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TARGETING DISSEMINATED MELANOMA WITH RADIOLABELLED METHYLENE BLUE

Comparative bio-distribution studies in man and animals

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Targeted radiotherapy for pigmented melanoma with 3,7-(dimethylamino) phenazathionium chloride [methylene blue (MTB)] labelled with Astatine-211 (^{211}At; α-particle emitter) proved to be very effective in animal model systems. Since the results justified an introduction of the treatment to the clinic, the aim of the bio-distribution studies using [^{123}I]-MTB and [^{131}I]-MTB in patients was to confirm selectiveness of radiolabelled MTB uptake in melanoma lesions. The investigations were carried out using planar and SPECT (single photon emission computed tomography) γ-cameras. A stable uptake of radioiodinated MTB was found in pigmented melanomas in man, with tumour/surrounding tissue and tumour/blood ratios amounting to 9 at 19 h after a single i.v. injection. A time-dependent kinetics of radioiodinated MTB distribution was similar to that observed in human melanoma-bearing athymic mice. Blood radioactivity decreased by about 90% during the first 2.5 min after i.v. injection of the compound ($T_{1/2\ biol} = 0.58$ min). Its retention time in various organs was either the same or very similar to that characteristic of the blood. A rapid uptake of radioiodinated MTB in the liver and kidneys confirmed the importance of these organs in excreting the compound: 25–30% of the radioactivity administered was expelled with urine over the first 24 h after the injection. There was no obvious retention of radioiodinated MTB in the brain over the observation period and in the eyes for at least the first 14 h.

Regardless of the cause(s) triggering growth of melanoma, this malignant neoplasm is rapidly increasing over the past two decades. A particular resistance of melanoma to all treatments routinely applied to other cancers urges a constant search for new methods.

Melanoma is a pigmented tumour with a variable level of melanin but its non-pigmented form is very uncommon. Melanin constitutes therefore a distinct target for melanoma-seeking compounds which, when labelled with a radioisotope(s), might deliver the radionuclides almost exclusively to the tumour cells and thus form a selectively localized source of radiation.

A class of compounds with a high affinity to melanin has been discovered fortuitously and well before formulating the idea of targeted radiotherapy (1–3). Most of these drugs do not exhibit toxic effects while administered systemically in doses required for such therapy. One of them, methylene blue (MTB) is well known in clinical practice due to its bactericidal and staining properties (4–6). Its affinity to melanin (7) made it the most attractive carrier

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for radioisotopes in targeted radiotherapy of melanoma. Although the mechanism underlying such high affinity has not been defined fully, a formation of charge-transfer complex (7–10) and van der Waals' forces occurring at the conjunctions of the aromatic rings of the compound and the aromatic indole-nucleus of melanin (11) seem to be predominantly responsible for the strong binding of methylene blue to this bio-polymer.

Our initial bio-distribution studies in vivo revealed a pigment-dependent uptake of [^{35}S]-labelled MTB, with the most effective accumulation in melanoma lesions, and confirmed that it is excreted through the liver and kidneys (12). The highest uptake in any normal tissue was observed in the pigmented eyes (12). Subsequent experiments in vitro (13, 14) showed not only a melanin-dependent uptake of radiolabelled methylene blue but also a precise distribution of the agent within malignant melanocytes. The localization coincided with that of melanosomes i.e. cellular organelles producing the pigment (13). These findings stimulated further our investigations concerning therapeutic effectiveness for melanoma of MTB labelled with various radioisotopes (13–17). Physical and radiobiological parameters required of radioisotopes to achieve the highest effectiveness in targeted *radiotherapy (18)* dictated a choice of three radionuclides: ^{35}S (β-emitter), ^{125}I (Auger electron emitter) and ^{211}At (α-particle emitter). Effectiveness of ^{211}At-methylene blue in controlling growth of melanoma exceeded that of two other MTB radioderivatives by two orders of magnitude (13). ^{211}At-methylene blue, therefore, was employed in subsequent studies using human melanoma xenografts. Astatine-211 features are very attractive: $T_{1/2} = 7.2$ h, 60 μm average range of α-particles in tissue and the mean energy of 6.8 MeV. These assure an almost optimal therapeutic efficacy of the radiation emitted without damage to normal tissues surrounding the tumour. Indeed, ^{211}At-methylene blue administered intravenously appeared to be very effective in preventing growth of metastases from single cells circulating with blood and of tumourlets, though a poorly pigmented melanoma was used to mimic a pattern of pigmentation of secondaries frequently observed in man (15). The treatment was also successful in inhibiting growth of solid cutaneous lesions and their spontaneous lymph node metastases. The overall effect depended on the degree of pigmentation and initial size of the cutaneous tumour, as well as fractionation régime employed (16). At the same time there was no indication of adverse effects caused by the treatment, either acute or chronic (16, 17).

The above results encouraged us to introduce the treatment to the clinic. Thus, detailed bio-distribution studies in man were necessary to establish to what extent the data obtained from animal investigations could be extrapolated to man and to enable calculations of radiation doses received from ^{211}At-methylene blue administered systemically. The current paper presents and discusses data obtained from first melanoma patients.

