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Development of a sensitive high-performance liquid chromatography method for the detection of 667 COUMATE in vivo☆

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Abstract

Steroid sulphatase inhibitors which decrease or prevent the biosynthesis of oestrogens, potentially have an important role in the treatment of breast cancer in postmenopausal women. The non-steroidal sulphatase inhibitor 667 COUMATE has been shown to be active both in vitro and in vivo. The pharmacokinetics of this drug have not been investigated. In preparation for the clinical evaluation of this agent, a sensitive and robust reversed phase high-performance liquid chromatography (HPLC) method was developed for the detection of 667 COUMATE in biological fluids. The sulphatase inhibitor was extracted from plasma with diethyl ether and separated from putative metabolites and endogenous plasma components with a C3-phenyl column. Using this method an extraction efficiency of 76 ± 5% and a limit of detection of less than 0.1 ng/ml was achieved. The stability of this agent was investigated under different pH conditions and during storage in plasma at room temperature or −20 °C. 667 COUMATE was found to be stable when stored in acidified plasma (pH 4.5) at −20 °C. In conclusion, the HPLC method developed is a reproducible and sensitive assay that will enable quantitation of the potent non-steroidal sulphatase inhibitor 667 COUMATE in biological fluids in the forthcoming Phase I clinical trial.

Keywords: 667 COUMATE; 667 COUMARIN; High-performance liquid chromatography

1. Introduction

Breast cancer is a major cause of death in western countries and there is an urgent requirement for novel treatment strategies. Many breast tumours are oestrogen dependent [1] and consequently inhibition of oestrogen synthesis offers a means of tumour management. One major source of oestrogen production is from oestrone sulphate by the catalytic action of steroid sulphatase [2]. Oestrone-3-*O*-sulphamate (EMATE) was developed as a potent sulphatase inhibitor. Administration of a single oral dose (10 mg/kg) of this agent to female *Wistar* rats resulted in a 93% decrease in hepatic sulphatase activity [3]. Subsequent studies with EMATE in ovariectomized rats revealed that this agent was itself oestrogenic [4]. The non-steroidal coumarin derivative, 667 COUMATE, was synthesised with the aim of retaining the sulphatase inhibitory activity of EMATE without its associated oestrogenicity. 667 COUMATE has been shown to inhibit sulphatase activity in vitro in a placental microsome assay. When a single oral bolus (10 mg/kg) of the sulphatase inhibitor was administered to female rats, hepatic sulphatase activity was decreased to less than 10% of the control values for 5 days [5]. These researchers also showed that 667 COUMATE was not oestrogenic, as indicated by its inability to stimulate uteri growth in the ovariectomised rat. Whilst there is considerable evidence for the efficacy of this agent in the rat, its pharmacokinetics are poorly understood. In order to investigate the disposition and metabolism of this agent in rats and ultimately in humans, a sensitive and reproducible HPLC method was developed for its detection in the biological matrix. In order to improve the reliability of the assay, the stability of the agent in human plasma was investigated both at room temperature and at −20 °C, storage conditions that are likely to be encountered during the clinical evaluation of this agent.

In contrast to many established breast cancer treatment regimens, for example fluorouracil or doxorubicin, which involve intra-venous routes of administration, 667 COUMATE is to be given orally. In order to gain an understanding of how the pH conditions of the gastrointestinal tract may impinge on the disposition of this chemotherapeutic agent, its

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stability was investigated at physiologically relevant pH conditions.

2. Materials and methods

2.1. Synthesis of 667 COUMATE

667 COUMATE was prepared by a Pechmann synthesis of the starting coumarin by reacting resorcinol with the corresponding five-membered cyclic β-ketoester (methyl-2-oxocycloheptane carboxylate) in the presence of concentrated sulphuric and trifluoracetic acids. The resulting phenolic compound was sulphamoylated to generate 667 COUMATE. The chemical structure of 667 COUMATE is shown in Fig. 1 and full details of the chemical synthesis are as described by Woo et al. [6].

