JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2004; **47**: 161–165.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.809

Research Article

Synthesis of estromustine, estramustine and estramustine phosphate labelled with deuterium

Danilo Giribone* and Erminia Fontana
Isotope Labelling Labs., Pharmacia Italia S.p.A, Viale Pasteur 10, 20014–Nerviano (Milan), Italy

Summary

The preparation of estromustine (EoM), estramustine (EaM) and estramustine phosphate (EMP) specifically labelled with deuterium is reported. A two-step, one-pot procedure based on the reaction of *N*,*N*-*bis*(2-chloroethyl)carbamoyl chloride with the commercially available [2,4,16,16-$^2H_4$]estrone (**1**) or [2,4,16,16,17-$^2H_5$]estradiol (**2**) afforded [2,4,16,16-$^2H_4$]estromustine (**3**) and [2,4,16,16,17-$^2H_5$]estramustine (**4**), respectively. The phosphorylation of **4** followed by hydrolysis gave [2,4,16,16,17-$^2H_5$]estramustine phosphate (**5**) in 65% chemical yield from **2**.

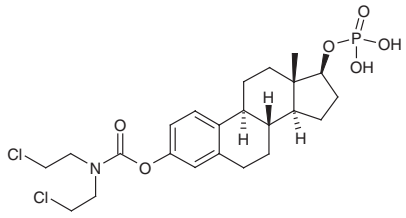
Key Words: deuterium; labelled; estradiol; estrone; estramustine; estromustine; emcyt®; estracyt®

Introduction

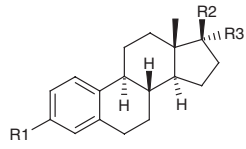
Estramustine phosphate (EMP; Estracyt®) is a derivative of estradiol-17β-phosphate in which a nornitrogen mustard is attached to the oxygen in position C-3 by means of a carbamate ester group. EMP is a cytotoxic agent having an additional hormonal effect and is used in the treatment of advanced prostate cancer.[1] The drug is rapidly dephosphorylated *in vivo* to estramustine (EaM) and its C-17 oxidized analog estromustine (EoM). The drug antimitotic effects are exerted by these two metabolites. Further metabolism of EaM and EoM involves carbamate ester cleavage with the formation of estradiol (E1) and estrone (E2), respectively, both of which are responsible for the estrogenic properties of EMP (see structures in Figure 1).[2,3] The promising results obtained with EMP in combination treatments with other antineoplastic agents[4] led to a renewed interest in this relatively old drug. In order to increase

*Correspondence to: D. Giribone, Isotope Labelling Labs., Pharmacia Italia S.p.A, Viale Pasteur 10, 20014–Nerviano (Milan), Italy. E-mail: danilo.giribone@pharmacia.com

Received 6 October 2003
Revised 14 November 2003
Accepted 18 November 2003



EMP



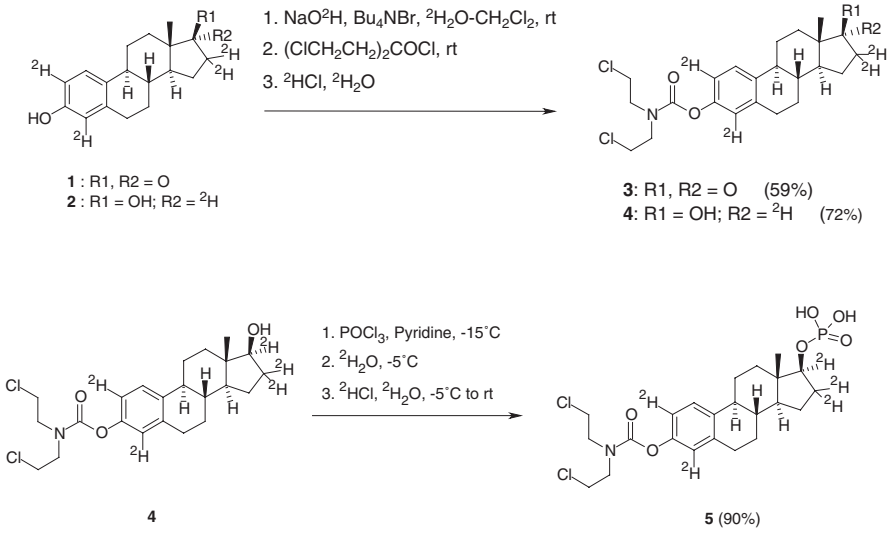
E1: R1 = R2 = OH; R3 = H
E2: R1 = OH; R2, R3 = O
EaM: R1 = $OC(O)N(C_2H_4Cl)_2$; R2 = OH; R3 = H
EoM: R1 = $OC(O)N(C_2H_4Cl)_2$; R2, R3 = O