Material and Methods

Human melanoma xenografts. Two human melanoma xenografts, poorly pigmented HX34 and a highly pigmented HX118, obtained by courtesy of Professor G. G. Steel of the Institute of Cancer Research, Sutton, U.K. were used for animal investigations. Both sub-lines derived from metastatic lesions of patients who had not undergone previous cytotoxic therapy. They were established in athymic mice by J. Mills of the same Institute and their properties described (19, 20). The obtained samples contained small pieces of tumour frozen at its fourth passage and previously grown s.c. in athymic mice. The material used for subsequent experiments derived from inguinal tumours passaged in vivo in 50–60-day-old female athymic mice ([Crl:nu-nu (CD/1^{TM})BR] supplied by Charles River, U.K.) every 3–4 weeks (HX34) or 5–6 weeks (HX118). Small tumour fragments obtained from either of the melanoma xenografts were suspended in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (both from Flow Laboratories Ltd., Irvine, U.K.) and inoculated subcutaneously into recipient mice (two approx. 0.5 mm^2 pieces suspended in 0.1 ml medium per site). Every experimental animal presented two cutaneous inguinal lesions.

Patients with malignant melanoma. Bio-distribution of radioiodinated MTB was studied in patients with disseminated melanoma, both female and male age 23–69 years. Although cerebral metastases dominated, localization of secondary lesions varied from lymph node deposits to the lung, liver, intestines, etc. All patients were extensively treated with surgery, chemo- and/or radiotherapy prior to radioiodinated MTB administration. Detailed examination of every patient with CT, ultrasounds and other methods preceded investigations with radioiodinated MTB.

Radioiodination of methylene blue. MTB was iodinated by electrophilic aromatic substitution according to the modified method designed by Blower & Carter (21) using 1% aqueous solution of MTB for injections (Bull Laboratories Ltd., Australia). Chemical stability of radioiodinated MTB remained unchanged over at least 6 days. ^{123}I was obtained from Medgenix Ltd., U.K. and ^{131}I from Amersham International Ltd., U.K., as Na[^{123}I] or Na[^{131}I] solution, free of buffers and reducing agents. A specific radioactivity of the final preparation of [^{123}I]-MTB or [^{131}I]-MTB did not exceed 150 MBq per mg MTB. A total activity of [^{123}I]-MTB or [^{131}I]-MTB administered to patients as an intravenous bolus injection varied between 135 and 559 MBq. The injected sample volume was 3–8 ml and contained less than 5 mg MTB.

Gamma-camera and single-photon emission computed tomography (SPECT) studies

^{123}I with an energy of emitted radiation more suitable for detection with γ-camera than that from ^{131}I was chosen for time-dependent bio-distribution studies in melanoma patients.

Data acquisition with gamma camera. The GE/CGR 400XCT gamma camera (General Electric Medical Systems, Milwaukee, USA) linked to a computer star 2000 and equipped with a parallel hole all purpose collimator and energy set at 159 keV, 20% window was used for acquisition.

a) Dynamic studies:

Twelve frames of 5 s each were acquired immediately after i.v. injection of [^{123}I]-MTB with a detector facing the thorax and abdomen of the supine patient. These were followed by 59 frames of 60 s each to make a total sequential acquisition time of 1 h. The acquisition matrix was always 128 × 128.

b) Whole-body scanning:

Anterior and posterior whole-body scans were obtained using 512 × 128 matrix. A speed of 10 min per meter was employed for scans taken between 1 and 15 h post injection, and 15–20 min per meter (depending on the count density) at 24 h after [^{123}I]-MTB administration.

c) Plain images:

When thought necessary, plain images (projection data) of the organs of interest such as the liver, lungs, limbs or head were acquired using 128 × 128 matrix. The acquisition time was as long as possible to obtain a minimum of 2×10^5 counts.

Data acquisition with SPECT. SPECT of the head was carried out on a single slice brain dedicated tomograph (SME 810; Strichman Medical Equipment Inc., Medfield, Massachussets, USA). Sequential investigations at a level of the basal ganglia, 4 cm above and parallel to the orbitomeatal line, as well as at different levels between cerebellum and vault of skull were obtained. A time of image collection was 5–10 min per slice.

Data analysis

a) Gamma camera:

Dynamic (sequential) acquisition data, as well as whole-body and plain images were analysed using the regions of interest (ROI) method. The obtained results were corrected for the radioisotope decay and a time-dependent accumulation kinetics of [^{123}I]-MTB for several organs was determined.

b) SPECT:

Data obtained from SPECT were processed, including attenuation corrections, by using the software supplied by the manufacturers (for detailed description of the programme see Ref. 22). As previously, the ROI method was employed to calculate time-dependent changes of [^{123}I]-MTB uptake in the regions of interest in the brain including corrections concerning ^{123}I decay.

Bio-distribution of radioiodinated methylene blue

Bio-distribution of [^{123}I]-MTB–animal studies. Experiments have been performed using 17 athymic mice bearing two highly pigmented HX118 tumours each and 10 athymic mice with two poorly pigmented HX34 melanoma per animal. 1 MBq of [^{123}I]-MTB was administered i.v. to one of the tail veins of melanoma-bearing mice 12 days after subcutaneous tumour implantation (mean diameter of the tumours = 3 mm). Two animals with either HX118 or HX34 melanoma were chosen randomly at 5 min and, subsequently, at regular time-intervals after the injection. Blood, as well as tumours and a number of organs (lungs, stomach, spleen, kidneys, skin, muscles, eyes, thyroid and lymph nodes) were taken. All tissues were rinsed with phosphate-buffered saline, blotted and weighed. Blood samples (0.5 ml) were centrifuged to separate red cells from plasma. The latter was carefully transferred with Pasteur pipette to another vial and its volume measured. ^{123}I-radioactivity present in every specimen was determined using scintillation gamma counter and subsequently expressed as ^{123}I-radioactivity per g of tissue. The results were compared with similar data obtained using the same methods as described above and derived from 76 mice bearing either human or B16 melanoma, as well as 33 hamsters with Bomirski's hamster melanoma (2 tumours/animal) injected with other radioderivatives of methylene blue, i.e., [^{35}S]-, [^{125}I]- or [^{131}I]-MTB ((12) and unpublished data).