2.2. HPLC analysis

667 COUMATE and its putative metabolite 667 COUMARIN were extracted from plasma (0.5 ml) with eight times the volume of diethyl ether (Fisher Chemicals Ltd., Leicestershire, UK) and the plasma was frozen using a dry ice/methanol (Fisher Chemicals Ltd., Leicestershire, UK) mixture. Where appropriate, 7-hydroxycoumarin (Sigma, Dorset, UK) was added to the plasma as an internal standard. The organic phase was decanted to a fresh tube and the solvent removed by centrifugal evaporation at room temperature. The residue was reconstituted in water, acetonitrile: water (1:1 (v/v)), tetrahydrofuran (THF) or acetonitrile (Fisher Chemicals Ltd., Leicestershire, UK). Each reconstituted solution also contained 0.1% trifluoroacetic acid (TFA), which was purchased from Fluka Chemicals Ltd. (Steinheim, Switzerland). The agent was analysed using an Agilent 1100 Solvent delivery module, autosampler and an UV-Vis photodiode array detector (Agilent Technologies, Stockport, Cheshire, UK). Separation of the 667 COUMATE from its putative metabolite and endogenous plasma components was achieved with a Prodigy C3-phenyl column (250 mm × 4.6 mm, 5 μm), purchased from Phenomenex (Cheshire, UK). A linear gradient of 5–95% acetonitrile in water over 30 min was used. The organic and aqueous modifiers of the mobile phase were acidified with TFA (0.1%).

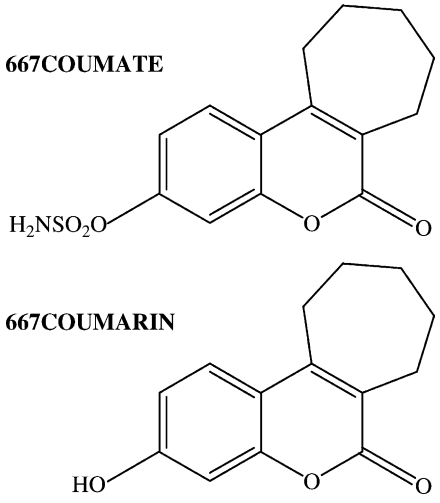

Fig. 1. Chemical structures of 667 COUMATE and 667 COUMARIN.

2.3. Stability studies in buffer and plasma

667 COUMATE (10 μM), dissolved in THF, was incubated in sodium phosphate buffer (0.2 M) and maintained at room temperature (24 °C) for 0.5, 1, 3, 4 h. Plasma was prepared by centrifugation of human blood (2800 × *g*, 4 °C, 15 min). The sulphatase inhibitor was incubated in plasma (0.5 ml) or plasma acidified by addition of 1 M citric acid (9:1 (v/v)). The final pH of the acidified plasma was 4.5. 667 COUMATE and its putative metabolites were extracted from buffer and plasma and analysed by HPLC as described in Section 2.2.

2.4. Stability studies of 667 COUMATE stored at −20 °C

667 COUMATE (10 μM), dissolved in THF, was maintained in either acidified or non-acidified plasma for 0, 1 and 4 weeks. The acidified plasma was prepared by addition of sodium citrate buffer (0.2 M, pH 3) to whole blood (1:9 (v/v)) and separated from other blood constituents by centrifugation (2800 × *g*, 4 °C, 15 min). The agent and its metabolites were extracted from plasma as described in Section 2.2.

3. Results

3.1. HPLC assay optimisation

The HPLC method described here separated the sulphatase inhibitor, 667 COUMATE from its putative metabolite 667 COUMARIN and the internal standard, 7-hydroxycoumarin (Fig. 2). The retention times of these analytes are shown in Table 1. The extraction efficiency of the agent from plasma was determined to be 76 ± 5% and the limit of detection of 667 COUMATE in plasma was less than 0.1 ng/ml. The coefficients of variation (inter-day and intra-assay) were found to be less than 10%. In order

Table 1
Retention times of 7-hydroxycoumarin, 667 COUMATE and 667 COUMARIN

Compound	HPLC retention time ± 0.5 min
667 COUMARIN	21.5
667 COUMATE	22.5
7-Hydroxycoumarin	14.7

For details of HPLC conditions see Section 2.

