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Accumulation of estramustine and estromustine in adipose tissue of rats and humans

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Summary. The tissue distribution of estramustine and estromustine, two cytotoxic lipophilic metabolites of estramustine phosphate (Estracyt, EMP) was studied in rats and humans. A single dose of [^{3}H]-estramustine was given i.v. to groups of rats. At 24 h after administration, the concentration of radioactivity in fat was about 20, 12, and 2 times that in muscle, plasma, and liver, respectively. Liquid chromatography verified that the radioactivity represented estramustine and estromustine. The clinical relevance of these results was investigated in pancreas cancer patients treated with a single oral dose of Estracyt at 12–16 h before surgery. As judged by gas chromatography, the concentration of estromustine, which is the main metabolite in man, was about 13 times higher in fat than in plasma and was also higher in adipose tissue than in muscle and liver. After 5 days of Estracyt treatment, the adipose uptake of estromustine was even higher, namely, about 40 times that in plasma and 8 times that in muscle and liver. Thus, our results demonstrate that estramustine and estromustine are stored in adipose tissue after the administration of EMP; this is important for the pharmacokinetics and, consequently for the therapeutic effects of Estracyt.

Introduction

Adipose tissue serves as a depot for several compounds in the body [2]. Consequently, fatty tissue can be of importance for the pharmacokinetics and, thus, for the pharmacological effects of the compounds. Although the mechanism of adipose uptake of various drugs is not yet quite clear, it appears that the uptake correlates with the lipid solubility of the compounds [2, 13].

Estramustine phosphate (Estracyt, EMP) is a nornitrogen mustard derivative of estradiol-17β-phosphate. The drug is an anti-mitotic agent with estrogenic properties and is widely used in the treatment of prostatic cancer [7, 8]. It is currently undergoing clinical trials against other forms of cancer. EMP is a prodrug that is extensively metabolized in the body (Fig. 1) [4]. Parent estramustine phosphate cannot be detected in plasma from cancer patients who have been treated orally with EMP, which indicates complete first-pass metabolism. EMP is dephosphorylated to estramustine, which is then oxidized in the 17th position to estromustine. Estramustine and estromustine, which are the main metabolites in plasma from cancer patients who have been treated with Estracyt, are responsible for the anti-tumour and anti-mitotic activity of the drug [12]. Estradiol and estrone are formed after cleavage of the carbamate ester of estramustine and estromustine and contribute to the estrogenic properties of the drug. EMP is a hydrophilic acid and exhibits a very small volume of distribution [4]. On the other hand, estromustine and estramustine are highly lipophilic compounds that display a large volume of distribution. In the prostate of the rat [3] and in human prostatic cancer [11], estramustine is enriched. This has been attributed to its binding to a specific intracellular protein, the so-called estramustine binding protein (EMBP).

The aim of the present investigation was to study the distribution of the different EMP metabolites in adipose tissue as compared with muscle, plasma and liver. Single doses of [^{3}H]-estramustine were therefore given to rats and the concentration of radioactivity was measured in the different organs. The identity of the radioactive material was investigated by liquid chromatography. Furthermore, single and repeated oral doses of Estracyt were given to pancreatic cancer patients before surgery to investigate the clinical relevance of animal results.

