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Distribution of estramustine in the BT4C rat glioma model

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Abstract Estramustine (EaM), a carbamate ester of 17β-estradiol and nor-nitrogen mustard, is a cytotoxic compound with antitumoral effect in malignant glioma in vitro and in vivo . However, knowledge of the pharmacokinetics of EaM in experimental glioma is limited. The objective of this study was therefore to investigate further the distribution of EaM in the BT4C rat glioma model. Assessment of EaM uptake and distribution was performed by quantitative whole-body autoradiography. In addition, the uptake of EaM and its metabolites estromustine (EoM), estradiol, and estrone were analyzed by gas chromatography. EaM was taken up from the circulation and was found to be the main product in glioma tissue. Whole-body autoradiography after [^{14}C]-EaM administration revealed a strong ^{14}C label simultaneously in tumor and normal brain tissue at 0.5 h after drug administration. In tumor tissue, sustained high levels of ^{14}C label were detected at 12 h after drug administration. In contrast to the tumor, radioactivity in normal brain tissue rapidly leveled off, indicating a retention of radioactivity in the tumor. The tumor/brain radioactivity ratio reached a peak of 4.5 at 12 h after drug administration. High levels of ^{14}C label were also found in pulmonary tissue. By gas chromatography, EoM was found to be the main metabolite in plasma. However, EaM reached higher levels in tumor tissue, with the mean tumor/plasma ratio being 11.7 as compared with 2.0 for EoM. Only low plasma levels of the estrogen metabolites were detected. In conclusion, EaM is taken up in the BT4C rat glioma tissue and is retained in the tumor as compared with normal brain tissue and plasma. EaM showed a greater selectivity for tumor tissue, exhibiting a high tumor/plasma ratio as compared with EoM. The distribution pattern after administration of EaM, as evaluated by both whole-body autoradiography and gas chromatography, supports the earlier suggestion that the uptake is related to a protein with EaM-binding characteristics.

Key words Estramustine · Glioma · Chemotherapy · Autoradiography · Gaschromatography

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Introduction

The treatment of malignant glioma remains one of the most challenging tasks in oncology. Patient survival after surgical resection and postoperative radiotherapy is only 10–12 months [27, 36] and long-term survivors are very rare [28]. Due to diffuse migration of tumor cells into the surrounding brain, locoregional treatment will almost never cure a malignant glioma, and chemotherapy has thus far been disappointing [36]. Therefore, a continuous search for chemotherapeutic agents that more effectively inhibit tumor progression is of utmost clinical importance.

Estramustine phosphate (EMP), a carbamate ester of 17β-estradiol and nor-nitrogen mustard, is currently in clinical use for the treatment of advanced prostate cancer. After oral delivery, EMP is rapidly dephosphorylated in the gastrointestinal tract to its main metabolites estramustine (EaM) and estromustine (EoM; Fig. 1) [3, 17]. Recent preclinical findings suggest that EMP/EaM is an active cytotoxic agent in experimental glioma in vitro as well as in vivo [4, 25, 29, 30, 33, 38, 40]. The mechanism of action is only partly understood, but one of the main effects seems to involve the microtubule system [11, 22, 34, 35, 39]. Furthermore, EaM has also been shown to exert cytotoxic effects on the DNA level through induction of apoptosis and interference with DNA synthesis [25, 38]. The uptake and action of EMP/EaM in prostatic carcinoma is closely related to a specific EaM-binding protein (EMBP) present

in high concentrations in human as well as murine prostate [10, 13, 15, 16]. In glioma a protein with similar binding characteristics for EaM has been demonstrated [5, 6, 32].

The pharmacokinetics of EMP/EaM in experimental as well as human prostatic cancer has previously been described [12, 18]. In glioma the information regarding EaM uptake and distribution is limited, and little is known about the spatial and temporal distribution in brain tissue and brain tumors. The role of estrogens as growth factors for malignant glioma has also been discussed, which emphasizes the importance of analyzing the distribution of the estrogen metabolites of EaM/EMP in a glioma model [24, 30, 31]. Furthermore, most side effects of EMP are related to the estrogenic metabolites of the compound. Therefore, we performed a study utilizing quantitative whole-body autoradiography and gas chromatography to describe the spatial as well as temporal uptake and distribution of EaM and its metabolites in the BT4C rat glioma model.

Materials and methods

In vivo glioma model and EaM treatment

The syngeneic intracerebral BT4C rat glioma model was used for this study [4]. Intracerebral transplantation of BT4C rat glioma cells was performed in inbred BDIX rats of both gender (BT4C cells were generously donated by R. Bjerkvig, Bergen, Norway). Prior to implantation, tumor cells were suspended in minimal essential medium (MEM; Flow Laboratories, Glasgow, Scotland) supplemented with 5% rat serum. Under stereotactic conditions, 20,000 tumor cells in a volume of 5 µl were deposited in the caudate nucleus 3.5 mm left of the bregma and at a depth of 4.5 mm using a microsyringe (22-gauge; Unimetrics, Shorewood, Ill., USA). Animals were housed under controlled conditions and fed ad libitum. The experiments were approved by the local ethics committee for animal research with the recommendation of using a limited number of animals.

