, –4.87, –4.90, –4.99. ATR-FTIR (solid/ cm^{-1}) 3340, 2939, 2892, 2861, 1664, 1598, 1467, 1417, 1369, 1252, 1155, 1085, 945, 830, 773, 695. MS (ESI) m/z $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{21}\text{H}_{38}\text{NO}_4\text{Si}$: 396.2570, found 396.16. HRMS (ESI) m/z $[\text{M} - \text{H}]^-$ calculated for $\text{C}_{21}\text{H}_{36}\text{NO}_4\text{Si}$: 394.2414, found 394.2406.

Conversion of **15** to **19**

Compound **15** (1 equiv, 20.70 mmol, 8.18 g) was dissolved in 33 mL of THF and 22 mL of DMPU, and the resulting solution was cooled to -75°C . Sodium bis(trimethylsilyl)amide (1 mol L^{-1} in THF, 1.1 equiv, 22.77 mmol, 4.15 g, 22.63 mL) was added dropwise, and the resulting reaction mixture was stirred for 15 min. After that, neopentyl chlorosulfate (1.4 equiv, 28.98 mmol, 5.39 g) was added to the reaction mixture, and the stirring was continued for 3 h. Then the reaction mixture was warmed to -10°C and stirred for 2 h. After completion of the reaction (indicated by TLC), ethyl acetate (20 mL) and saturated aqueous



Figure 3. HPLC chromatograms of ES, NS and OS.

Table 1. Mass accuracy study by LC-HRMS

Compound name	Molecular formula	Calculated mass (m/z)	Experimental mass (m/z)	Diff (Tgt, ppm)
Octopamine sulfate	$\text{C}_8\text{H}_{11}\text{NO}_5\text{S}$	233.0358	232.0285	–3.61
Norfenefrine sulfate	$\text{C}_8\text{H}_{11}\text{NO}_5\text{S}$	233.035	232.0285	–4.35
Etilefrine sulfate	$\text{C}_{10}\text{H}_{15}\text{NO}_5\text{S}$	261.066	260.0598	–4.49

NaHCO₃ solution were added (at −10 °C), and then the reaction system was allowed to reach room temperature. After that, it was extracted with EtOAc (20 mL × 4). Over anhydrous Na₂SO₄ the organic extract was dried and evaporated under reduced pressure to obtain the crude product, which was directly used in the next step.

To a solution of the crude reaction mixture in THF at 0 °C was added dropwise *tetra-n*-butylammonium fluoride (70% in water, 1.5 equiv, 31.05 mmol, 8.10 g, 11.5 mL), and the reaction mixture was stirred for 1 h. Then, the reaction mixture was warmed to room temperature, and the stirring continued for 3 h. After completion of the reaction (indicated by TLC), THF was evaporated under reduced pressure, and 20 mL of water was added to the reaction mixture. It was then extracted with EtOAc (20 mL × 4), and anhydrous Na₂SO₄ was used to dry it. Under reduced pressure, the solvent was evaporated to obtain the crude product, which was purified by column chromatography on silica gel (EtOAc/hexane = 3:7) to afford **19** (75% yield, 6.76 g) as a colorless sticky solid. ¹H NMR (400 MHz, CDCl₃) δ 7.37 (t, *J* = 7.8 Hz, 1H), 7.35–7.26 (m, 2H), 7.24–7.17 (m, 1H), 4.93 (dd, *J* = 7.4, 3.1 Hz, 1H), 4.08 (s, 2H), 3.41–2.91 (m, 3H), 1.47 (s, 9H), 0.99 (s, 9H), 0.05 (s, 5H). ¹³C NMR (100 MHz, CDCl₃) δ 150.50, 145.40, 129.94, 124.81, 120.07, 118.74, 83.55, 80.83, 74.16, 56.02, 44.58, 32.04, 28.50, 26.08, 13.57, 1.14. MS (ESI) *m/z* [M + Na]⁺ calculated for C₂₀H₃₃NNaO₇S: 454.1875, found 454.12.