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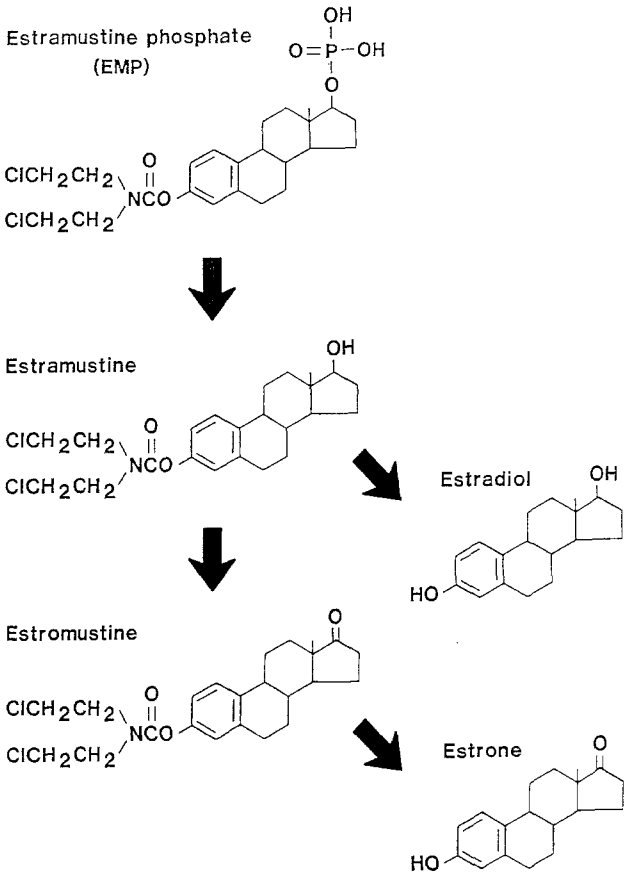

Fig. 1. Metabolic pathways for estramustine phosphate

Patients, materials, and methods

Animal studies

Animals. Specific pathogen free (SPF) male Sprague-Dawley rats were obtained from Anticimex Laboratory Animals Breeding, Sollentuna, Sweden. The animals were approximately 7 weeks old and weighed about 220 g at the start of the study. The rats were fasted for 16 h before dosing. After dosing, they had free access to water, and food was offered at 4 h after drug administration.

Test compound. [^{3}H]-Estramustine (2, 4, 6, 7-^{3}H) was synthesized at Pharmacia LEO Therapeutics AB, Helsingborg, Sweden (sp. act., 6.6 mCi/mmol). The chemical and radiochemical purity determined by thin-layer chromatography and liquid chromatography was $\geq 95\%$.

Treatment. The rats were randomly divided into groups of four animals each. The animals were injected i. v. in the tail vein with 5 mg/kg [^{3}H]-estramustine dissolved in polyethylene glycol 400 (1 ml/kg). Groups of rats under ether anesthesia were killed by exsanguination via the aorta at 0.5, 1, 4, 8, 24, and 48 h after drug injection. Blood and tissue samples were collected.

Analysis of radioactivity. Total radioactivity was determined by combustion of aliquots (200 µl) of whole blood and samples of tissues (200–400 mg) in a Packard Tri-Carb Sample Oxidizer (Model 506). The combustion products were then submitted to a liquid scintillation spectrometer (Packard Tri-Carb Liquid Spectrometer, Model 2650) or a Philips PW 4510-00 Liquid Scintillation Analyzer. Corrections for quenching were made by the external-channels ratio method. The tissue samples were analysed in triplicate. The mean recovery of tritium in the combustion procedure was 102% (range, 95%–113%). Aliquots of plasma samples were determined by dilution in Instagel and counted in a liquid scintillation spectrometer.

Liquid chromatography. Tissues (0.7–2 g) were extracted using 10–30 vol. methanol. The extracts were analysed by liquid chromatography as described elsewhere [9]. The liquid chromatography system consisted of a Model 6000 A pump, a guard column, a U6K injector, a Model 440 UV detector (254 nm; Waters Associates, Milford, Mass., USA) and a Radial-Pak C18 cartridge column. The chromatograms were recorded on a W+W Tarkan recorder 600, and the outlet of the detector was connected to an LKB 7000 Ultrorac fraction collector. The mobile phase consisted of acetonitrile-water (60:40, v/v) and 5 ml was collected; it was then changed to acetonitrile-water (75:25, v/v) and about 20 ml was collected. The positions of references were revealed by the addition of authentic compounds to the methanol extract. The concentrations of EMP metabolites were determined in duplicate and mean values were used for calculations. In a separate experiment, the identities of the chromatographic peaks were verified by gas chromatography.