The EaM dose given in this study was obtained from previous studies [4] demonstrating that 20 mg/kg EaM exerts a significant antitumoral action and also shows minimal signs of toxicity in the BT4C rat glioma model.

Quantitative whole-body autoradiography

A total of 12 rats with intracerebral BT4C tumors were chosen for autoradiography studies performed on day 24 after tumor implantation. [^{14}C]-EaM (Pharmacia AB, Helsingborg, Sweden) was obtained by substitution of all carbon atoms in the nitrogen mustard part of the molecule with ^{14}C. The specific activity of the compound was 28 µCi/mg and the radiochemical purity was 96%. Animals were put under light anesthesia using a 1:1 mixture of Hypnorm (fluanisonum at 10 mg/ml and fentanylum at 0.2 mg/ml) and Dormicum (midazolam at 5 mg/ml) given at 1.8 ml/kg as a single i.p. injection. To obtain a safe route of administration the central tail artery was freed and cannulated with a heparinized polyethylene catheter (PE 50, outer diameter 0.965 mm; Clay-Adams, USA), after which 20 mg/kg [^{14}C]-EaM was injected into the tail artery as a bolus injection. The animals were euthanized with an anasthesia overdose at 0.5, 2, 4, 12, or 24 h after [^{14}C]-EaM injection.

The technique of whole-body autoradiography has been described elsewhere [37]. In brief, all samples were rapidly frozen in hexane cooled with dry ice (–78 °C). Most animals were mounted in a gel of carboxymethylcellulose (CMC) for sagittal whole body sectioning. Some animals were postmortally decapitated and the heads were mounted for frontal sectioning. From each rat, 20-µm-thick sagittal sections were cut at different levels with a cryomicrotome (LKB 2250 PMV Cryomicrotome) at a temperature of –20 °C. The sections obtained were mounted on tape (Minnesota Mining and Manufacturing Co., number 810). After being freeze-dried at –20 °C for at least 24 h, the sections containing the ^{14}C-radioactivity were apposed to imaging plates for 2 days and measured by radioluminography (RLG). The imaging plates were analyzed using a bioimaging analyzer system (BAS 2000 Fuji, Japan). In this system the radiation energy stored on the imaging plate is emitted as photostimulated luminescence (PSL), having an intensity proportional to the radiation energy stored [1]. The PSL values are transformed to concentration units by means of radioactive ^{14}C-standards (Amersham, UK). The picture obtained was displayed on a monitor, and various regions of interest (ROI) were delineated for calculation of average concentrations expressed in nanogram equivalents of EaM per gram tissue.

Gas chromatography

Ten tumor-bearing rats were selected for gas chromatography analysis of EaM, EoM, estradiol, and estrone. Uptake was analyzed after euthanization at 2, 4, 12, and 24 h after i.p. injection of 20 mg/kg EaM. In addition, two animals were given EaM at 20 mg/kg i.p. daily for 5 consecutive days, and drug uptake was analyzed at 24 h after the last injection. The rats were decapitated and their brains were taken out and carefully dissected under a microscope. Tumor tissue and contralateral normal brain tissue were frozen in liquid nitrogen. Blood collected from cervical vessels at decapitation was centrifuged and serum was frozen in liquid nitrogen and stored at below –70 °C until analyzed.

Serum samples and tissue specimens were analyzed using gas chromatography as previously described [2, 4]. Serum was analyzed for EaM, EoM, estradiol, and estrone using gas chromatography. Prior to gas chromatography the samples were purified by a C18 solid-phase and liquid-liquid extraction procedure and derivatized with BSTFA [bis(trimethylsilyl)trifluoroacetamide]. The reagent solution of each sample was divided into two parts and evaporated. One part of the residue was dissolved in xylene, and EoM and EaM were quantified by gas chromatography (HP 5890A) with nitrogen-phosphorus (NP) detection. The other part was dissolved in toluene, and estrone and estradiol were quantified by gas chromatography with selected ion monitoring (Varian 3400-Finnigan Mat INCOS 500). As internal standards the chemical analogues PNU-212176 and PNU-210510 were used in the EoM/EaM assay and deuterated estrone and estradiol were used in the estrogen assay. Seven-point calibration graphs were used for the quantitations. The method was selective and the linearity was good for all metabolites. The limit of quantitation (LOQ) was determined to be 13 ng/g for EoM, 13 ng/g for EaM, 3 ng/g for estrone, and 2 ng/g for estradiol. The limit of detection (LOD) was determined to be 3 ng/g for EoM, 3 ng/g for EaM, 1.3 ng/g for estrone and 0.5 ng/g for estradiol.The precision expressed as the coefficient of variation (CV) was 9.6% for EoM, 9.4% for EaM, 9.7% for estrone, and 13.1% for estradiol. The accuracy was 99.2% for EoM, 112.0% for EaM, 100.3% for estrone, and 107.2% for estradiol.

