Biochemical Pharmacology, 1966, Vol. 15, pp. 215–219. Pergamon Press Ltd., Printed in Great Britain.

FLUORESCENCE TECHNIQUE APPLIED TO WHOLE BODY SECTIONS FOR DISTRIBUTION STUDIES OF TETRACYCLINES

L. BLOMQUIST and Å. HANNGREN

Dept. of Pharmacology, Kungl. Veterinärhögskolan and Department of Medicine,

Karolinska Sjukhuset, Stockholm, Sweden

Received 1 *January* 1965; *accepted* 1 *June* 1965)

Abstract—The fluorescence technique has been applied to whole body tape sections to study the distribution of 6 commercially available tetracyclines. The tape sectioning technique described by Ullberg for autoradiography of labeled chemical compounds has proved to be of value also in distribution studies of fluorophors. The distribution patterns of the tetracycline compounds have been studied as well as the influence of different routes of administration. The fluorescence distribution picture of tetracycline has been compared to the autoradiogram of the radioactively labeled compound. The influence of pH on the fluorescence has been investigated. Advantages and limitations of the method are discussed.

TETRACYCLINES as tracers have achieved increasing interest because of their fluorescence properties and their affinity for newly forming bone,[1–5] mineralizing cartilage[6] and various pathological tissues.[7–20] Studies based upon the fluorescence technique have been performed with different tetracycline analogues. Almost all agree as far as qualitative bone accumulation is concerned while in studies of soft tissues varying results are reported.[13, 21–23] This may be due to different fluorescence intensity of the various tetracyclines and/or to real differences in their distribution. In favor of the latter possibility is the finding that radioactive tetracycline and chlortetracycline accumulate differently in bone.[2]

In order to study tetracycline fluorescence of tumour inplants in mice it was thought necessary first to investigate the fluorescence characteristics of the commercially available tetracyclines in normal tissues. At our laboratory André[24] studied the distribution of tritium-labeled tetracycline using whole-body tape sectioning combined with autoradiography. Whole body autoradiography has the advantage of having most organs available on the same section, thus permitting comparative studies of drug concentration in these organs and also in different parts of these organs. It was thought that the fluorescence technique applied to whole-body tape sections may give information of differences in the fluorescence pattern between the compounds Furthermore it was hoped to elucidate the fluorescence capacity by comparing fluorograms to autoradiograms.

The present study describes the application of the fluorescence technique in whole body sections and a comparison of the fluorescence of six tetracycline analogues in different non-pathological tissues in mice.

METHODS

Material

The following tetracyclines were investigated : tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), demethylchlortetracycline (DMCTC), N-(pyrrolidinyl-1-methyl)-tetracycline (PTC), and lysine-conjugated tetracycline (LTC).* They were administered intravenously or intramuscularly to white mice weighing 20–28 g in a single dose of 30 mg/kg body wt. or orally in a dose of 10 mg/kg body wt. three times a day for three days. For the i.v. and i.m. routes standard commercial parenteral preparations were used. For oral administration the contents of commercial capsules were dissolved in water and injected into the esophagus by means of a special cannula with a blunt tip. DMCTC and PTC, were obtainable only as oral and intravenous preparations, respectively, and thus had to be given in the same form for all three routes of administration. For each tetracycline eight mice were used, being sacrificed at 1, 5 and 12 hr after i.v. and i.m. injection, and at 5 and 12 hr after the last oral. dose. To get a direct comparison to the autoradiograms of Andre[24], four additional mice were killed at 5, 20, 80 and 320 min respectively after intravenous injection of TC.

Tape-sectioning technique

Killing of the mice was accomplished by immersion into a mixture of solid carbon dioxide and acetone (−75°). At −10° sagittal sections through the animals were cut according to the tape-sectioning technique described by Ullberg.[25] For fluorescence studies 60μ-thick sections were taken, and at each level an additional 10μ section was obtained for later histological staining. A number of tapes have been tested for fluorescence,† among which Scotch Magic Mending Tape No. 810 was the most suitable because of its low autofluorescence. After drying at −10° for two days, the sections may be transferred to room temperature.

Examination and registration technique

The light source was a long wave mercury u.v. lamp (Philips HPW 125 W). This lamp emits u.v. between 3000 and 4000 Å with a preponderance of the mercury lines at 3650–3663 Å. Although the tetracyclines have absorption maximums at 3750–3900 Å, this device is sufficient to produce good fluorescence of the drugs.

Photographic registration was obtained with Kodachrome-X daylight color film.‡ To prevent the u.v. light from entering the camera we first used a 5 mm-thick liquid filter consisting of a 0·5% (v/v) solution of $Ce(NO_3)_4 \cdot 2NH_4NO_3$ in 1% (v/v) sulfuric acid, which gives an excellent recording of the yellow fluorescence of the tetracyclines. However, since this solution is rather unstable, we changed to Kodak Wratten filter 2 E, which gave almost comparable but more reproducible colour photographs. These filters pass nearly all visible light including the blue auto-fluorescence of the tissues not containing tetracycline, thus yielding a contrast effect.