Conversion of **19** to **22**

In a round-bottom flask fitted with a reflux condenser, compound **19** (1 equiv, 15.6 mmol, 6.76 g) was dissolved in 40 mL of DMF, and then sodium azide (1.5 equiv, 23.4 mmol, 1.52 g) was added. The reaction mixture was stirred at 70 °C in an oil bath for 24 h. After completion of the reaction (indicated by TLC), the DMF was evaporated under reduced pressure, and the crude reaction

mixture was directly subjected to column chromatography on silica gel (EtOH/DCM = 2:8) to afford **22** (84% yield, 5.01 g) as a white solid. ¹H NMR (500 MHz, D₂O) δ 7.26 (t, *J* = 7.6 Hz, 1H), 7.15–7.01 (m, 3H), 4.76 (d, *J* = 5.2 Hz, 1H), 3.39 (ddd, *J* = 75.4, 13.3, 6.8 Hz, 2H), 3.06–2.85 (m, 2H), 1.22 (s, 3H), 1.16 (s, 6H), 0.99 (q, *J* = 7.0 Hz, 1H), 0.88–0.77 (m, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 156.91, 151.89, 129.40, 123.78, 121.29, 119.22, 114.17, 80.09, 72.42, 53.56, 43.57, 29.81, 28.44, 22.80, 14.25. MS (ESI) *m/z* [M – Na][−] calculated for C₁₅H₂₂NO₇S: 360.1117, found 360.1.

Conversion of **22** to **6**

To a stirring solution of compound **22** (1 equiv, 13.08 mmol, 5.01 g) in 40 mL of DCM, CF₃COOH (6 equiv, 78.48 mmol, 8.94 g, 6.00 mL) was added dropwise, and the resulting reaction mixture was stirred at room temperature for 8 h. After completion of the reaction (indicated by TLC), DCM was evaporated under reduced pressure. Then to the crude reaction mixture, EtOAc 20 mL was added, resulting in the formation of a white precipitate, which was purified by simple filtration and washed with EtOAc (10 mL × 4) to afford pure product **6** (97% yield, 3.50 g) as a white solid. ¹H NMR (500 MHz, D₂O) δ 7.28 (t, *J* = 7.9 Hz, 1H), 7.15 (d, *J* = 7.4 Hz, 2H), 7.10 (d, *J* = 7.1 Hz, 1H), 4.87 (dd, *J* = 9.1, 3.5 Hz, 1H), 3.18–3.05 (m, 2H), 2.95 (q, *J* = 7.2 Hz, 2H), 1.09 (t, *J* = 7.3 Hz, 3H). ¹³C NMR (126 MHz, D₂O) δ 151.30, 141.49, 130.32, 123.61, 121.69, 119.03, 68.43, 52.38, 43.06, 10.25. ATR-FTIR (solid/cm^{−1}) 3485, 3047, 2835, 1443, 1389, 1286, 1210, 1138, 1044, 958, 883, 837, 804, 730, 696, 598, 578. MS (ESI) *m/z* [M + H]⁺ calculated for C₁₀H₁₆NO₅S: 262.0749, found 262.0.

HPLC study

A reversed-phase HPLC system (M/s Agilent Technologies series, 1260 infinity HPLC-II system) was used. The HPLC system was composed of a G7129A 1260 autosampler, column compartment