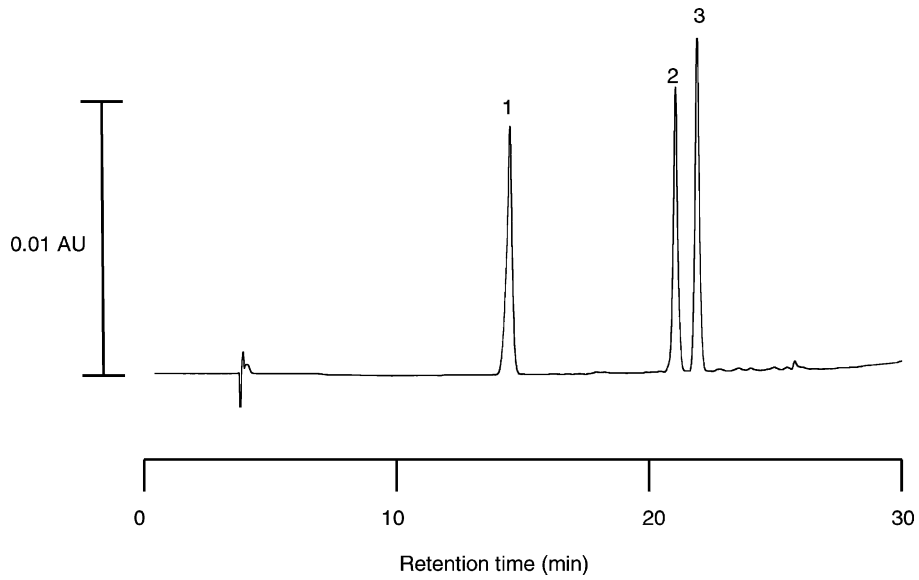

Fig. 2. High-performance liquid chromatogram with detection at 320 nm of authentic standards dissolved in acetonitrile. The solvent contained 0.1% TFA. The internal standard, 7-hydroxycoumarin, 667 COUMARIN and the sulphatase inhibitor, 667 COUMATE are represented by peaks 1–3, respectively. For conditions of HPLC analysis and the retention times of the analytes see Section 2.2 and Table 1, respectively.

to investigate the stability of the 667 COUMATE in the autosampler, the agent was dissolved in water, acetonitrile: water (1:1 (v/v)), THF or acetonitrile and maintained at 24 °C. Each solvent contained 0.1% TFA. When 667 COUMATE was dissolved in acidified water, 10% of the agent was found to degrade to 667 COUMARIN in 4 h (data not shown). The sulphatase inhibitor was also unstable in acetonitrile: water (1:1 (v/v)). In contrast, 667 COUMATE was stable in acidified acetonitrile and THF at room temperature for at least 16 h.