Figure 1. Structures of estramustine phosphate (EMP) and its metabolites estradiol (E1), estrone (E2), estramustine (EaM) and estromustine (EoM)

the sample throughput, the development of a new, specific, robust and validated liquid chromatography–mass spectrometry (LC–MS) method for the simultaneous determination of EMP and its four metabolites in biological fluids was needed. A method for the determination of EMP and its metabolites in human plasma was recently reported, using liquid chromatography with fluorescence detection and gas chromatography.[5] In this paper, analogs of EMP, EaM and EoM with different nornitrogen mustard groups were used as internal standards. As it is generally agreed that in most cases the stable isotopically labelled analogues with the same molecular structure of a compound are the best internal standards for an LC–MS assay, a stable labelled form of the compound under investigation (EMP) and its metabolites E1, E2, EaM and EoM were required. In this paper the preparation of EMP, EaM and EoM specifically labelled with deuterium and starting from the commercially available deuterated estrone (**1**) and estradiol (**2**) is described.

Results and discussion

Standard requirements of an acceptable internal standard usually include a molecular weight at least three mass unit higher than that of the non-labelled material and stability of the labels during sample preparation. Preliminary information from the new LC–MS assay conditions indicated that the steroid skeleton was the most suitable moiety to label. The introduction of more than 3 carbon-13 atoms in the steroid skeleton would have required considerable effort. On the other end the ready availability of [2,4,16,16-$^2H_4$]estrone **1** and [2,4,16,16,17-$^2H_5$]estradiol **2** prompted us to consider a synthetic approach to the stable isotope versions of EoM, EaM and EMP involving the use of these deuterium-labelled steroids as the starting materials. The synthetic pathways followed are depicted in Scheme 1. In the literature an interesting method was



Scheme 1. Synthesis of deuterium-labelled estromustine (3), estramustine (4) and estramustine phosphate (5)

described to prepare the carbon-11 labelled EMP.[6,7] Following a similar procedure, the attachment of the mustard moiety to the labelled estrone **1** and estadiol **2** was successfully achieved by reacting the corresponding freshly prepared tetrabutyl ammonium-3-olates with *N*,*N*-*bis*(2-chloroethyl)-carbamoyl chloride. The conversion to the deuterated EMP was then performed by phosphorylation of the labelled EaM with a slight excess of phosphorous oxychloride in the presence of dry pyridine at $-15^{\circ}C$ under nitrogen, followed by immediate hydrolysis with deuterated water at $-5^{\circ}C$. The possibility of an isotopic exchange of deuterium with hydrogen during reactions and/or work-ups was also considered. Isotopic exchange might affect the deuterium enrichment of the final compounds, thereby making them unsuitable as internal standards. In order to avoid this, all the reaction conditions were strictly controlled by using only deuterated protic reagents (i.e. ^2H_2O, ^2HCl, NaO^2H) and anhydrous reagent/solvents when performing crucial reactions as well as work-ups. By applying these precautions, the chemically pure [2,4,16,16-$^2H_4$] estromustine **3** was obtained in 59% yield from the commercially available [2,4,16,16-$^2H_4$]estrone **1**. [2,4,16,16,17-$^2H_5$]Estramustine **4** and [2,4,16,16,17-$^2H_5$]estramustine phosphate **5** were prepared starting from the commercially available [2,4,16,16,17-$^2H_5$]estradiol **2** in 72% and 65% yield, respectively. These compounds were found to be suitable for use as internal standards for LC–MS determination of EMP and its metabolites in biological fluids.

Experimental

General methods

All solvents and reagents were of analytical grade and were used without purification unless otherwise indicated. Compounds **1** and **2** (>98 at% ^2H) were purchased from CDN Isotopes Inc. Sodium deuteroxide (40 wt% in ^2H_2O, >99.9 at% ^2H) and deuterated water (>99.990 at% ^2H) were purchased from Aldrich Chemical Co. Deuterated hydrochloric acid (38 wt% in ^2H_2O, 99.9 at% ^2H) was purchased from Carlo Erba Reagenti. *N*,*N*-*bis*(2-chloroethyl)carbamoyl chloride was purchased from TCI Tokyo Kasei. All the reactions were performed under nitrogen using oven-dried glassware. Chemical purities were determined by HPLC using a series-200 pump (Perkin-Elmer) equipped with a series 200 solvent degasser (Perkin-Elmer), a series AS-950 autosampler (Jasco), an LC-295 UV detector (Perkin-Elmer) and Turbochrom 4.0 software (Perkin-Elmer). The following conditions were used: SymmetryShield RP-8 column (100 × 4.6 ID mm, 3.5 μm particle size, Waters) at 40°C eluting with acetonitrile : water : trifluoroacetic acid 75 : 25 : 0.1 by volume (A) and acetonitrile: water : trifluoroacetic acid 10 : 90 : 0.1 by volume (B) mixtures: 30 min from 100% A to 0% A in 13 min then 4 min at 100% B; flow rate: 1.0 ml/min; analytical wavelength: 220 nm.