Human studies

Patients. A total of 13 subjects aged 44–78 years (8 men and 5 women) underwent surgery for cancer of the pancreatic head. After thorough investigation, all were found to be fit for major surgery, which implies that their cardiac, renal, and hepatic capacity was considered to be good. At surgery, the cancer was found to be resectable in nine cases and a radical procedure was carried out. In four cases, cancer spread outside the regional lymph nodes was found and a palliative procedure was performed.

Study design. Eight patients were given single oral doses of 280 mg Estracyt at 12–16 h before surgery. This period lies well beyond the termination of the absorption an distribution phases for EMP and its metabolites [4]. Five patients were treated orally with Estracyt in two daily doses of 280 mg for 5 days before surgery. As in the single-dose study, samples were taken at 12–16 h after the last dose. At surgery, samples of fat (1–3.5 g), liver (0.5–1 g), and muscle (0.2–0.6 g) were obtained and a blood specimen (10 ml) was taken. Plasma was separated by centrifugation at 1,300 *g* and 4° C for 10 min. The samples were immediately frozen and stored at –20° C until their analysis. The study was approved by the Ethics Committee of Lund.

Gas chromatography. Plasma was analysed for estramustine, estromustine, estrone, and estradiol using a selective chemical method based on gas chromatography with selected ion monitoring (GC-SIM) [1]. For determination of the concentrations of the four compounds in tissues, the method was used in a modified form as described elsewhere [11]. The limit of quantification was about 150 ng/g for estramustine and estromustine and 15 ng/g for estrone and estradiol when 0.2 g tissue was extracted and analysed. In plasma, the limit was 15 ng/ml for estramustine and estromustine and 3 ng/ml for estrone and estradiol. The concentrations of EMP metabolites were determined in duplicate and mean values were used for the calculations.

Results and discussion

The pharmacokinetic properties and, consequently, the pharmacological effects of a drug are often influenced by storage of the drug and/or its metabolites in various tissues in the body. Adipose tissue is an important potential storage site; it is well known that many compounds that are highly lipophilic can be stored in fat [2, 13].

EMP (Estracyt) is used in the treatment of prostatic cancer [7, 8] and is currently undergoing clinical trials against other forms of cancer. The drug is metabolized in the body to the two cytotoxic metabolites estramustine and estromustine [4]. Both of these metabolites are highly lipophilic, and the present study was therefore initiated to evaluate the possible accumulation of these compounds in