* TC = Achromycin (Lederle), CTC = Auromycin (Lederle), OTC = Terramycin (Pfizer), DMCTC = Ledermycin (Lederle), PTC = Syntodecin (Astra), LTC = Tetralysal (Carlo Erba).

† Scotch Magic Mending Tape No. 810, Scotch Cellulose Tape No. 600, Scotch Klebeband Düsseldorf No. 688 and Scotch Electrical Tapes No. 5, 58 and 59.

‡ This film proved to give a better recording of the fluorescence than other films tested, comprising the day-light color films Kodachrome II, Kodachrome II A and Ectachrome-X.

It is also possible to eliminate most of the non-yellow light by using a monochromatic filter such as Zeiss Monokromatfilter A, but the exposure time must be increased from a few seconds to several minutes.

To convert the color photographs to black-and-white paper prints the diapositives were rephotographed on Adox KB 14 film through the above mentioned monochromatic filter. In positive prints from this film the fluorescent areas stand out white against a black background.

RESULTS AND DISCUSSION

Fluorescence in vitro. If the 6 tetracyclines investigated are tested for fluorescence *in vitro* at different pH by placing drops of the same concentration on filter paper, certain differences between the compounds are easily seen (Fig. 1). DMCTC has the widest pH-range and the strongest yellow fluorescence, whereas OTC has the weakest. The fluorescence becomes stronger with increasing pH for all compounds, but DMCTC has the lowest pH maximum (pH 7). Furthermore, DMCTC has an orange-yellow tinge which makes it easier to detect in low concentrations.

Autofluorescence of tissue sections. On the whole-body section of a mouse not given tetracyclines, most tissues show blue autofluorescence; the exceptions are blood, bile, Harderian gland and the fundus part of the stomach. The blood does not fluoresce at all, since, as is well known, hemoglobin quenches the fluorescence of different compounds including tetracycline. Thus the contents of heart, lungs and vessels are always dark under the u.v. light. The Harderian gland behind the eye, which only exists in rodents, and the fundus part of the stomach fluoresce in a red color. The bile usually has a fluorescence similar to that of the tetracyclines, and also the liver may show a yellow tinge. Thus, in the interpretation of the tetracycline-induced fluorescence one must bear in mind the autofluorescence of the tissues, especially in the liver and bile (Fig. 6 a).

Tissue fluorescence of intravenously injected mice. Figure 2 demonstrates the yellow fluorescence of a whole body section 20 min after intravenous injection of TC. At this time there is fluorescence in bone, teeth, skin, tracheal cartilage, liver, intestinal wall, salivary glands (especially the mucous ones), kidney, and lymphatic organs (lymph glands, thymus, white pulp of the spleen). In spite of the autofluorescence of the tissues and the quenching of tetracycline fluorescence by hemoglobin, the fluorogram shows a striking similarity to the autoradiogram of corresponding time and dose (Fig. 3). However, there are some quantitative differences. If the accumulation in bone is used as reference, liver, salivary glands and lymphatic organs show a higher intensity on the fluorogram than on the autoradiogram, whereas in kidney and intestines the opposite is seen. The importance of fluorescence quenching by hemoglobin resulting in dark areas of heart, lungs and vessels is in this case minimized by the rapid extravascular distribution giving a low concentration in the blood. In the liver the faint yellow autofluorescence is immaterial since the accumulation of tetracycline there at this time is very high. The similarity between the fluorogram and autoradiogram is also seen at 80 min after intravenous injection (Figs. 4 and 5), both showing the persistence of tetracycline in the skeleton, liver and intestines. At

the other intervals used by André (5 and 320 min) the correspondence is quite obvious also. However, the high concentration of radioactive tetracycline in the thyroid and especially in parathyroid found by André does not correspond to fluorescence findings.

All the other tetracyclines show qualitatively the same distribution pattern after intravenous injection as TC, but quantitatively the intensity of the fluorescence in the organs varies somewhat. In the skeleton TC shows stronger and more persisting fluorescence than the other compounds; DMCTC shows almost as strong fluorescence as TC. In the kidney OTC and DMCTC are the most intense.

In the liver CTC and DMCTC show consistently fainter fluorescence than the others though the individual variations in autofluorescence make evaluation difficult. Apart from the liver, persistence in the soft tissues is not observed 12 hr after administration. Passage into the central nervous system is not observed for any of the compounds.

Tissue fluorescence of intramuscularly-injected mice

Compared to intravenous administration a fainter fluorescence was obtained using the intramuscular route with the same dose, for all three intervals and for all the tetracyclines. Thus, an instant high blood concentration of the drugs may be better than a lower but more persisting one to obtain fluorescence. This in accordance with the findings of Wallman and Hilton,[26] who studied tetracycline induced discolouration of teeth in infants and found the total dose more important than the duration of treatment. With intramuscular administration TC again showed the highest fluorescence of the tetracyclines at 12 hr.