Tissues were extracted for EoM, EaM, estradiol, and estrone determination according to a method described by Andersson et al. [2] and modified for methanol extracts. Tissue samples were homogenized with methanol. The extract was evaporated and the residue was dissolved in water, extracted with hexane, and separated on an aluminum oxide column. The eluate was evaporated and derivatized with BSTFA. The reagent solutions were assayed as described for the serum samples. The limit of quantitation estimated in this study was 15 ng/sample for EoM, 10 ng/sample for EaM, 2 ng/sample for estrone, and 1 ng/sample for estradiol. The detection limits were calculated to 3.5 ng/sample for EoM, 2.3 ng/sample for EaM, 0.8 ng/sample for estrone, and 0.3 ng/sample for estradiol using the ratio LOD/LOQ obtained for the serum method. The precision of the method, determined in a preliminary validation step, was 11% for EoM at 45 ng/ml methanol homogenate, 18% for EaM at 45 ng/ml, 54% for estrone, at 7 ng/ml, and 39% for estradiol at 7 ng/ml. Samples were analyzed in duplicate or triplicate and the median value was used for further evaluation.

Table 1. Tissue radioactivity

	0.5 h	2 h	4 h	12 h	24 h
Tumour	2142 ± 354	1738 ± 238	1268 ± 379	2071 ± 1508	571 ± 202
Left brain	1875 ± 227	976 ± 103	411 ± 25	417 ± 161	214 ± 0
Right brain	1589 ± 227	1071 ± 186	411 ± 25	339 ± 126	196 ± 25
Liver	9964	12020 ± 379	16140	11680 ± 505	8000
Lung	1607	1339 ± 227	1214	1464 ± 606	1000
Muscle	1893	982 ± 25	536	357 ± 101	250

Tissue radioactivity expressed as (ng-Eq/g) for tumor, brain (left and right hemisphere), liver, lung and muscle 0.5 h, 2 h, 4 h, 12 h and 24 h after administration of [^{14}C]-EaM i. a. Values given as mean ± SD (if possible)

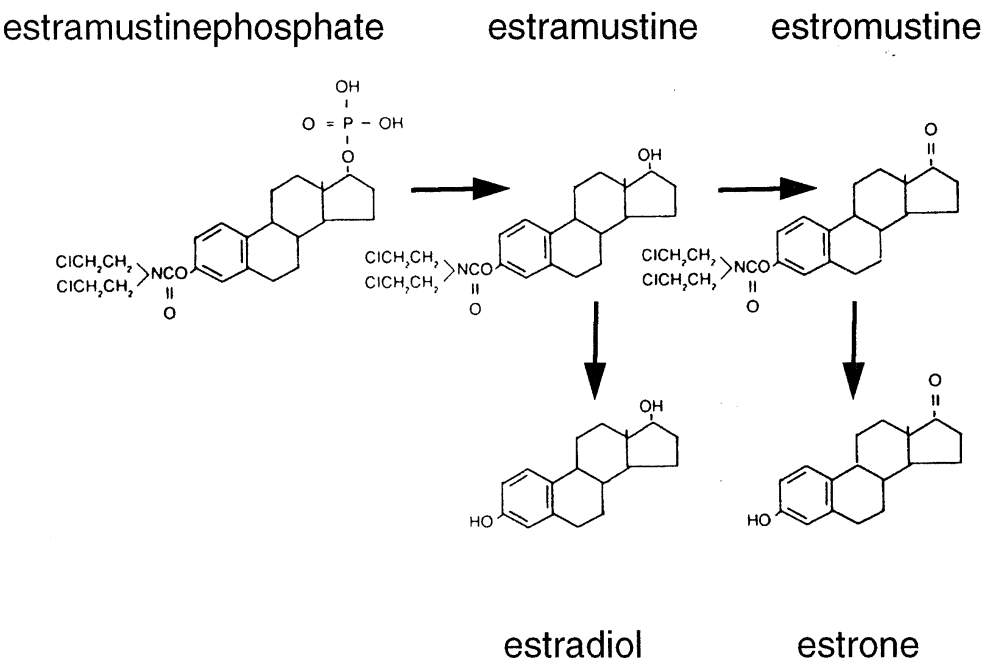

Fig. 1 The metabolism of EMP to its main metabolites EaM, EoM, estradiol, and estrone

For comparison of obtained by gas chromatography with the autoradiography data the sum of the EaM and EoM concentrations from the gas chromatography analyzis were compared with the EaM-equivalent concentrations given by quantitative autoradiography. This procedure is based on the assumption that both [^{14}C]-EaM and [^{14}C]-EoM contribute to the ^{14}C radioactivity seen in the autoradiograms.