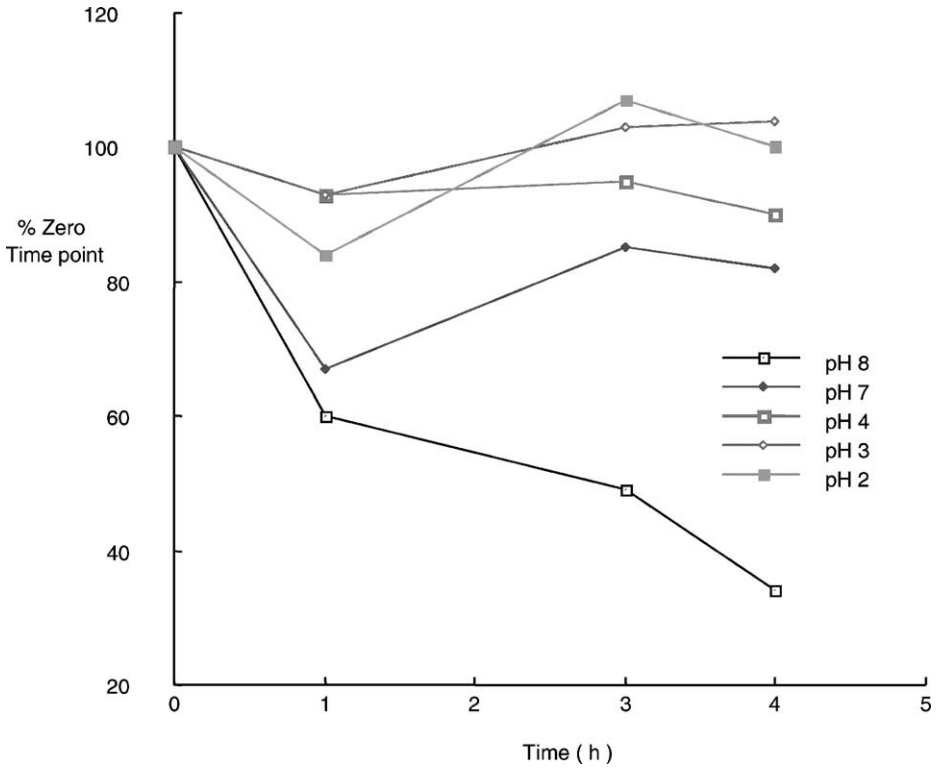

Fig. 3. Time course of effect of pH on stability of 667 COUMATE in sodium phosphate buffer. The agent was maintained at room temperature (24 °C). For details of the extraction of 667 COUMATE from sodium phosphate buffer and HPLC analysis see Section 2.

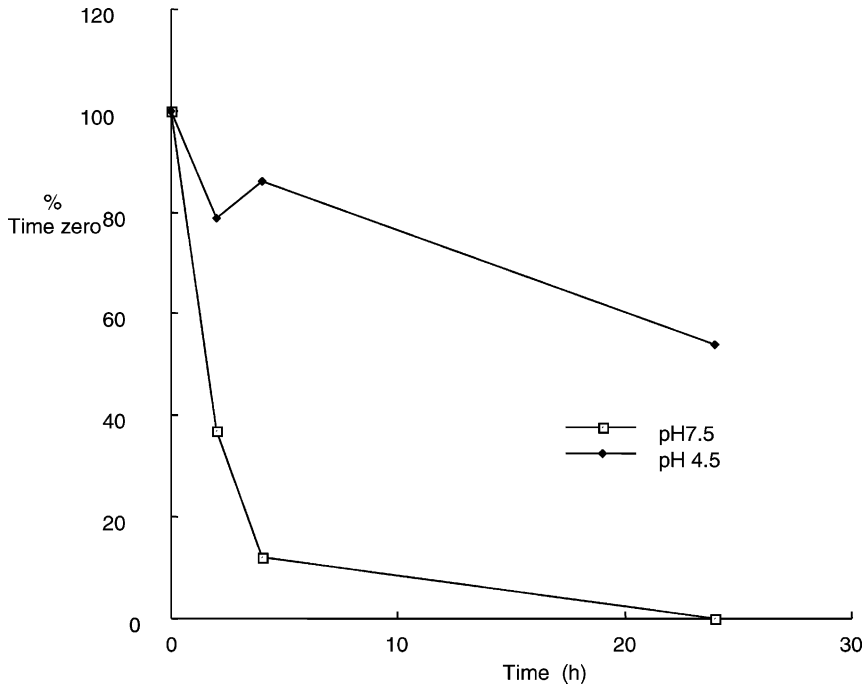

Fig. 4. Time course of effect of pH on stability of 667 COUMATE when maintained either in plasma or acidified plasma (pH 4.5) at room temperature (24 °C). For details of the preparation of plasma, extraction of 667 COUMATE and HPLC analysis see Section 2.

3.2. Stability of 667 COUMATE in buffer and plasma

The stability of 667 COUMATE was investigated under different pH conditions (Fig. 3). These data demonstrate that the stability of the sulphatase inhibitor in phosphate buffer is increased at lower pH values. In order to determine whether the stability of 667 COUMATE in human plasma could also be increased by lowering the pH, the agent was incubated at room temperature with non-acidified plasma or acidified plasma (pH 4.5) for up to 24 h (Fig. 4). When 667 COUMATE was maintained in acidified plasma, more than 80% of the agent remained intact after 4 h (Fig. 5C). In contrast, less than 20% of the agent was recovered intact following incubation in non-acidified plasma for 4 h (Fig. 5D).