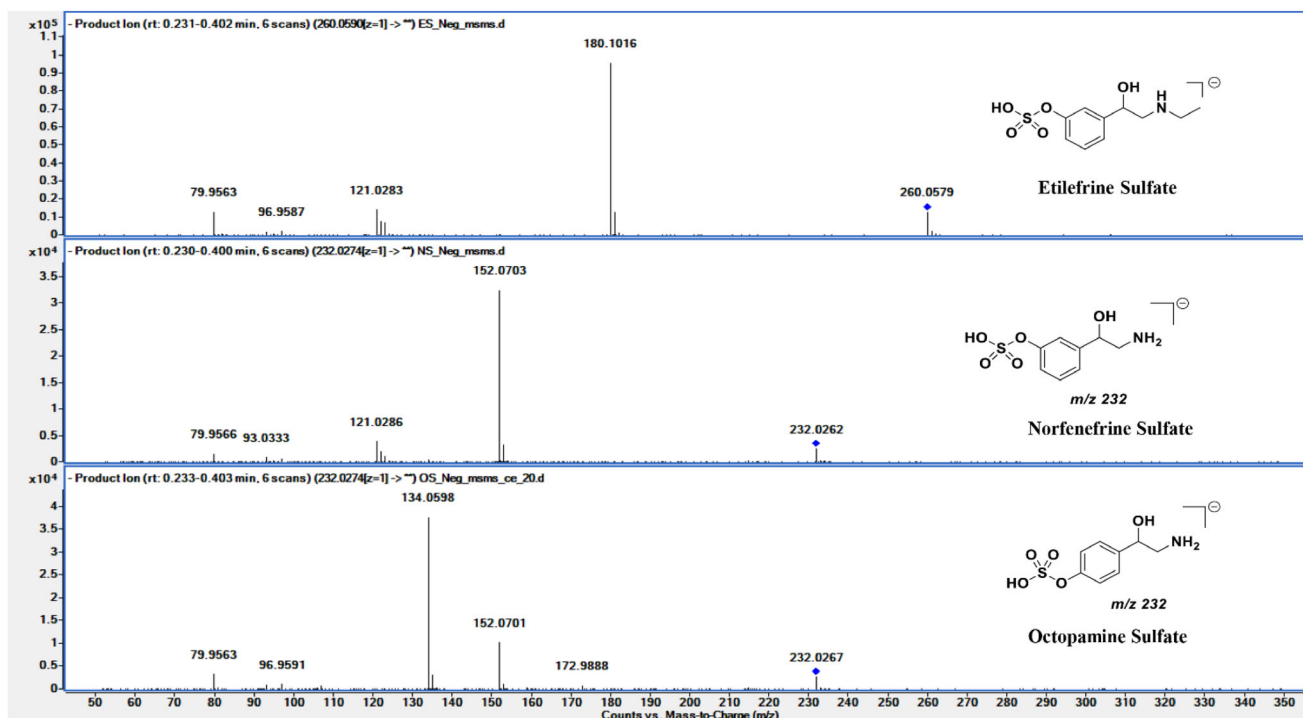
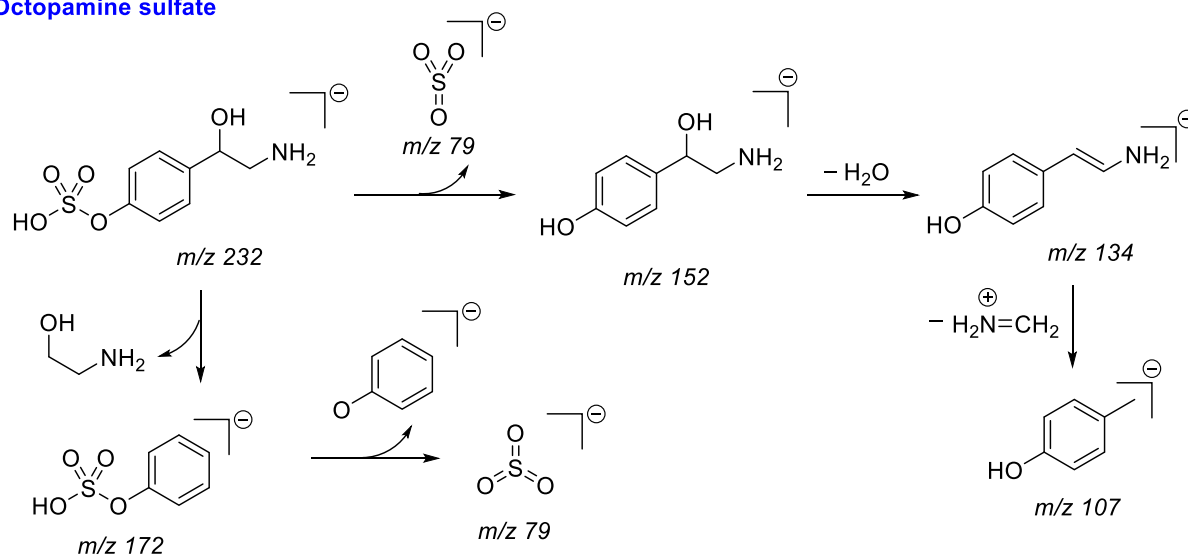
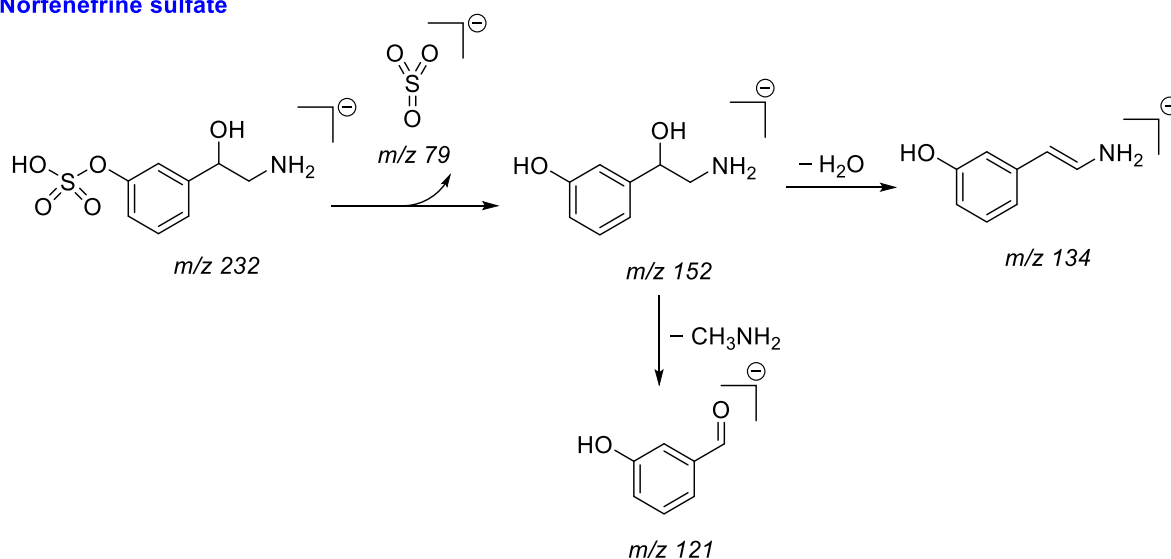