Results

Quantitative autoradiography

Autoradiography analyzis of ^{14}C radioactivity are presented quantitatively in Table 1 and Fig. 2(a–d). Autoradiographs are presented in Figs. 3(a–e) and 4. In summary, quantitita-tion of the autoradiograms revealed a rapid and high uptake of ^{14}C radioactivity in the tumor as well as normal brain tissue. Tumor radioactivity remained high at 12 h after drug administration but leveled off thereafter. Brain radioactivity initially reached the same magnitude as tumor radioactivity but, in contrast to the latter, the brain radioactivity rapidly leveled off and reached low levels at 12 h after [^{14}C]-EaM injection. For measurement of the tumor accumulation of ^{14}C radioactivity the uptake ratio tumor/brain was calcu-lated. Initially the ratio was found to be close to 1, indicating homogeneous uptake in the brain and tumor tissue. However, the ratio increased and reached a ratio maximum of 4.5 after 12 h indicating a retention of ^{14}C radioactivity in tumor tissue.

In muscle tissue a situation comparable with that ob-served in normal brain tissue was seen, involving an initially high uptake and a rapid fall to low levels. Liver displayed a high and sustained level of uptake that re-mained elevated for 24 h after drug administration. In lung tissue a high sustained level of radioactivity and a kinetic profile similar to that observed in the tumor was noted.

Gas chromatography

Plasma and tissue concentrations of estramustine and its metabolites as analyzed by gas chromatography are pre-sented in Table 2 and Fig. 5(a–d). Concentrations between the LOD and the LOQ were used in the calculations of median values as they contribute, not inconsiderably, to the information extracted from the study. These data show that EaM was taken up by tumor tissue at a concentration higher than that seen in normal brain tissue. EoM was also taken up in the tumor, but not to the same extent as EaM. However, in plasma the concentrations of EoM were twice as high as those of EaM. The mean EaM tumor/