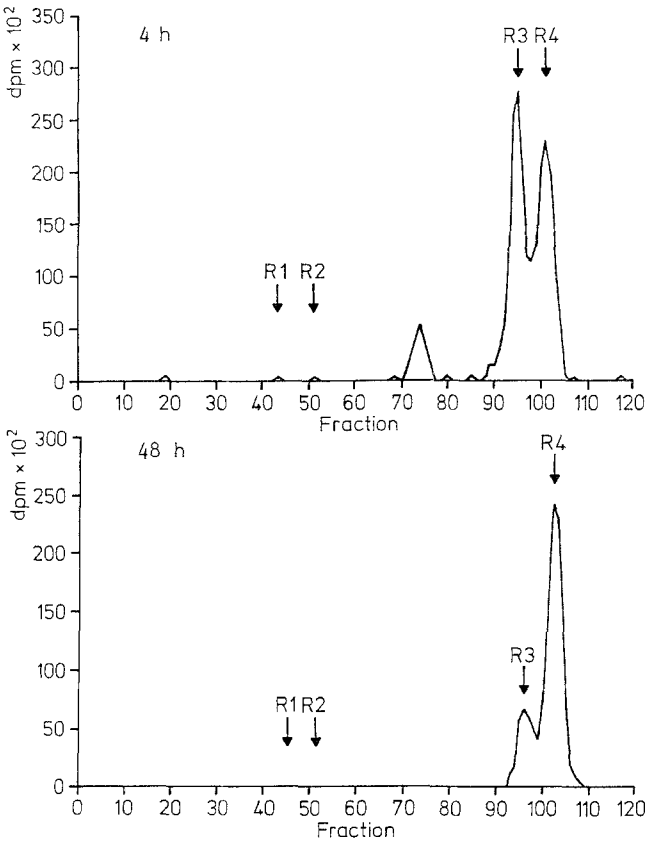

Fig. 2. Chromatograms of adipose tissue extracts obtained using liquid chromatography at 4 and 48 h after a single dose of 5 mg/kg [^{3}H]-estramustine. References: *R1*, estradiol; *R2*, estrone; *R3*, estramustine; *R4*, estromustine

fatty tissue. For purposes of comparison, the distribution of two estrogen metabolites, estradiol and estrone, in adipose tissue was also studied.

First we studied the distribution of [^{3}H]-estramustine after its i.v. administration to rats. After oral administration in humans, EMP is well absorbed and then completely dephosphorylated to estramustine pre-systemically. However, in rats, the absorption of EMP is incomplete after oral administration (Gunnarsson, unpublished results); therefore, in the present rat study we chose to use i.v. infusion of the dephosphorylated metabolite estramustine. Table 1 summarizes the concentration of radioactivity in plasma, muscle, liver, and fat. It can be seen that the concentration not only in the liver but also in fat was high as compared with that in plasma and muscle. At 24 h after administration, the concentration of radioactivity in fat was about 20, 12, and 2 times that in the muscle, plasma, and liver, respectively.

Table 2. Tissue concentrations of EMP metabolites after a single oral dose of 280 mg Estracyt in eight cancer patients

Tissue	Estromustine	Estrone
Plasma	54 (<20–318)	6 (2– 21)
Muscle	270 (140–500)	<40
Fat	690 (270–1,690)	32 (28– 64)
Liver	430 (220–4,800)	38 (32–420)

Tissue samples were taken at 12–16 h after the Estracyt dose. Estradiol and estramustine levels were below the limit of detection in all samples. Data represent median values expressed in nanograms of metabolite per gram of tissue; ranges are shown in parentheses

To identify the radioactivity taken up in fat, we extracted aliquots of the tissue with methanol and analysed the extracts using liquid chromatography. Figure 2 demonstrates that most of the radioactivity could be accounted for as parent estramustine and its metabolite estromustine, with only minor amounts representing estradiol and estrone. Since our results gave support to the hypothesis that adipose tissue might contribute to the pharmacokinetic profile of estramustine, we initiated experiments in patients to clarify the clinical significance of this finding.

The clinical efficacy of Estracyt in pancreas cancer was investigated in a phase II clinical trial. Because some of these patients also underwent surgical treatment, an opportunity arose to study the uptake of EMP metabolites in different tissues. One group of eight patients were given a single oral dose of Estracyt at 12–16 h before surgery. This interval was chosen to ensure that the sampling time lay well beyond the termination of the absorption and distribution phases for EMP and its metabolites [4]. The concentration of EMP metabolites were determined in fat, plasma, liver, and muscle. A selective method based on GC-SIM for determination of the metabolites in plasma was used, with some modifications. Estromustine and estrone could be quantified in most of the samples, whereas estramustine and estradiol levels were below the limit of detection in all samples after a single dose of Estracyt. As can be seen in Table 2, the median concentration of estromustine was 13 times higher in fat than in plasma and was also higher in adipose tissue than in muscle and liver. Estrone also accumulated to some extent in fat, but levels of this metabolite in adipose tissue were only 6 times those in plasma.