Figure 4. HRMS/MS of OS, NS and ES.

Octopamine sulfate



Norfenefrine sulfate



Etilfrine sulfate

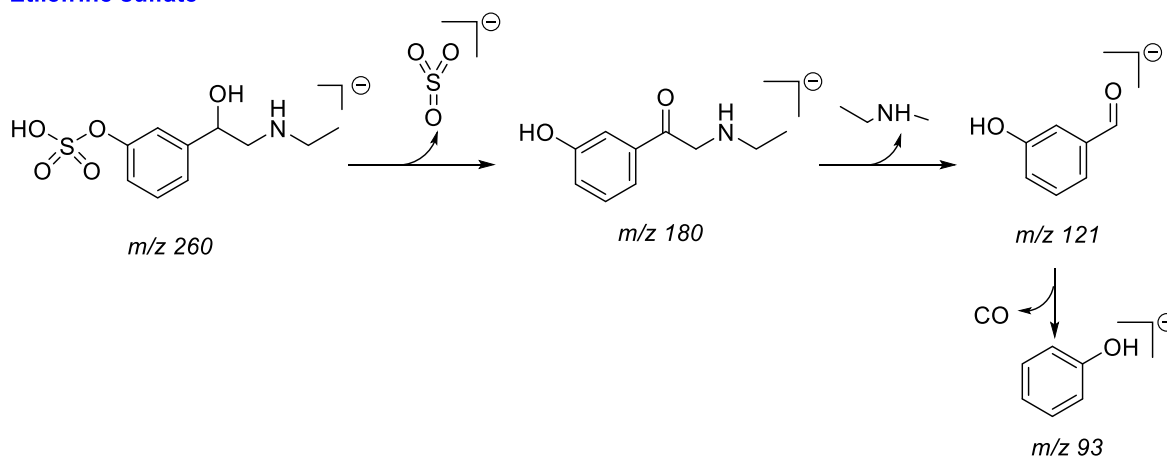


Figure 5. Fragmentation of OS, NS and ES.

and a 1260 DAD. HPLC separation was achieved at 30 °C using a Luna Hypersil C8 GOLD (150 mm × 3.0 mm, 5 μ) for OS separation, and a C18 column (250 mm × 4.6 mm, 5 μm) for NS and ES separation. The data were collected using control panel software connected to a 21CFR part 11 enabled central server.^{38–40} The mobile phase was composed of water and acetonitrile in isocratic elution (50:50) with flow rates of 0.7 mL min^{−1} for OS and 1.2 mL min^{−1} for NS and ES. The wavelength for the analysis was kept at 212 nm for the three compounds. A sample was dissolved in water–acetonitrile in (50:50) composition and made into a 1 mg mL^{−1} solution. Further dilution was made to 100 ppm with methanol and run through HPLC. Injection volume and the column temperature were kept at 10 μL and 30 °C.^{21–23}

LC-HRMS study

For the LC-HRMS study, an Agilent G6545B LC-QTOF was used where the sheath gas temperature was 350 °C and the drying gas flow rate was 8 mL min^{−1}. Full-scan mass range: 100–400 *m/z*, resolution: 40 000 (full MS); MS/MS was scanned in the range of 50–350 *m/z* with a collision energy for OS and ES of 20 V, whereas, for NS, the collision energy was 25 V. The mass spectrometric properties of the analytes were investigated using ESI in the negative ion mode. The full scan and MS/MS were done through union, and 0.1% formic acid in water and acetonitrile was used as the mobile phase. The data analysis was done using Agilent software qualitative analysis 10.0 version.^{42,44,45}