Bio-distribution of [^{123}I]-MTB and [^{131}I]-MTB—human studies. Eight patients with disseminated melanoma and the thyroid blocked by potassium iodate (170 mg daily for 5 days starting 2 days prior to radioiodinated MTB administration) were injected i.v. with a single dose of 135–559 MBq of the compound. Monitoring of the heart, liver and kidney(s) with γ-camera was carried out in patients immediately after [^{123}I]-MTB administration and lasted for 1 h, while a whole body (anterior and posterior) scanning was performed at 1, 2, 3, 4, 15 and 23/24 h after the injection. Every patient was given laxatives (twice daily) to clean the bowels.

Blood samples (5 ml each) were collected from patients over the initial 3 h (the additional sample was taken from some patients at 13 and 24 h). Urine was collected from patients for approx. 24 h after [^{123}I]-MTB or [^{131}I]-MTB administration. The radioisotope content in the blood and urine was assessed using scintillation gamma counter with the results expressed as a percentage of the total radioactivity administered.

SPECT was used to investigate a time-dependent distribution of radioiodinated MTB in the brain. In patients with

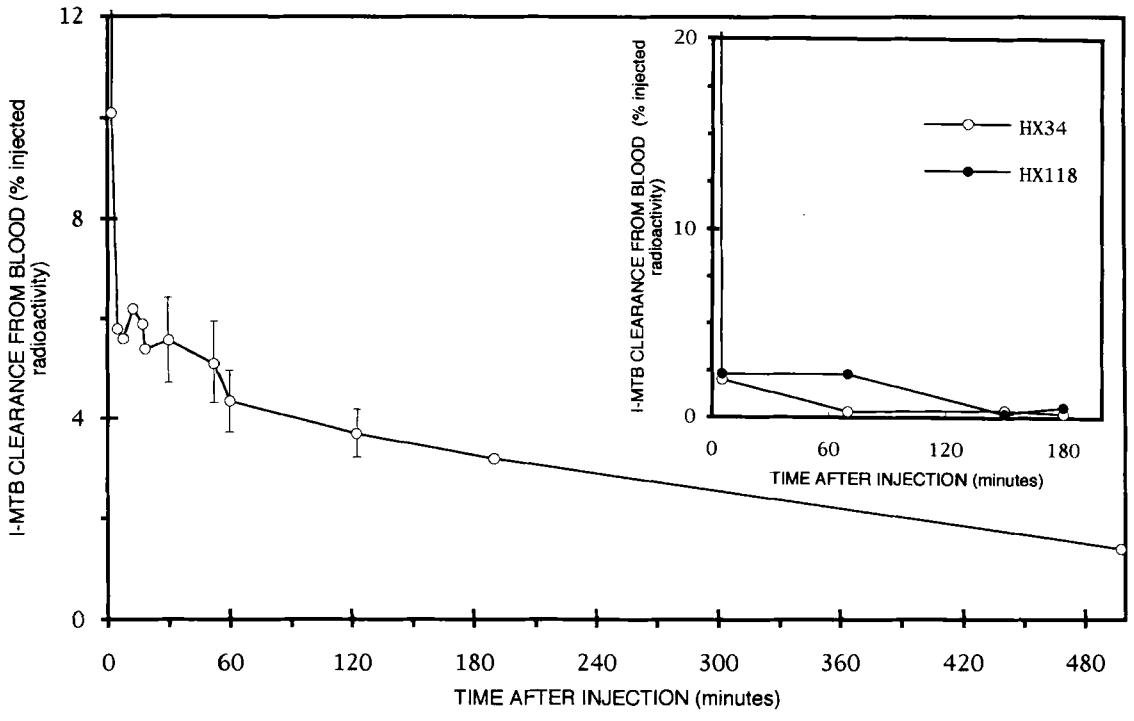

Fig. 1. Time-dependent kinetics of [^{123}I]-MTB and [^{131}I]-MTB in the blood of melanoma patients after a single i.v. injection of the compound. Insertion: the same parameter in athymic mice bearing a highly pigmented HX118 human melanoma (●) or a poorly pigmented HX34 human melanoma (○). Error bars: ± S.D.

cerebral metastases [^{123}I]-MTB uptake and clearance from the organ was monitored at the visual cortex level immediately after the radioisotope injection for 2 h. Subsequent images of [^{123}I]-MTB distribution have been taken in either thirteen 1 cm thick slices of the brain at 3 h after the injection and 2.5 h later in nine 0.5 cm thick slices, or alternatively, at 2.5 and 15/16 h after [^{123}I]-MTB administration.

Biopsy of cutaneous lesions with their diameter varying from 2–10 mm has been taken whenever possible at 19 and 26 h after [^{123}I]-MTB injection. The tumours were separated carefully from the surrounding tissues, weighed and the radioisotope content in both tumour and normal tissue measured using scintillation gamma counter. The tumour/skin and tumour/blood ratios were calculated.

Simultaneously with every patient 2 athymic mice bearing two HX118 human melanoma tumours each were injected with radioiodinated MTB given to the patient. The animals were sacrificed 30 h later and the radioactivity content in tumours, blood, as well as several normal organs determined in scintillation counter. The ratios: tumour/normal tissue, tumour/blood and normal tissue/blood were calculated and compared with previous results to confirm chemical stability of the radiolabelled compound used and its high affinity to melanoma.