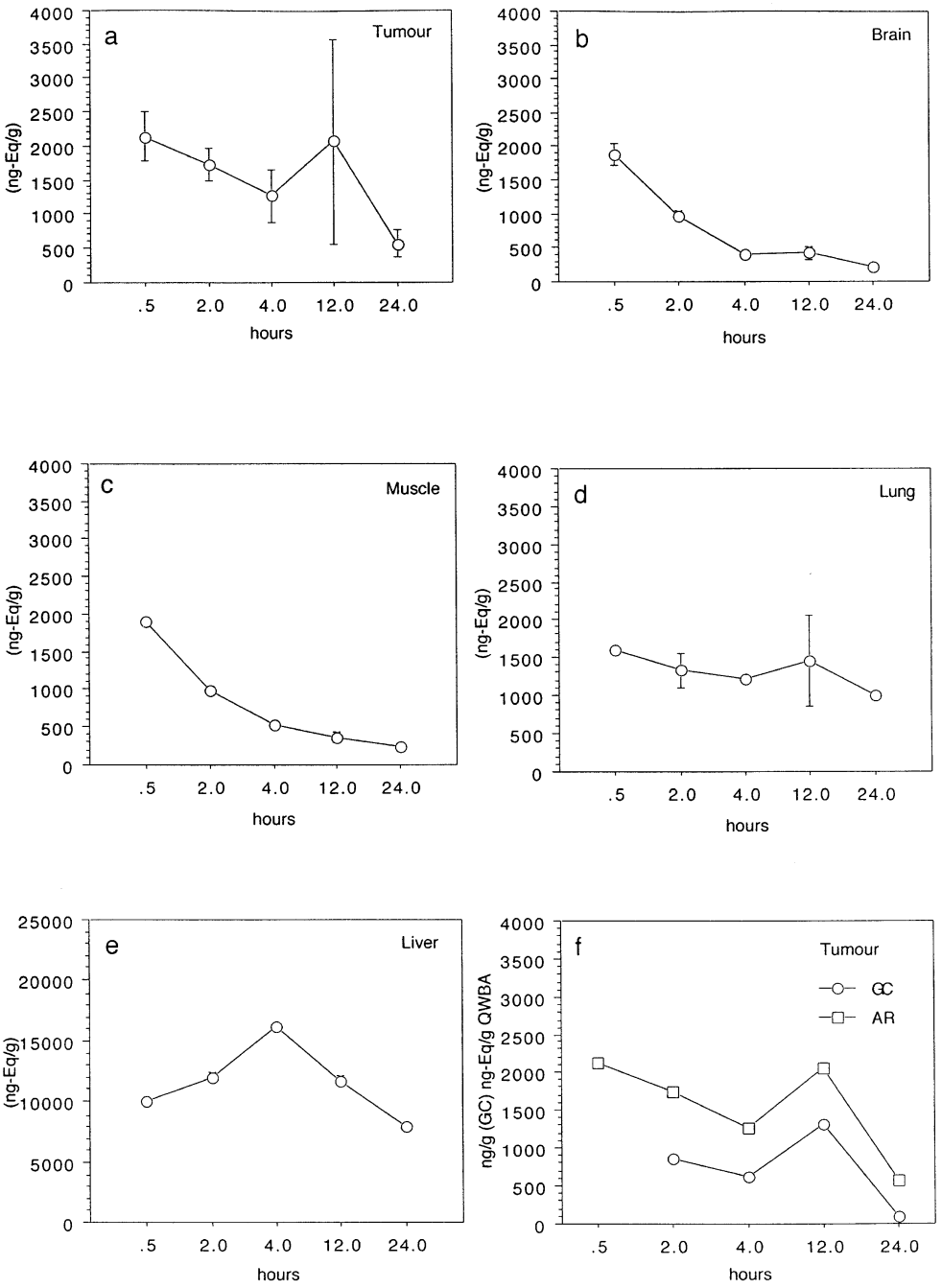

Fig. 2a–d Organ radioactivity (ng-Eq/g) determined at 0.5, 2.0, 4.0, 12.0, and 24 h after i.a. administration of [^{14}C]-EaM. Results are shown for tumor (**a**), left normal brain (**b**), lung (**c**), muscle (**d**), and liver (**e**). Data represent mean values ± SD (when possible). **f** Comparision of autoradiography (*AR*) and gaschromatography (*GC*) estramustine tumor-uptake profiles

plasma ratio of 11.7 ± 5.8 exceeds the mean EoM tumor/plasma ratio of 2.0 ± 0.4, indicating that tumor binding of EaM is stronger than that of EoM. A cumulative accumulation of EaM and EoM in tumor and brain tissue was apparent after 5 consecutive days of treatment at 24 h after drug administration. Again, the EaM tumor/plasma ratio was higher than the EoM ratio. Plasma and tissue levels of the estrogen metabolites of EaM and EoM were found to be low. Plasma concentrations of estrone were slightly higher than the estradiol levels measured. Treatment for 5 days resulted in a moderate accumulation of estradiol and estrone in all three of the compartments serum, tumor, and brain.

A comparison of autoradiography data with the concentrations obtained by gas chromatography showed similar uptake profiles for the cytotoxic metabolites EaM and EoM in tumor (Fig. 2f) and brain tissue (data not shown). Gas chromatography generally indicated concentrations lower than those seen by autoradiography.

Discussion

EMP has been shown by autoradiographic distribution studies to display a significant degree of drug uptake in rat ventral prostate [26], and subsequent trials have demon-