3.3. Stability of 667 COUMATE at −20 °C

Due to the rapid degradation of 667 COUMATE in human plasma at room temperature (Fig. 4), it was vital to develop a method of acidifying human blood immediately upon removal from the patient. Whole blood was acidified with sodium citrate buffer to give a final pH of 4.5, from

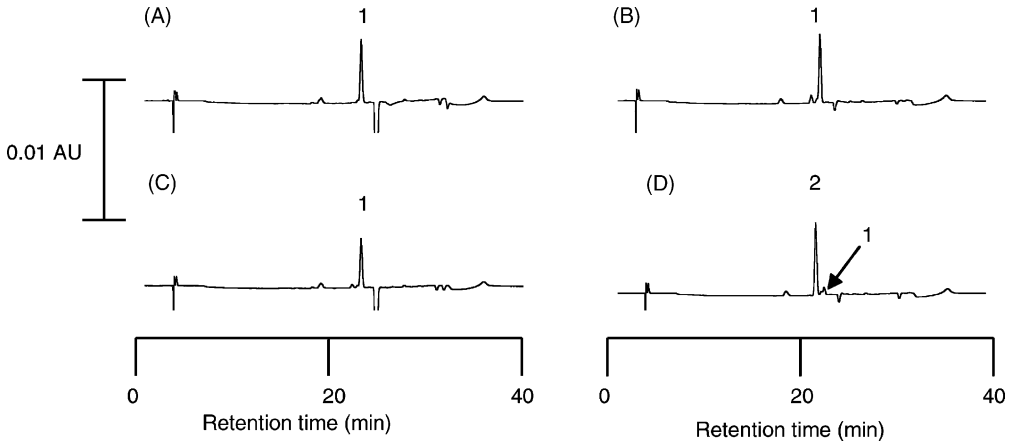

Fig. 5. High-performance liquid chromatogram with detection at 320 nm of plasma extracts. 667 COUMATE was added to acidified plasma (A and C) or plasma (B and D) and maintained for either 0 h (A and B) or 4 h (C and D) at room temperature. 667 COUMATE and 667 COUMARIN are represented by peaks 1 and 2, respectively. For details of the preparation of plasma, extraction of 667 COUMATE and HPLC analysis see Section 2.

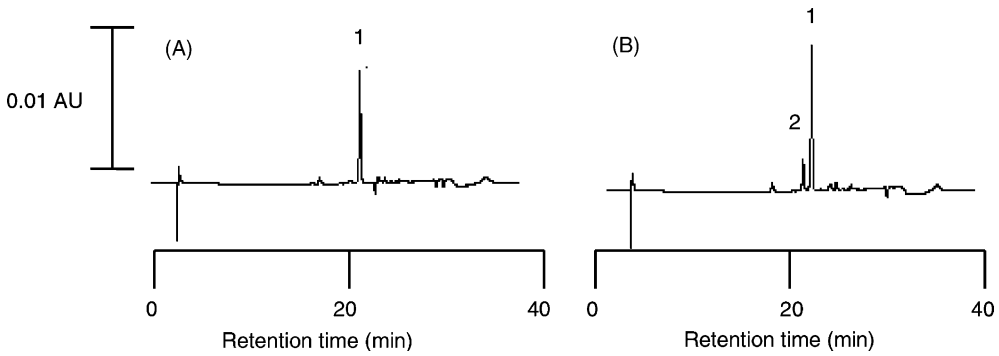

Fig. 6. High-performance liquid chromatogram with detection at 320 nm of plasma extracts. 667 COUMATE was maintained in plasma prepared from acidified blood (A) or non-acidified blood (B) for 4 weeks at −20 °C. 667 COUMATE and 667 COUMARIN are represented by peaks 1 and 2, respectively. For details of the preparation of plasma, extraction of 667 COUMATE and HPLC analysis see Section 2.

which plasma was prepared. Using this acidified plasma and non-acidified plasma, levels of 667 COUMATE were compared after storage at −20 °C for up to 4 weeks (Fig. 6). When stored at −20 °C in plasma prepared from acidified whole blood, no degradation of 667 COUMATE was detected at 4 weeks (Fig. 6). However, when the drug was added to non–acidified plasma a small amount of degradation of 667 COUMATE to 667 COUMARIN was detected.