Calculations of absorbed doses

Estimations of doses received by tumour, critical organs and blood from [^{211}At]-MTB to be used for targeted radiotherapy of disseminated melanoma have been calculated using the method described by Kassis et al. (23). To simplify the calculations, a homogeneous distribution of the radiolabelled MTB was assumed within both normal and malignant tissues. A pattern of biological decay of the radiolabelled MTB in every tissue of interest and the compound's biological half-life(s) were determined from biokinetic curves obtained from biodistribution studies presented in this paper.

Results

Bio-distribution of radioiodinated methylene blue: Animal investigations

A bio-distribution of [^{123}I]-MTB in athymic mice bearing human melanoma was similar to that of [^{35}S]-MTB observed previously in hamsters with Bomirski's melanoma (12), as well as [^{125}I]- and [^{131}I]-MTB in mice bearing either B16 or human melanomas (unpublished data).

[^{123}I]-MTB uptake in normal tissues. The radioactivity in blood diminished within the first 5 min to about 4% of that administered, and to below 0.5% over the next 2–3 h after [^{123}I]-MTB injection (Fig. 1 – insertion). The highest uptake of [^{123}I]-MTB in normal organs was observed in the liver and kidneys (Fig. 2 and Table 1) which metabolize and excrete methylene blue, respectively (24). Both accumula-

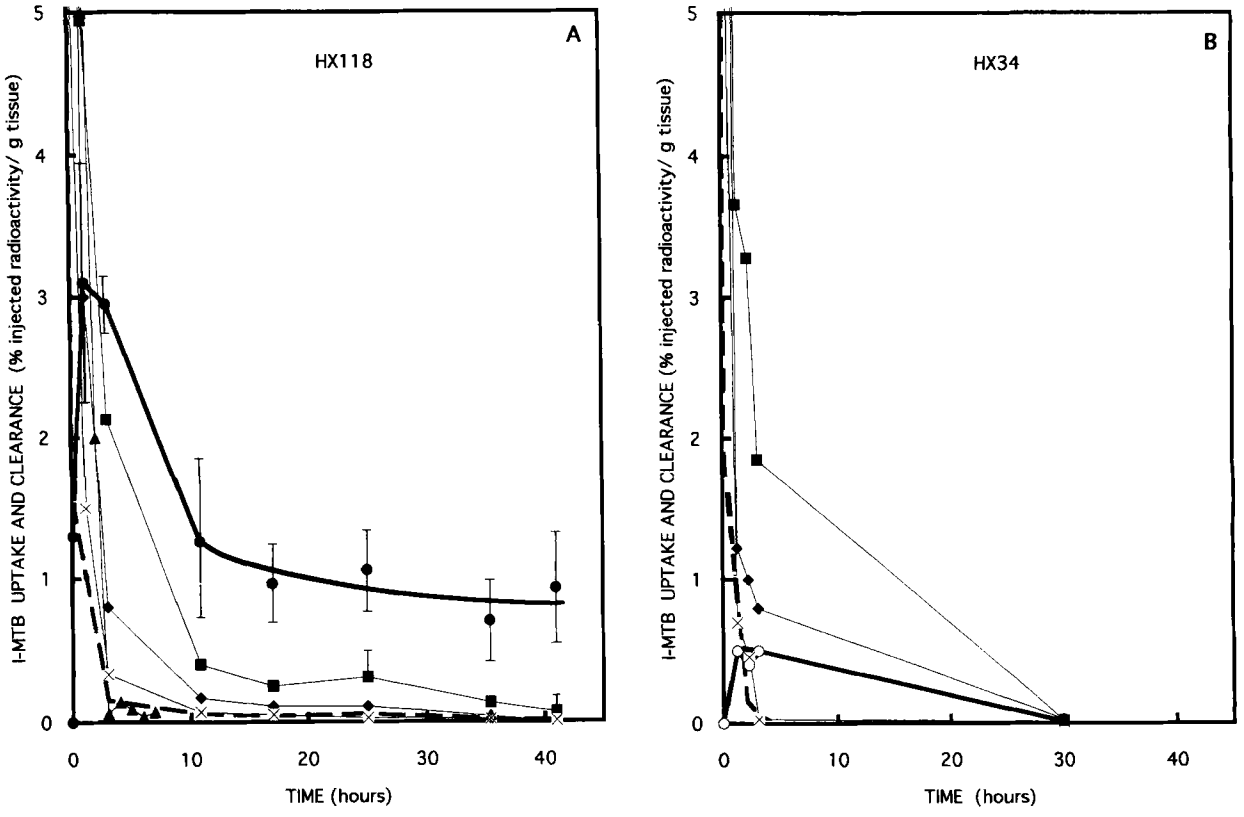

Fig. 2. A time-dependent bio-distribution of [^{123}I]-MTB in melanoma lesions and normal tissues of athymic mice bearing highly pigmented (A) or poorly pigmented (B) human melanoma after a single i.v. injection of the compound: ● —highly pigmented tumour; ○ —poorly pigmented tumour; ▲ —spleen, ■ —liver; ◆ —kidney; × —lung; dashed line-blood.

tion and decrease of the radioactivity in the organs were rapid: a maximum uptake occurred at less than 5 min after [^{123}I]-MTB injection and the radioisotope level diminished subsequently with $T_{1/2biol}$ of approximately 30 min (Fig. 2).