*[2,4,16,16-$^2H_4$]Estromustine (**3**)*

^2H_2O (1.5 ml), Bu_4NBr (88 mg, 0.273 mmol, 1.1 eq) and NaO^2H (170 μl, 2.5 mmol, 10 eq) were added under nitrogen at room temperature to a suspension of **1** (69.6 mg, 0.254 mmol) in dry CH_2Cl_2 (1.5 ml). The clear two-phase mixture was vigorously stirred for 30 min, to which $(ClCH_2CH_2)_2NCOCl$ (84 μl, 0.5 mmol, 2 eq) were then added. After 2 h, the reaction was complete (as determined by HPLC). After dilution with ^2H_2O (1.5 ml) the mixture was extracted with CH_2Cl_2. All the organic extracts were pooled, and successively washed with ^2H_2O (3 × 0.7 ml), ^2HCl (0.5 N in ^2H_2O, 3 × 0.7 ml), water (3 × 10 ml) and finally dried over anhydrous Na_2SO_4. After filtration and evaporation to dryness from CH_3OH, crude **3** was obtained as a white solid; unreacted carbamoyl chloride was the major impurity. The crude material was purified by flash-chromatography on a SiO_2 column (17 × 5.5 cm) using a mixture of ethyl acetate: *n*-hexane 1:3 by volume as eluting solvent system. The collected fractions were combined as appropriate and after evaporation to dryness from CH_3OH, **3** was obtained (67 mg, 0.15 mmol) as a white solid, >96% chemically pure (by HPLC; Rt = 12.82 min). The yield of this step was approximately 59%. Mass spectrum (EI): *m*/*z* 441 ($[M]^+$).

*[2,4,16,16,17-$^2H_5$]Estramustine (**4**)*

Compound **2** (73.3 mg, 0.264 mmol) was reacted with $(ClCH_2CH_2)_2NCOCl$ using the same procedure previously described for compound **1**. After work-up crude **4** was recovered as a white solid. It was purified by flash-chromatography on a SiO_2 column (17×5.5 cm) using a mixture of ethyl acetate: *n*-hexane 1:2 by volume as an eluting solvent system. The collected fractions were combined as appropriate and after evaporation to dryness from CH_3OH, **4** was obtained (85 mg, 0.19 mmol) as a white solid, >98% chemically pure (by HPLC; Rt = 12.54 min). The yield of this step was approximately 72%. Mass spectrum (ESI^+): *m/z* 445.5 ($[M+H]^+$).

*[2,4,16,16,17-$^2H_5$]Estramustine phosphate (**5**)*

To a cooled (−15°C) solution of $POCl_3$ (15 µl, 0.16 mmol) in dry pyridine (200 µl), a cooled (0°C) solution of **4** (45 mg, 0.10 mmol) in dry pyridine (800 µl) was slowly added. After 1 h of stirring at −15°C, the starting material was no longer present (as determined by HPLC). To the obtained whitish suspension, ^2H_2O (500 µl) was added and the temperature increased to −5°C. After 1 h of stirring at −5°C a yellowish solution was obtained, to which a cooled (0°C) solution of ^2HCl (25 wt% in ^2H_2O, 1.3 ml) was slowly added with vigorous stirring. A white suspension was formed that was stirred at room temperature for 1.5 h. The solid was filtered and the white sticky residue successively washed with 0.5 N HCl (3 ml) and water (2×3.5 ml) before dissolving in a mixture of ethanol: ethyl acetate 1:1 by volume (30 ml). After evaporation to dryness from a mixture of ethyl acetate: *n*-hexane 1:20 by volume (20 ml) **5** was obtained (48 mg, 0.09 mmol) as a white solid, >98% chemically pure (by HPLC; Rt = 10.07 min). The yield of this step was approximately 90%. Mass spectrum (ESI^-): *m/z* 523.3 ($[M\text{-}H]^-$).

References

1. Jonsson G, Hogberg B, Nilsson T. *Scand J Urol Nephrol* 1977; **11**: 231–238.
2. Gunnarsson PO, Plym-Forsell G, Fritjofsson A, Norlen B- J. *Scand J Urol Nephrol* 1981; **15**: 201–206.
3. Andersson SB, Gunnarsson PO, Nilsson T, Plym-Forsell G. *Eur J Drug Metab Pharmacokinet* 1981; **6**: 149–154.
4. Hamilton SA, Stanford JL, Gilliland FD, Albetsen PC, Stephenson RA, Hoffman RM, Eley JW, Harlan LC, Potosky A. *J Clin Oncol* 2001; **9**: 2509–2516.
5. Edman K, Svensson L, Eriksson B, Gunnarsson PO. *J Chrom B* 2000; **738**: 267–279.
6. Lidström P, Bonasera TA, Nilsson S, Langström B. *J Labelled Cpd Radiopharm* 1997; **40**: 788–789.
7. Lidström P, Bonasera TA, Marquez-M M, Nilsson S, Bergström M, Langström B. *Steroids* 1998; **63**: 228–234.