4. Discussion

The novel HPLC method described in this paper was developed in order to allow detection of 667 COUMATE and its putative metabolite, 667 COUMARIN in biological samples. Initially, a procedure was used in which 667 COUMATE was extracted from plasma with dichloromethane following a protocol developed for the extraction of EMATE [7]. However, an emulsion was formed between the organic and water phases which resulted in poor extraction efficiency (50%) of the agent from plasma. An alternative extraction method was developed using diethyl ether to extract 667 COUMATE from plasma and an extraction efficiency of 76% was achieved. This method also allowed rapid extraction of the drug from plasma because the lower aqueous layer could be frozen and the organic layer decanted.

667 COUMATE was separated from its putative metabolites and other endogenous plasma components with a C3-phenyl column. This column was chosen because the phenyl side groups are able to participate in π–π interactions which should provide an enhanced selectivity for aromatic compounds. The selectivity of the column for the COUMATE moiety means that any other metabolic products, for example conjugates of the drug generated in vivo, should also be detected.

Breakdown of 667 COUMATE to 667 COUMARIN was observed when samples were maintained in aqueous solutions for prolonged periods (e.g. overnight in the autosampler). The instability of 667 COUMATE in aqueous, basic medium is probably a corollary of hydrolytic attack of the sulphamate group together with a competing beta elimination mechanism ($ROSO_2NH_2 \rightleftharpoons ROSO_2NH^- + H_2O \rightarrow ROH + H_2NSO_3^-$). The stability of the agent can be improved by removal of water or lowering the pH of the solution (Fig. 3). Extracted plasma samples for HPLC analysis were routinely prepared in 100% acetonitrile acidified with 0.1% TFA. Under these storage conditions breakdown could not be detected after maintaining the agent for 16 h at room temperature. It is important for HPLC studies with this compound that liquid samples which are to not be analysed immediately (e.g. queuing in an autosampler) be stored in an acidified organic matrix. The agent can be stabilised in plasma by acidification (Fig. 4). However, it may more appropriate to acidify the whole blood prior to preparation of plasma, as this will minimise the time delay consequent of centrifugation of the blood. We found that sodium citrate buffer at pH 3 could be used to reduce the pH of the blood to 4.5, without causing red blood cell lysis or coagulation. After acidification, plasma samples could be stored for up to 4 weeks at −20 °C with minimal loss of 667 COUMATE (Fig. 6). In conclusion, the acidification of the whole blood, storage at −20 °C and final extraction into an acidified organic phase followed by HPLC analysis provides a feasible and practical method for the pharmacokinetic analysis of 667 COUMATE with minimal losses.

The pH stability of 667 COUMATE was examined because the drug is proposed to be administered as an oral formulation and hence the compound will be exposed to the low pH of the stomach. Over a range of pH from 2 to 8, it was clear that acidic conditions enhanced the stability of the drug. Consequently, the agent should be stabilised under the acidic conditions of the upper gastrointestinal tract. It will be interesting to investigate the absorption of 667 COUMATE under acidic conditions using cells derived from the human gastrointestinal tract, e.g. Caco-2 cells [8–10].

667 COUMATE has previously been shown to cause regression of rat mammary gland tumours induced with nitrosomethylurea [3]. The agent has also been shown to inhibit

oestrone sulphatase activity in vivo in female rats and in vitro in a placental microsome assay with an IC_{50} of 8 nm. The sensitive, reproducible and robust assay described in this paper will allow plasma levels of 0.1 ng/ml 667 COUMATE to be detected in human plasma. This method should allow quantitation of this agent and potentially its metabolites in human samples generated during clinical trials.

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