Initial accumulation of [^{123}I]-MTB in the lungs was also significant (Fig. 2). However, its time-dependent kinetics appeared to parallel that of blood, suggesting the latter as a cause of the transiently high level of the radioisotope in the organ.

[^{123}I]-MTB uptake in the eyes remained low over the period of investigation (Table 1), unlike that previously observed in hamsters (12). However, it should be pointed out that eye pigmentation in athymic mice is

Table 1

A time-dependent biodistribution of [^{123}I]-methylene blue in normal tissues of athymic mice bearing human melanoma after a single i.v. injection of the compound

Time (hours)	[^{123}I]-MTB uptake in various tissues (%/g)*				
	Lymph nodes	Stomach	Skin	Muscles	Eyes
0.08	2.0 ± 0.62	4.2 ± 0.43	1.2 ± 0.16	2.0 ± 0.03	1.1 ± 0.27
1.2	1.2 ± 0.17	0.9 ± 0.12	1.4 ± 0.06	0.6 ± 0.05	0.25 ± 0.04
3.0	0.3 ± 0.04	0.4 ± 0.06	0.6 ± 0.05	0.2 ± 0.07	0.04 ± 0.01
11.0	0.06 ± 0.01	0.05 ± 0.001	0.07 ± 0.025	0.05 ± 0.006	0.02 ± 0.005
17.0	0.05 ± 0.01	0.06 ± 0.005	0.03 ± 0.002	0.02 ± 0.002	0.02 ± 0.003
25.0	0.01	0.03 ± 0.01	0.02 ± 0.0008	0.01	0.06
35.5	0.01	0.02	0.01	0.01	0.01
41.0	0.01	0.01	0.01	0.01	0.01

(*) [^{123}I]-MTB uptake expressed as percentage of injected radioactivity per g of tissue (mean value ± S.D.)

Table 2

Tumour/normal tissue ratios obtained after a single i.v. injection of 1 MBq [^{123}I]-MTB to athymic mice bearing a highly pigmented HX118 human melanoma

Time after [^{123}I]-MTB (injection)	Ratio		
	Tumour/ Blood	Tumour/ Muscle	Tumour/ Skin
5 min	0.7	0.6	1.0
1 h	2.0	5.2	2.2
3 h	20.0	14.0	5.0
10 h	22.0	25.0	19.0
17 h	30.0	42.0	28.0
40 h	52.0	215.0	72.0

negligible. The compound accumulation in the whole thyroid gland amounted to 0.07% of injected [^{123}I]-MTB as compared to 1–3% of free radioiodine mixed with MTB (measurements were carried out 24 h after i.v. administration of the preparations). Since a high affinity of iodine and its radioisotopes to the thyroid gland is well known, a lack of specific uptake of [^{123}I]-MTB in this organ confirms binding stability of the radioiodine to MTB in vivo.

A time-dependent accumulation of [^{123}I]-MTB in other organs including the spleen followed that found in blood (Fig. 2 and Table 1). It should be emphasized that the bio-distribution of radiolabelled MTB observed in normal tissues was independent of the pigmentation of the tumours borne by the animals.

[^{123}I]-MTB uptake in melanomas. Maximum uptake of [^{123}I]-MTB in highly pigmented HX118 tumours occurred approximately 1 h after administration of the compound and was followed by 65% decrease over the next 7 h (Fig. 2A). Subsequently, the level of [^{123}I]-MTB remained almost stable until the end of the observation period (40 h). Consequently, values of the tumour/blood and tumour/surrounding tissue (i.e. skin) ratios increased with time elapsed after [^{123}I]-MTB injection from 0.7 and 1.0 at 5 min to 52 and 72 at 40 h respectively (Table 2).

The magnitude and pattern of [^{123}I]-MTB uptake in poorly pigmented HX34 melanoma followed those found for blood and an average normal organ (Fig. 2B).

Bio-distribution of radioiodinated methylene blue: Human investigations

A pattern of radioiodinated MTB uptake and retention in man, as well as their time-dependent kinetics, were comparable to those found in the animals.

Radioiodinated MTB uptake in normal tissues. Radioactivity found in whole blood diminished to about 10% of that injected with $T_{1/2biol}$ of 0.58 min in the first 2.5 min after [^{123}I]-MTB or [^{131}I]-MTB administration, and to 5% over the next 5 min (Fig. 1). Further decrease in blood radioactivity was continuous but slow (Fig. 1). There was no difference between the uptake in the spleen and in the blood itself as revealed by simultaneous monitoring of both the spleen and the heart for 1 h after the injection of the compound. This suggested that the spleen had not possessed its own affinity to MTB (Fig. 3A).

The highest accumulation was observed in the liver, thoracic organs and kidneys. Maximum uptake in the liver occurred at 8 min after the injection (Fig. 3A). Thereafter, the hepatic biological $T_{1/2}$ amounted to approx. 1.5 h (Fig. 3B). This rapid clearance from the liver resulted in an increase of the compound in the bowel. (The latter was eliminated effectively with laxatives.)

Similarly to the liver, a high and rapid accumulation of radioiodinated MTB (approx. 10% of the injected radioactivity at 1 h after its administration) was found in the thorax. However, a subsequent clearance from the organ with $T_{1/2biol}$ of approx. 1 h excluded any affinity to the tissue and suggested that the initially high level of radioactivity found should be attributed to the thoracic blood pool amounting to 20% (10% in the lungs and the heart each) of the total blood volume.