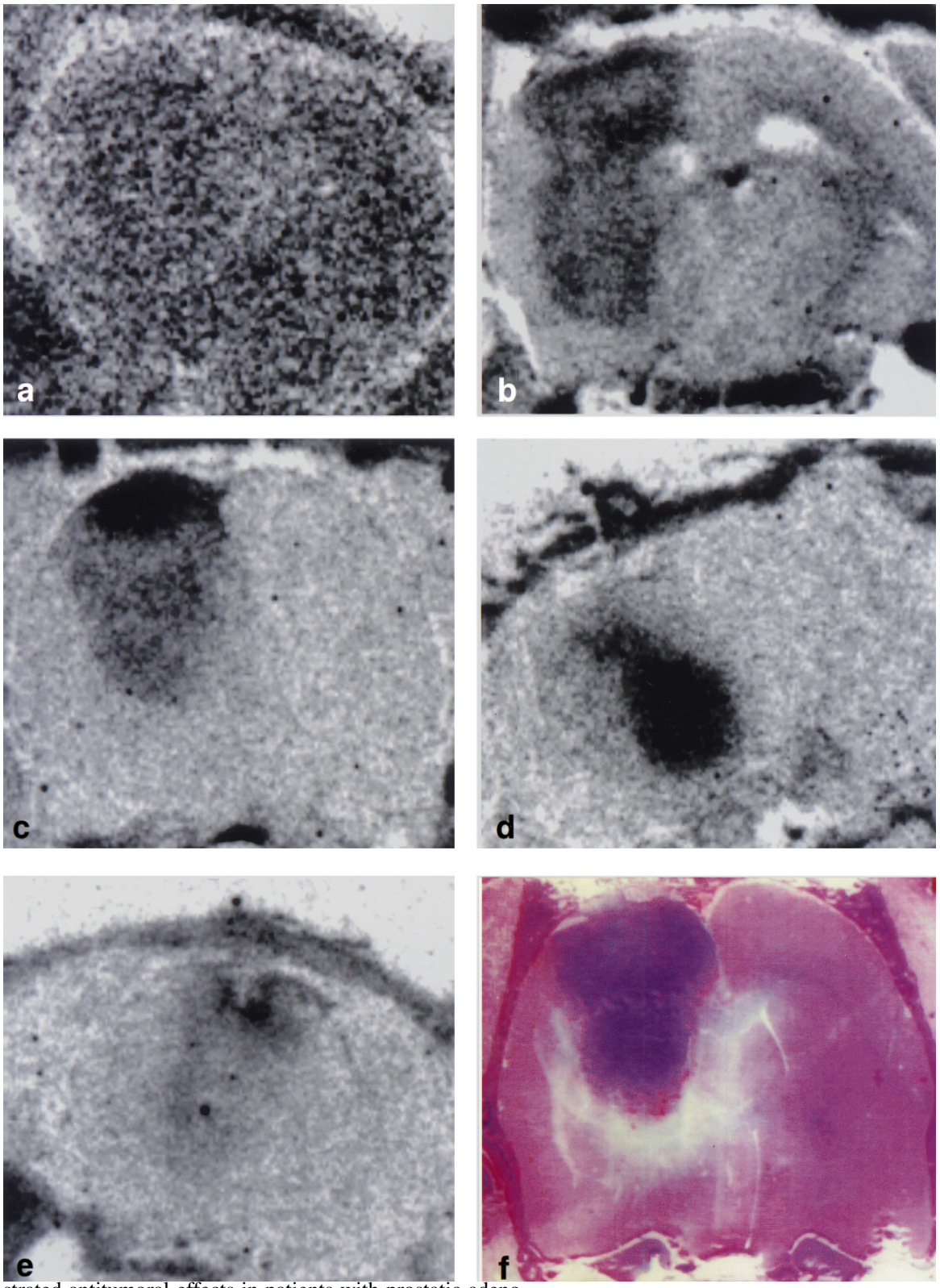

Fig. 3a–f Coronal autoradiograms of whole rat brain obtained at 0.5 (**a**), 2.0 (**b**), 4.0 (**c**), 12.0 (**d**), and 24 h (**e**) after i.a. administration of [^{14}C]-EaM. **f** Hematoxylin-eosin stain of the same section illustrated in **c**, showing the tumor as a densly stained area in the right hemisphere, corresponding well to the ^{14}C autoradiogram

strated antitumoral effects in patients with prostatic adenocarcinoma [20, 21]. The present study now adds the information that EaM also selectively accumulates in an experimental glioma, a finding that may be of considerable clinical interest.

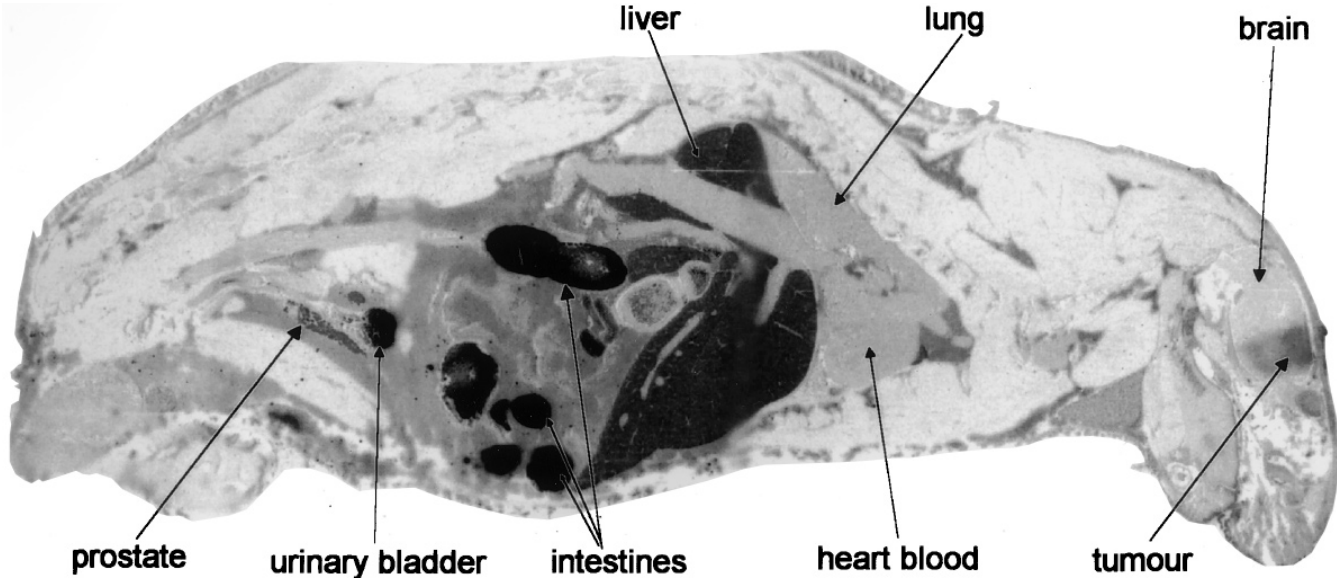

Fig. 4 Whole-body autoradiogram of animal R4 at 12 h after i.a. administration of [^{14}C]-EaM. Note the high tumoral uptake, visible as a *dark field* in the frontal lobe. A high degree of uptake is also clearly visible in the liver, subcutaneous adipose tissue, and prostate

This study describes for the first time the spatial distribution of EaM in a rat glioma model. Autoradiography after [^{14}C]-EaM administration clearly shows that ^{14}C radioactivity is taken up in glioma tissue and that a significant retention of radioactivity in tumor tissue is noted as compared with normal brain tissue. Gas chromatography data indicate that EaM is the major metabolite found in glioma tissue after parenteral drug administration. Estrogen metabolites were found in the tumor as well as in normal brain tissue but the levels were low as compared with those of the parental compound.