In a second group of five subjects, we studied the accumulation of the metabolites after 1 week of treatment with

Table 1. Tissue concentrations of radioactivity following a single i.v. dose of 5 mg/kg [^{3}H]-estramustine in rats

Tissue	Time after dosing (h)					
	0.25	1	4	8	24	48
Plasma	2.91 ±0.27	1.93 ±0.16	1.42 ±0.38	0.72 ±0.11	0.35 ±0.03	0.3 ±0.03
Liver	23 ±0.99	20.3 ±1.5	18.2 ±4.6	9.72 ±1.36	1.8 ±0.14	1.31 ±0.27
Muscle	4.64 ±0.19	2.02 ±0.25	0.64 ±0.06	0.36 ±0.04	0.21 ±0.036	0.15 ±0.022
Fat	7.04 ±1.51	12.8 ±0.89	14 ±3.24	8.86 ±0.86	4.05 ±0.66	2.14 ±0.42

Data represent mean values ± SD ($n = 4$) expressed in nanogram equivalents of estramustine per gram of tissue

Table 3. Tissue concentrations of EMP metabolites after treatment of five cancer patients with 280 mg Estracyt two times daily for 1 week

Tissue	Estromustine	Estrone	Estramustine	Estradiol
Plasma	116 (27– 153)	18 (11– 29)	<15	<3
Muscle	580 (270– 8,030)	22 (<18–112)	<150	<15
Fat	4,700 (1,480–16,100)	180 (140–370)	325 (114–820)	<15
Liver	610 (190– 1,680)	86 (19–170)	310 (<190–650)	29 (19–124)

The last dose was given at 12–18 h prior to surgery. Data represent median values expressed in nanograms of metabolite per gram of tissue; ranges are shown in parentheses

Estracyt. As the half-life of estromustine in man is 10–20 h [4], 1 week of treatment should guarantee that steady-state conditions are obtained. Table 3 demonstrates that the accumulation of estromustine after repeated administration was even higher than that after a single dose. The median concentration in fat was 40 times that in plasma and 8 times that in muscle and liver. This experiment also indicated that estrone is accumulated as well, but the accumulation factor was only about 10. A median value for estramustine but not estradiol in fat could be determined after repeated EMP administration. None of the compounds could be quantified in plasma, but as judged by the limit of quantification of estramustine in plasma, the ratio between fat and plasma could be estimated to be at least 20. Thus, although the levels of estramustine in patients were at least 10 times lower than those of estromustine, the accumulation in adipose tissue appeared to be of the same magnitude. Estromustine is the main metabolite in humans, whereas it is far less pronounced in rats; this observation indicates an obvious species difference in estramustine metabolism.

Thus, our results demonstrate that the two cytotoxic metabolites of EMP, estramustine and estromustine, are stored in adipose tissue. These two compounds have log *P*-values that are >4 (Andersson, unpublished results). Interestingly, it has been suggested that there is a threshold value for log *P* at around 2.5, above which limit adipose storage occurs [2].

As mentioned above, the plasma half-life of estromustine and estramustine in cancer patients is 10–20 h [4]. The half-life of the metabolites estradiol and estrone lie in the same order of magnitude as that of estromustine, which indicates that elimination of these metabolites is governed by the rate of their formation from estramustine and estromustine. The half-life of estradiol and estrone after administration of the compounds as such is $\leqslant 4$ h in man [10].

The uptake of estramustine and estromustine in the prostate of rats and cancer patients has previously been thoroughly studied [3, 11]. These studies indicate that the cytotoxic metabolites of EMP are retained in the prostate by selective binding to a specific cytosolic protein, the so-called estramustine-binding protein (EMBP). EMBP is not present in adipose tissue, but our results suggest that partition of estramustine and estromustine into adipose tissue plays an additional role in the pharmacokinetics and, thus, in the clinical efficacy of EMP metabolites. The adipose accumulation may act as a reversible depot, which continuously provides the prostate with the cytotoxic metabolites estramustine and estromustine. This may be of clinical significance, because pharmacology studies have suggested that the anti-mitotic effect of the drug is not only dose-dependent but also time-dependent [6].

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