Maximum uptake in the kidneys was found at 2–3 min after radioiodinated MTB administration to the patients (Fig. 3A). Subsequently, a slope of the curve illustrating excretion from the organ became almost parallel to that characterizing the retention in the blood (Fig. 3A). Of the initially injected radioactivity 25–30% was excreted with urine over the first 24 h. Radioiodinated MTB uptake monitored by γ-camera was higher in the face than in the brain (Fig. 3B). However, the rates of disappearance of the radioisotope from both did not differ. More detailed investigations carried out with SPECT confirmed a rapid uptake and almost equally fast clearance of [^{123}I]-MTB from the brain. A maximum accumulation took place between 10 and 20 min after [^{123}I]-MTB injection and, subsequently, the activity diminished with $T_{1/2biol} = 0.8$ h (Fig. 4A).

Radioiodinated MTB uptake in melanoma lesions. A simultaneous, time-dependent monitoring of the brain and a cerebral metastasis with SPECT showed in detail the pattern of the compound's uptake and clearance/retention in both normal and malignant tissue (Fig. 4). While the radioactivity diminished rapidly in the brain ($T_{1/2biol} =$ 0.8 h), [^{123}I]-MTB clearance/retention in metastatic lesion occurred in a bi-phasic manner similar to the pattern observed in pigmented melanomas grown in animals (compare Figs. 4B and 2A). Rapid accumulation in the tumour

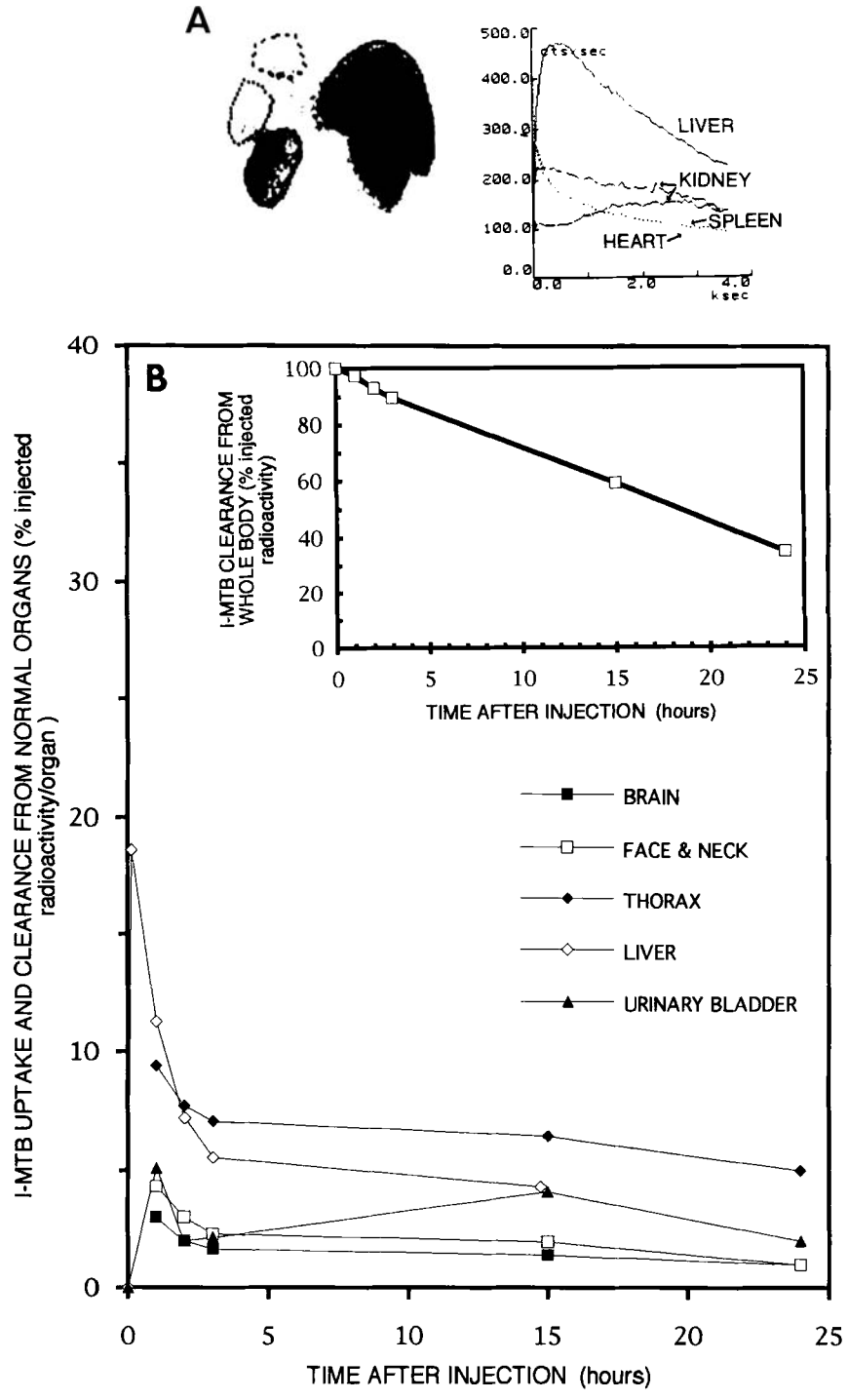

Fig. 3. A. Detailed monitoring of [^{123}I]-MTB uptake and retention in the liver, kidneys, spleen and heart during the first 60 min. after a single i.v. injection of the compound. *B.* Bio-distribution of [^{123}I]-MTB in melanoma patients after a single i.v. injection of the compound. Insertion: Whole-body clearance of [^{123}I]-MTB in melanoma patients after a single i.v. injection of the compound. All data obtained from planar γ-camera.