RESULTS AND DISCUSSION

Synthetic chemistry

It is apparent from the above discussion that developing a reliable and efficient methodology for the synthesis of these metabolites is a significant task. To achieve this goal, we envisage exploring neopentyl chlorosulfate as a promising sulfating agent⁵¹ to procure the conjugated sulfate metabolites of octopamine, norfenefrine and etilefrine. The presence of multiple reaction sites in the parent drug molecules makes the synthesis challenging and complicated. In order to achieve the selective sulfation of the phenolic hydroxyl group, a protecting/deprotecting group strategy is sought. As depicted in Fig. 2(b), initially, we intend to protect the active amine group, and subsequently, both the phenolic and benzylic hydroxyl groups will be protected.⁴⁷ Next, selective deprotection of the phenolic hydroxyl group and sulfation will be carried out. Finally, after benzylic hydroxyl and amine group deprotection, the desired sulfate conjugated metabolites would be afforded.^{9,36–38,41,43,47–50}

To validate our hypothesis, we began the synthesis of OS (**4**) from octopamine hydrochloride (**1**). As shown in Scheme 1, initially, *tert*-butoxycarbonyl (Boc) protection of the amine group of **1** was carried out to get compound **7** in 98% yield. Next, phenolic and benzylic hydroxyl groups in **7** were protected with *tert*-butyldimethylsilyl (TBS) to get compound **10** in 94% yield. Then the aromatic hydroxyl group was selectively deprotected using Cs₂CO₃ at room temperature to afford compound **13** in 76% yield. The sulfate ester of compound **13** was subsequently prepared by reacting it with neopentyl chlorosulfate in the presence of NaHMDs. TBS deprotection in the resulting compound **16** (70% yield) was achieved by *tetra*-butylammonium fluoride (TBAF), which produced compound **17** in 94% yield. NaN₃ deprotected the neopentyl group to get compound **20** in 97% yield, from which the desired OS **4** was obtained in 98% yield after trifluoroacetic acid (TFA)-assisted Boc deprotection. The overall yield of the

process after seven steps is 42%. A similar strategy was adopted to synthesize NS (**5**) and ES (**6**). It is worth noting that while preparing the sulfate ester of compound **14** by reacting it with neopentyl chlorosulfate in the presence of NaHMDs, TBS deprotection of benzylic hydroxyl group also co-occurs to produce compound **18** in 45% yield. NS (**5**; 26% overall yield) was obtained after two deprotecting steps of neopentyl and Boc groups. In the case of ES synthesis, the crude sulfate ester was treated with TBAF to achieve TBS deprotection of benzylic hydroxyl group to get compound **19** in 75% yield from which ES was obtained after two steps of neopentyl and Boc group deprotections in 54% overall yield. It is worth mentioning that Gerk *et al.* also attempted to synthesize ES using sulfur trioxide pyridine complex as a sulfating agent but with a lower yield of only 8%.⁴⁶ All the NMR spectra have been mentioned in Figs S1–S38 in the supporting information.

HPLC analysis

Purity assessment of OS, NS and ES is essential to ensuring the quality of synthesized reference materials using HPLC-DAD. HPLC is the most common technique to ensure peak purity by spectral analysis. The authors developed a novel HPLC-DAD method for OS, NS and ES purity assessment. The purity of the three compounds was >99% as shown in Fig. 3. The chemical purity (% w/w) of synthesized OS, NS and ES was calculated using Eqn (1), and the loss on drying and ignition was studied using TGA and a muffle furnace, respectively. OS, NS and ES chemical purity was 95.56%, 98.97% and 98.91% (w/w), respectively. HPLC method validation data are presented in the supporting information (Figs S39–S44), and the TGA results are shown in Fig. S45.

$$\begin{aligned} \text{Chemical purity (potency)} &= 100\% & (1) \\ &-(\% \text{Impurities by HPLC}) \\ &+ \text{Loss on drying}\% \\ &+ \% \text{Residue on Ignition}) \dots \end{aligned}$$

LC-HRMS analysis

In order to determine the mass accuracy of OS, NS and ES, LC-HRMS and LC-HRMS/MS were used. The three metabolites were all well ionized in the negative ionization mode ($[M - H]^-$). A full scan was performed at 100–400 *m/z*, where the deprotonated precursor ion for OS and NS was 232.0285 *m/z*, and that for ES was 260.0598 *m/z*, as shown in Table 1. The error value of the three synthesized compounds was less than 10 ppm. Further, the authors have studied LC-HRMS/MS of OS, NS and ES at collision energies of 20–30 eV. The product ions of the three compounds are shown in Fig. 4, and it was observed that product ion *m/z* 79 was common in the three metabolites, product ion *m/z* 152 was common in OS and ES, and common *m/z* 121 was found in NS and ES. Figure 5 illustrates the fragmentation patterns of OS, NS and ES.