Tissue fluorescence after oral administration.

Oral administration gave somewhat inconsistent results, indicating difficulties in obtaining reproducible absorption when using this route in mice. Apart from the gastro-intestinal tract only the skeleton was found to fluoresce in these animals, and here CTC had the highest intensity, whereas PTC had the lowest. This is in accordance with the findings of Buyske *et al.*[2] who found a higher concentration of CTC than of TC in bone after oral administration. Incomplete absorption of the latter compound when given orally may be the reason for this finding.

Tissue fluorescence in mice given a large dose intravenously

If tetracycline is given in a dose of 100 mg/kg body wt, which is approximately one third of the LD_{50}, a much stronger and more persisting fluorescence is seen in all organs. However, the central nervous system never shows fluorescence. Nor in testis or in cartilage—except tracheal cartilage—is fluorescence observed. Figure 6 b shows the distribution of DMCTC in a pregnant mouse 5 hr after administration of 100 mg/kg body wt. Fluorescence in the foetal skelton indicates placental passage. No other organs of the foetus have taken up the drug. Figure 6 c demonstrates the fluorogram converted to black-and-white paper print and Fig. 6 a the autofluorescence of a control animal.

The findings of fluorescence in the foetal skelton are in accordance with the autoradiograms of André[24] and with the observations by Douglas[5] of fluorescent deciduous teeth in children whose mothers had received long term treatment with tetracycline during pregnancy.

REFERENCES

1. R. A. MILCH, D. P. RALL and J. E. TOBIE, *J. Nat. Cancer Inst.* **19**, 87 (1957).
2. D. A. BUYSKE, H. J. EISNER and R. G. KELLY, *J. Pharmac. exp. Ther.* **130**, 150 (1960).
3. P. J. VANDERHOEFT, L. F. A. PETERSON and P. J. KELLY, *Proc. Staff Meet. Mayo Clin.* **37**, 229 (1962).
4. A. HULTH and S. OLERUD, *Acta Soc. Med. Upsal.* **67**, 219 (1963).
5. A. C. DOUGLAS, *Br. J. Dis. Chest.* **57**, 44 (1963).
6. H. M. FROST, A. R. VILLANEUVA and H. ROTH, *Stain Techn.* **35**, 135 (1960).
7. D. P. RALL, T. L. LOO, M. LANE and M. G. KELLY, *J. Nat. Cancer Inst.* **19**, 5 (1957).
8. T. L. LOO, E. D. TITUS and D. P. RALL, *Science* **126**, 253 (1957).
9. I. HÄKKINEN, *Acta physiol. scand.* **42**, 282 (1958).
10. I. HÄKKINEN and K. HARTIALA, *Ann. Med. exp. Biol. Fenniae* **37**, 115 (1958).
11. J. F. MCLEAY, *Am. J. Surg.* **96**, 415 (1958).
12. P. S. VASSAR, A. M. SAUNDERS and C. F. A. CULLING, *A.M.A. Archives of Pathology* **69**, 25/613 (1960).
13. P. MÁLEK, J. KOLC and F. ZÁK, *Antimicr. Ag. Annual* p. 206, (1960).
14. R. A. MILCH, J. E. TOBIE and R. A. ROBINSON, *J. Histochem. Cytochem.* **9**, 261 (1961).
15. R. W. BAILEY and P. D. LEVIN, *Surg. Forum* **12**, 455 (1961).
16. N. B. ACKERMAN and A. S. MCFEE, *Surgery* **53**, 247 (1963).
17. P. MÁLEK, J. KOLC, VL. ZÁSTAVA, F. ZÁK and B. PELESKA, *Cardiologia* **42**, 303 (1963).
18. F. L. LOCK, D. M. LASKIN and M. B. EUGEL, *J. Pharmac. exp. Ther.* **141**, 131 (1963).
19. I. HÄKKINEN and I. LINDGREN, *Acta Path. Microbiol. Scand.* **59**, 428 (1963).
20. A. CABRERA, J. JURADO, S. DE LA PAVA and J. W. PICKREN, *New York State J. Med.* **64**, 981 (1964).
21. S. HELANDER and L. E. BÖTTIGER, *Acta Med. Scand.* **147**, fasc. I, 71 (1953).
22. L. E. BÖTTIGER, *Acta Med. Scand.* **151**, fasc. V, 343 (1955).
23. L. E. BÖTTIGER, *Antibiotics & Chemother.* **5**, 332 (1955).
24. T. ANDRE, *Acta Radiol.*, suppl. 142 (1956).
25. S. ULLBERG, *Acta Radiol.* suppl. 118 (1954).
26. I. S. WALLMAN and H. B. HILTON, *Lancet I*, 827 (1962).