was followed by 54% decrease of the radioactivity over the next 50 min and, subsequently, the level of radioiodinated MTB remained almost stable until the end of the observation time (Fig. 4B). An unknown weight of this very small lesion (it was revealed on the CT scan only 5 months after investigations with [^{123}I]-MTB prevented calculation of the tumour/normal organ ratio per g of tissue. Instead, the ratio of radioactivity accumulated in the entire lesion to that present in the whole brain was determined and illustrated in the insertion to Fig. 4B. After a plateau

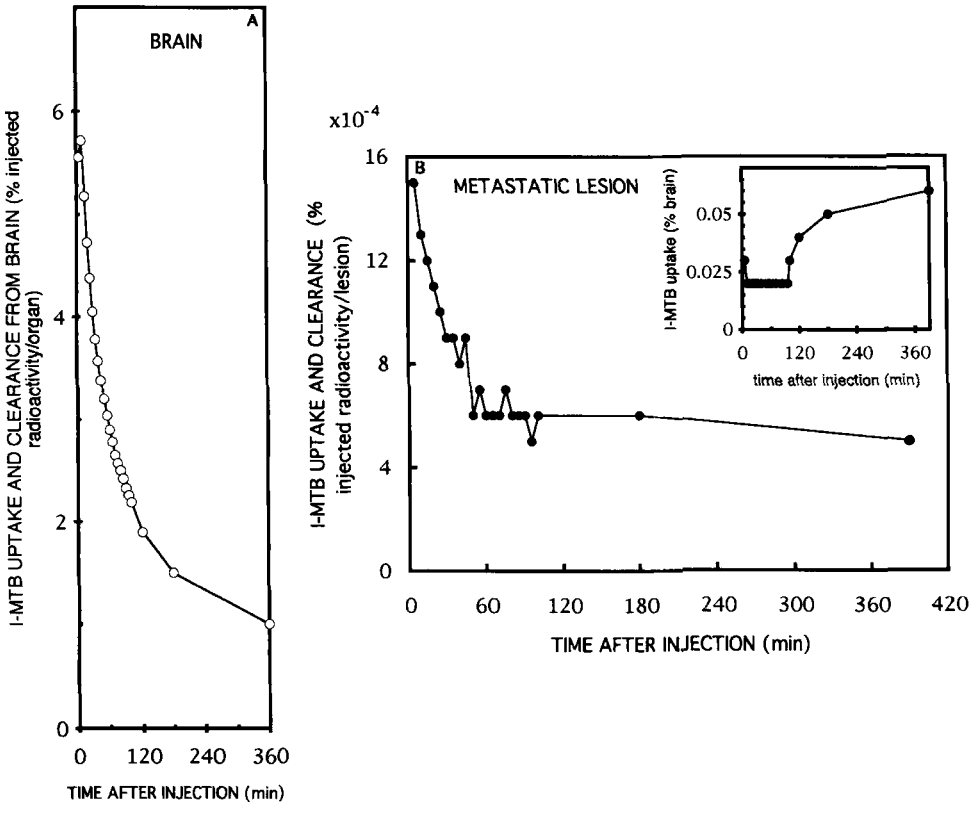

Fig. 4. Detailed analysis of the uptake and retention of [^{123}I]-MTB in the brain (A) and cerebral metastasis (B) during the first 6 h after i.v. injection of the compound to melanoma patients. Insertion: Time-dependent changes of radioactivity present in the brain and cerebral metastasis expressed as the tumour/brain ratio.

over the initial period of rapid clearance of radioiodinated MTB from blood (approx. 1.75 h) the tumour/brain ratio increased 3-fold within 4 h. (Such increase in the tumour/surrounding tissue ratio was also observed in the animal model—see Table 2.) Consequently, cerebral lesions (verified subsequently with routinely used methods) were detected easily with planar γ-camera in all investigated patients with brain metastases even a few days after [^{131}I]-MTB administration.

Direct measurement of radioactivity was carried out in three cutaneous melanomas characterised by various pigmentation and biopsied from two patients at 19 and 26 h after administration of radioiodinated MTB. The tumour/skin ratio for highly pigmented lesions amounted to 8.5 at 19 h and 6.6 at 26 h, whereas 1.1 was the value for poorly pigmented tumour. The ratios were irrespective of the initial radioactivity of the injected compound (Table 3). Since the vasculature develops equally well in melanomas regardless of tumour pigmentation (16) the observed difference in radioiodinated MTB uptake in the lesions can only be accounted for the discrepancy in their melanin content.

Discussion

Detailed bio-distribution studies of radiolabelled MTB in man have been initiated after encouraging therapeutic results achieved by us, particularly with [^{211}At]-MTB in targeted radiotherapy of disseminated melanoma in animal model systems (15–17) justified an introduction of this treatment to clinical trials. Similarities in the time-dependent distribution of the compound in melanoma patients and human melanoma-bearing animals are suggestive of the effectiveness of [^{211}At]-MTB therapy in man. The data enabled an estimation of the absorbed doses received from [^{211}At]-MTB in human organs with the highest risk from the accumulated [^{211}At]-MTB (Table 4) and assured their safety in the therapeutic procedures on the basis of animal toxicological investigations (17).

Results presented in this paper confirm a highly selective uptake of radiolabelled methylene blue in pigmented melanomas in man: tumour/surrounding tissue ratio amounted to approximately 9 at 19 h after a single intravenous injection of radioiodinated MTB. At the same time, there was no obvious affinity of the compound to any normal organ.