CONCLUSION

The Phase II metabolites OS, NS and ES were successfully synthesized and characterized using ¹H NMR, ¹³C NMR, LC-HRMS/MS and TGA. It is intended to synthesize the sulfate conjugates of octopamine, norfenefrine and etilefrine, which are prohibited in sports under the category of S6 stimulants. The reference materials were obtained with high overall yields (26–54%). The new HPLC-DAD

method has been developed using C8 and C18 HILIC columns. The developed materials' HPLC-PDA purity was greater than >99%. These developed reference materials will be used by WADA-accredited laboratories to maintain clean sports missions across the globe. Developed high-quality reference materials will provide a ray of hope to WADA laboratories in routine testing to maintain mandatory testing guidelines to stop doping in sports.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

- Berry DA, The science of doping. *Nature* **454**:692–693 (2008).
- Scientific Research | World Anti Doping Agency. Available from: <https://www.wada-ama.org/en/data-research/scientific-research> [3 May 2023].
- Sliwoski G, Kothiwale S, Meiler J and Lowe EW, Computational methods in drug discovery. *Pharmacol Rev* **66**:334–395 (2014).
- Mackay LG and Rymantas K, The importance of reference materials in doping control analysis. *Anal Bioanal Chem* **401**:483–493 (2011).
- Mazzoni I, Barroso O and Rabin O, The list of prohibited substances and methods in sport: structure and review process by the World Anti-Doping Agency. *J Anal Toxicol* **35**:608–612 (2011).
- Landry GL and Kokotailo PK, Drug screening in the athletic setting. *Curr Probl Pediatr* **24**:344–359 (1994).
- Marclay F, Mangin P, Margot P and Saugy M, Perspectives for forensic intelligence in anti-doping: thinking outside of the box. *Forensic Sci Int* **229**:133–144 (2013).
- Kamber M and Mullis P-E, The worldwide fight against doping: from the beginning to the World Anti-Doping Agency. *Endocrinol Metab Clin North Am* **39**:1–9 (2010).
- Murray TH, Doping in sport: challenges for medicine, science and ethics. *J Intern Med* **264**:95–98 (2008). <https://doi.org/10.1111/j.1365-2796.2008.01994.x>.
- McGrath JC and Cowan DA, Drugs in sport. *Br J Pharmacol* **154**:493–495 (2008). <https://doi.org/10.1038/bjpp.2008.171>.
- Dvorak J, Therapeutic use exemption. *Br J Sports Med* **40**:i40–i42 (2006). <https://doi.org/10.1136/bjbm.2006.027490>.
- Gómez-Guerrero NA, González-López NM, Zapata-Velásquez JD, Martínez-Ramírez JA, Rivera-Monroy ZJ and García-Castañeda JE, Synthetic peptides in doping control: a powerful tool for an analytical challenge. *ACS Omega* **7**:38193–38206 (2022).
- Pöppel K, Efficient ways to combat doping in a sports education context? A systematic review on doping prevention measures focusing on young age groups. *Front Sport Act Living* **3**:1–19 (2021).
- TD2022DL | World Anti Doping Agency. Available from: <https://www.wada-ama.org/en/resources/lab-documents/td2022dl> [3 May 2023].
- Thevis M, Kuuranne T and Geyer H, Annual banned-substance review: analytical approaches in human sports drug testing 2019/2020. *Drug Test Anal* **13**:8–35 (2021). <https://doi.org/10.1002/dta.2969>.
- de Oliveira A, de Paula M, Comar J, Vilela V, Peralta R and Bracht A, Adrenergic metabolic and hemodynamic effects of octopamine in the liver. *Int J Mol Sci* **14**:21858–21872 (2013).
- C.R. Ganellin. *Dictionary of Pharmacological Agents*. Available from: <https://www.routledge.com/Dictionary-of-Pharmacological-Agents/Ganellin-Triggler/p/book/9780412466304>.
- Falany CN, Enzymology of human cytosolic sulfotransferases. *FASEB J* **11**:206–216 (1997). <https://doi.org/10.1096/fasebj.11.4.9068609>.
- Simonin J, Gerber-Lemaire S, Centeno C, Seghezzi C, Iglesias K, Abid K *et al.*, Synthetic calibrators for the analysis of total metanephries in urine: revisiting the conditions of hydrolysis. *Clin Chim Acta* **413**:998–1003 (2012 Jun).