The uptake profiles obtained by autoradiography and gas chromatography showed good correlation in the tumor (Fig. 2f) and brain tissue (data not shown). In general, gas chromatography indicated lower concentrations of the cytotoxic metabolites, which may be explained partly by the different routes of administration and partly by the different natures of the analysis techniques. However, the similar uptake kinetics seen with the two methods further validate the present data.

EMP is rapidly dephosphorylated in the gastrointestinal tract when given orally [18]. The main metabolites EaM and EoM are, after absorption, further metabolized to estradiol and estrone in relatively small amounts (Fig. 1). EoM is the main metabolite in plasma, occuring at considerably higher concentrations relative to EaM [3, 12, 18]. Since EaM is the first downstream metabolite of EMP and we wanted a safe and reproducible delivery of the drug, we chose to give EaM parenterally in this study. Our finding of EoM as the main metabolite in plasma in the rat confirmed the validity of this approach. In glioma patients given 280 mg EMP orally at 14 h prior to surgery, EoM was shown to be the main plasma metabolite [3]. The EaM tumor/plasma ratio was found to be 16.1, whereas the tumor/plasma ratio recorded for EoM was only 0.76,

Table 2. Gas chromatography

		2 h	4 h	12 h	24 h	5 d
EaM	Serum	77 ± 90	128 ± 175	116 ± 2.5	8.8 ± 2.5	57 ± 31
	Tumour	600 ± 848	480 ± 254	761 ± 237	69 ± 34	261 ± 93
	Brain	239 ± 261	399 ± 555	420 ± 44	26 ± 37	202 ± 147
EoM	Serum	202 ± 230	263 ± 342	288 ± 128	14 ± 8.0	219 ± 214
	Tumour	258 ± 364	153 ± 216	558 ± 240	15 ± 2.2	143 ± 41
	Brain	167 ± 187	275 ± 350	315 ± 46	15 ± 11	116 ± 90
estradiol	Serum	6.3 ± 8.3	3.4 ± 4.6	6.6 ± 4.2	0.5 ± 0.7	3.2 ± 1.3
	Tumour	17 ± 2.8	5.5 ± 7.8	9.0 ± 2.8	1.4 ± 2.0	9.5 ± 3.5
	Brain	2.2 ± 3.2	4.5 ± 5.7	5.2 ± 3.9	1.2 ± 1.1	3.0 ± 4.2
estrone	Serum	34 ± 42	25 ± 33	20 ± 11	1.1 ± 1.6	11 ± 9.9
	Tumour	10 ± 15	13 ± 18	18 ± 3.5	3.6 ± 5.1	16 ± 4.2
	Brain	5.8 ± 0.4	8.0 ± 11	18 ± 0	4.2 ± 3.9	12 ± 8.5

Estramustine (EaM), Estromustine (EoM), estradiol and estrone concentrations (ng/g sample) in serum, tumour and normal contralateral brain analysed 2 h, 4 h, 12 h and 24 h after drug administration. Uptake also analysed 24 h after last administration in a 5 d daily treatment period. Concentrations given as mean ± SD