- Chapman E, Best MD, Hanson SR and Wong C-H, Sulfotransferases: structure, mechanism, biological activity, inhibition, and synthetic utility. *Angew Chem Int Ed* **43**:3526–3548 (2004). <https://doi.org/10.1002/anie.200300631>.
- Lynch KL, Toxicology: liquid chromatography mass spectrometry. *Mass Spectrom Clin Lab*:109–130 (2017). <https://www.sciencedirect.com/book/9780128008713/mass-spectrometry-for-the-clinical-laboratory>.
- Thevis M and Schänzer W, Current role of LC–MS(/MS) in doping control. *Anal Bioanal Chem* **388**:1351–1358 (2007). <https://doi.org/10.1007/s00216-007-1131-4>.
- Ray E, Vaghasiya K, Sharma A, Shukla R, Khan R, Kumar A *et al.*, Autophagy-inducing inhalable co-crystal formulation of niclosamide-nicotinamide for lung cancer therapy. *AAPS PharmSciTech* **21**:260 (2020). <https://doi.org/10.1208/s12249-020-01803-z>.
- Mishra P, Handa M, Ujjwal RR, Singh V, Kesharwani P and Shukla R, Potential of nanoparticulate based delivery systems for effective management of alopecia. *Colloids Surf B* **208**:112050 (2021).
- Pawar SD, Kumar GJ, Chikkondra A, Lal B, Radhakrishnanand P, Murty US *et al.*, Synthesis, characterization, method development, and validation of nor-ethylmorphine hydrochloride reference material using established analytical techniques for dope control analysis. *Drug Test Anal* **14**:1–5 (2021).
- Joseph A, Kumar GJ, Pawar SD, Hirlekar BU, Bharatam PV, Konda S *et al.*, Analytical developments of p-hydroxy prenylamine reference material for dope control research: characterization and purity assessment. *Drug Test Anal* **14**:224–232 (2021). <https://doi.org/10.1002/dta.3171>.
- Kumar GJ, Pawar SD, Pawar SR, Khatib AM, Saini A, Das P *et al.*, Process development for the total synthesis of the novel drug metabolite carboxy toremifene as a standard reference material along with characterization and purity assessment for the antidoping quality control purposes. *Drug Test Anal* **14**:2002–2006 (2022).
- Pawar SD, Kumar GJ, Chikkondra A, Lal B, Radhakrishnanand P, Murty US *et al.*, Synthesis, characterization, method development, and validation of nor-ethylmorphine hydrochloride reference material using established analytical techniques for dope control analysis. *Drug Test Anal* **14**:388–392 (2022).
- Joseph A, Kumar GJ, Pawar SD, Hirlekar BU, Bharatam PV, Konda S *et al.*, Analytical developments of p-hydroxy prenylamine reference material for dope control research: characterization and purity assessment. *Drug Test Anal* **14**:224–232 (2022).
- Jeong ES, Kim S-H, Cha E-J, Lee KM, Kim HJ, Lee S-W *et al.*, Simultaneous analysis of 210 prohibited substances in human urine by ultrafast liquid chromatography/tandem mass spectrometry in doping control. *Rapid Commun Mass Spectrom* **29**:367–384 (2015). <https://doi.org/10.1002/rcm.7113>.
- Hoshino J, Park E-J, Kondratyuk TP, Marler L, Pezzuto JM, van Breemen RB *et al.*, Selective synthesis and biological evaluation of sulfate-conjugated resveratrol metabolites. *J Med Chem* **53**:5033–5043 (2010). <https://doi.org/10.1021/jm100274c>.
- Schamasch P and Rabin O, Challenges and perspectives in anti-doping testing. *Bioanalysis* **4**:1691–1701 (2012). <https://doi.org/10.4155/bio.12.145>.
- Nicoli R, Guillaume D, Leuenberger N, Baume N, Robinson N, Saugy M *et al.*, Analytical strategies for doping control purposes: needs, challenges, and perspectives. *Anal Chem* **88**:508–523 (2016). <https://doi.org/10.1021/acs.analchem.5b03994>.
- Robinson N, Sottas PE and Schumacher YO, The athlete biological passport: how to personalize anti-doping testing across an athlete's career? *Med Sport Sci* **62**:107–118 (2017).
- Mazzarino M, Bragan MC, De La Torre X, Molaioni F and Botrè F, Relevance of the selective oestrogen receptor modulators tamoxifen, toremifene and clomiphene in doping field: endogenous steroids urinary profile after multiple oral doses. *Steroids* **76**:1400–1406 (2011). <https://doi.org/10.1016/j.steroids.2011.06.005>.

- 36 Parr MK, Wuest B, Naegele E, Joseph JF, Wenzel M, Schmidt AH et al., SFC-MS/MS as an orthogonal technique for improved screening of polar analytes in anti-doping control. *Anal Bioanal Chem* **408**: 6789–6797 (2016).
- 37 Mazzarino M, Fiacco I, De La Torre X and Botrè F, A mass spectrometric approach for the study of the metabolism of clomiphene, tamoxifen and toremifene by liquid chromatography time-of-flight spectroscopy. *Eur J Mass Spectrom* **14**:171–180 (2008).
- 38 Padakanti AP, Pawar SD, Kumar P and Chella N, Development and validation of HPLC method for simultaneous estimation of erlotinib and niclosamide from liposomes optimized by screening design. *J Liposome Res*:1–15 (2023). <https://doi.org/10.1080/08982104.2022.2162540>.
- 39 Jat S, Preparation and characterization of amoxapine- and naringin-loaded solid lipid nanoparticles: drug-release and molecular-docking studies. *Nanomedicine* **17**:450 (2023).
- 40 Pawar SD, Gawali K, Kulhari H, Murty US and Kumar P, Amoxapine-loaded solid lipid nanoparticles with superior preclinical pharmacokinetics for better brain delivery: LC-MS/MS and GC-MS analysis. *ACS Chem Neurosci* **14**:1388–1398 (2023). <https://doi.org/10.1021/acscchemneuro.2c00673>.
- 41 Aasmundstad T, Xu B, Johansson I, Ripel A, Bjorneboe A, Christophersen A et al., Biotransformation and pharmacokinetics of ethylmorphine after a single oral dose. *Br J Clin Pharmacol* **39**:611–620 (1995).
- 42 Nogueira R, do Rego ECP, Sousa MVB, Wollinger W, da Silva TE, Moreira GF et al., Development studies of captopril certified reference material. *Braz J Pharm Sci* **47**:339–350 (2011).
- 43 Quan C, Su F, Wang H and Li H, Development of anabolic-androgenic steroids purity certified reference materials for anti-doping. *Steroids* **76**:1527–1534 (2011).
- 44 Arbouche N, Bottinelli C, Gerace E and Gheddar L, Interest of HRMS systems in analytical toxicology: focus on doping products. *Toxicol Anal Clin* **34**:42–68 (2022).
- 45 Mazzarino M, Di Costanzo L, Comunità F, Stacchini C, De La Torre X and Botrè F, UHPLC–HRMS method for the simultaneous screening of 235 drugs in capillary blood for doping control purpose: comparative evaluation of volumetric and non-volumetric dried blood spotting devices. *ACS Omega* **7**:31845–31868 (2022).
- 46 Zhang Z, Zhang Y and Gerk PM, Preparation of phenylephrine 3-O-sulfate as the major in vivo metabolite of phenylephrine to facilitate its pharmacokinetic and metabolism studies. *J Pharmacol Toxicol Methods* **95**:66–69 (2019).
- 47 de Almeida AF, Moreira R and Rodrigues T, Synthetic organic chemistry driven by artificial intelligence. *Nat Rev Chem* **3**:589–604 (2019).
- 48 Moradi S, Kundu S, Rezazadeh M, Yeddu V, Voznyy O and Saidaminov MI, High-throughput exploration of halide perovskite compositionally-graded films and degradation mechanisms. *Commun Mater* **3**:1–5 (2022).
- 49 Yang J, Muckel F, Baek W, Fainblat R, Chang H, Bacher G et al., Chemical synthesis, doping, and transformation of magic-sized semiconductor alloy nanoclusters. *J Am Chem Soc* **139**:6761–6770 (2017). <https://doi.org/10.1021/jacs.7b02953>.
- 50 Gómez-Guerrero NA, González-López NM, Zapata-Velásquez JD, Martínez-Ramírez JA, Rivera-Monroy ZJ and García-Castañeda JE, Synthetic peptides in doping control: a powerful tool for an analytical challenge. *ACS Omega* **7**:38193–38206 (2022). <https://pubs.acs.org/doi/full/10.1021/acsomega.2c05296>.
- 51 Simpson LS and Widlanski TS, A comprehensive approach to the synthesis of sulfate esters. *J Am Chem Soc* **128**:1605–1610 (2006). <https://doi.org/10.1021/ja056086j>.