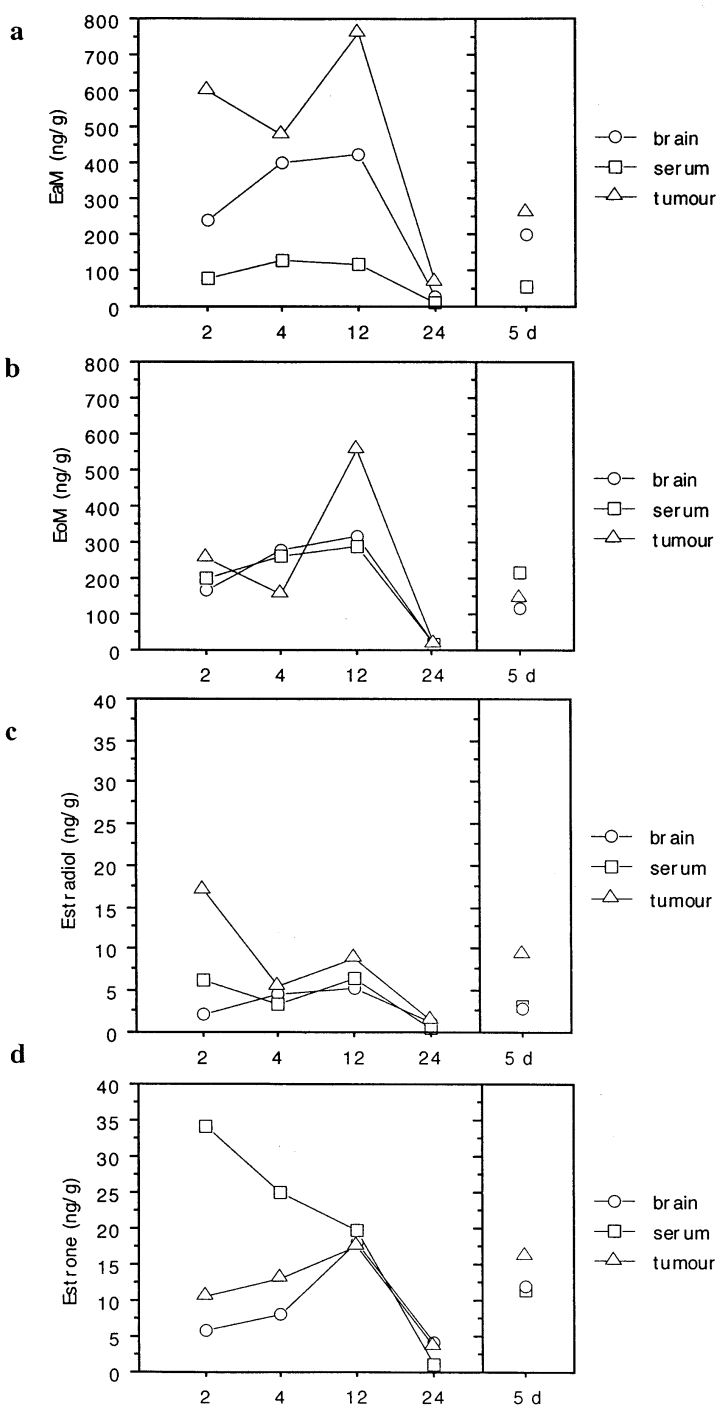

Fig. 5a–d Gas chromatographic analyzis of estramustine and its metabolites in serum, tumor and normal brain tissue. EaM, EoM, estradiol, and estrone concentrations are given as mean concentrations (ng/g) measured at 2, 4, 12, and 24 h after a single i.p injection of EaM. The mean concentration determined at 24 h after the last drug administration in a 5-day (120-h) treatment period is shown separately in each panel. Data represent values only (for SD, refer to Table 2)

suggesting that EaM is the main metabolite actively accumulated in human glioma tissue. These data correlate well with the tumor/plasma ratios of 11.7 and 2.0, respectively, obtained in the present study for the BT4C rat glioma.

A 3-fold increase in EaM uptake in tumor tissue was clearly seen after 5 days of consecutive treatment (Fig. 5a). EoM also showed a similar cumulative accumulation in tumor tissue (Fig. 5b) as compared with that resulting from single-dose administration. However, its higher tumor/brain and tumor/serum ratios identify EaM as the metabolite most actively retained in glioma tissue. This is of both clinical and scientifical interest since it indicates that higher intratumoral drug concentrations may be reached with prolonged treatment schedules and further strengthens the proposal of EaM as the active metabolite in malignant glioma.

The uptake and action of EMP/EaM in prostatic carcinoma is closely related to a specific EaM-binding protein (EMBP) [10, 14, 15, 23]. In glioma tissue an intracellular protein has been recognized by antibodies against EMBP in experimental models as well as in patients [7, 6, 29, 32]. This EMBP-like protein displays binding characteristics for

EaM similar to those of prostatic EMBP [5, 16, 19] but partial characterization reveals structural differences [5]; hence, the proposal of the term EMBP-like antigen [8]. In normal human brain tissue the expression of this EMBP-like antigen has been found to be low as compared with that in glioma tissue, which further strengthens the assumption that the uptake of EaM we observed in glioma tissue, is related to this EMBP-like antigen [32]. Studies on the cytotoxicity of EaM have also revealed that EaM induces apoptosis in rat glioma tissue but not in normal rat brain tissue [38]. In addition, the expression of EMBP-like antigen also seems to correlate positively with a poor prognosis in astrocytoma patients [7].

In liver and pulmonary tissue we also found a high degree of uptake of EaM, but the uptake in skeletal muscle was low, a finding that is consistent with earlier reports [16]. A sustained uptake of [^{14}C]-EaM was noted in lung tissue, an interesting finding in the light of earlier reports showing the expression of an EMBP-associated protein in several lung-cancer cell lines [9].

In serum, tumor and normal brain tissue the levels of estrogen metabolites were low as compared with the concentration of the parental drug. In the clinical situation the side effects of EMP are generally regarded as estrogenic [8], and doubts have been raised concerning the possibility for estrogen to stimulate growth in glioma, thus counteracting the effects of EMP. However, even if estradiol has been demonstrated in astrocytoma tissue, it seems that estradiol receptors are not present [24] and that there is no stimulatory or cytotoxic effect of estradiol on glioma cells in vitro [30]. Also in vivo, estradiol did not affect the growth of glioma in the BT4C model (Johansson et al., unpublished data). In addition, in an evaluation of the binding characteristics of EMBP it was found that estradiol had a low degree of affinity, although it was considerably lower than determined for EaM [16].

In this study we demonstrated an uptake and retention of EaM in glioma tissue in a rat model using two different pharmacokinetic techniques. EaM seems to be the main metabolite in glioma tissue, whereas EoM is the main metabolite in plasma. Estradiol and estrone were also detected, albeit at low levels in both glioma tissue and normal brain tissue. The distribution pattern of EaM might further support the earlier suggestion that the uptake is related to a specific protein-binding (EMBP-like antigen).

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