Parallel studies carried out in animals enabled a comparison of the data obtained from melanoma patients with those in mice bearing human melanoma xenografts. Detailed investigations concerning a time-dependent kinetics of [^{123}I]-MTB accumulation and retention in pigmented tumours grown in animals showed a gradual uptake of the

Table 3

Tumour/normal tissue ratios obtained from cutaneous biopsies taken from two melanoma patients to whom radioiodinated MTB was administered as a single i.v. injection

Time after I-MTB injection	Ratio		Ratio of absorbed dose from [^{211}At]-MTB (*)	
	Tumour/Blood	Tumour/Skin	Tumour/Blood	Tumour/Skin
Highly pigmented tumours				
19 h	8.9	8.6	229	211
26 h	—	6.6	—	167
Poorly pigmented tumour				
19 h	1.2	1.1	30	28

(*) To calculate doses absorbed by cutaneous tumours $T_{1/2\ biol}$ = const was applied, and $T_{1/2\ biol}$ = 330 min for the blood and the skin.

compound and, subsequently, a comparably fast decrease of the radioactivity to the level of approximately 35% of its maximum. The latter remained stable afterwards throughout the time of investigation. Similar pattern of the compound accumulation and retention was found in metastatic melanoma in man. MTB, being a very small molecule, can penetrate easily through plasma membranes resulting in a uniform distribution of the compound within tissue. Therefore, there should be an equilibrium between the MTB level in the blood and tissue unless the compound were bound to a cellular structure(s) or involved in biochemical processes. Since MTB forms a strong complex with melanin (7), the compound is bound to the bio-polymer once it enters melanosomes. Taking into account that approximately 40% of a tumour tissue consists of cells (in case of melanoma—melanocytes) and melanosomes are restricted to the melanocytes—a total volume of these organelles must be smaller than that of melanocytes themselves, i.e. 40% of the total volume of the tumour. Consequently, a final and stable MTB level in melanoma lesion should be in a range of 40% of its maximum uptake and should depend on a degree of pigmentation of the tumour. The above explains the observed pattern of radioiodinated MTB retention in highly pigmented lesions, as well as a comparable level of the compound in very poorly pigmented melanoma and in blood found both in man and animals. A significant, time-dependent increase in the tumour/blood and tumour/surrounding tissue ratios observed for the highly pigmented lesions validates the reasoning.

A further confirmation of the stability of radiolabelled MTB binding in highly pigmented lesions in man came from biopsies of cutaneous melanomas. The radioactivity found in these tumours (as determined per g of the tissue—data not shown), as well as the tumour/skin ratios were almost independent of the radioactivity administered to the patients and the time that elapsed between injection of the compound and the surgery (i.e., 19 h and 26 h).

A comparison of radioiodinated MTB bio-distribution in man and mice revealed the same pattern of uptake and retention of the compound in organs of both host types. Slopes of most curves were either the same or very similar to those characteristic of the blood, thus confirming that the blood-flow rather than a specific affinity of radioiodinated MTB to these tissues was responsible for the presence of radioactivity in them. Since a fast clearance of radiolabelled MTB from the blood assures that low radiation doses are delivered by [^{211}At]-MTB (Table 4), all these organs should not be affected by targeted radiotherapy with [^{211}At]-MTB. Indeed, toxicological studies performed in human melanoma-bearing mice did not reveal serious adverse effects caused by [^{211}At]-MTB treatment in normal organs (17). Particularly high and rapid uptake of the compound in the liver and kidneys showed once again that these two organs are most important in metabolising and excreting MTB (24) and, additionally, that the introduction of the radioisotope to the structure of the compound did not change the above pattern. On the other hand, such elevated uptake of MTB labelled with ^{211}At might result in radiation doses delivered during [^{211}At]-MTB therapy too high for these organs. However, combination of a rapid clearance of the compound from the structures, a short half-life of ^{211}At and an exceptionally small range of emitted α-particles prevents

Table 4

Estimations of absorbed doses expected in critical organs and blood from ^{211}At-methylene blue if distributed homogeneously within the tissues

Organ	$T_{1/2\ biol}$ (min)	Dose (cGy/MBq)
Blood	0.58	0.0011
Thorax	60	0.075
Liver	90	0.073
Kidney	68	0.28

deposition of excessive radiation doses (Table 4) and damage to the organs (17) during [^{211}At]-MTB treatment.

The pattern of radioiodinated MTB uptake and retention in the brain was also very similar to that of blood suggesting an existence of the blood-brain barrier impenetrable to radiolabelled MTB (such barrier does not apply to cerebral metastases). An appearance of neurotoxic effects after intrathecal administration of MTB, but not after an intravenous injection of the compound (25, 26), confirms the existence of such a barrier. The difference in toxicity depending on the route of administration could not be explained by high MTB doses delivered intrathecally as compared with the intravenous ones; severe neurological disorders distant from the site of MTB injection occurred after a single dose not exceeding 60 mg. The highest non-toxic dose of MTB administered intravenously amounts to 7 mg/kg body weight (= 490 mg per 70 kg) (24). Since the cerebro-spinal blood volume equals approximately 18% of the total blood pool, the MTB dose delivered to the brain and the spinal cord after an intravenous injection would amount to approximately 88 mg. Therefore, the compound should induce similar neurological symptoms to those observed after its intrathecal injections in the absence of the blood-brain barrier. However, this is not the case. The above information is of particular importance since highly pigmented structures of the brain such as substantia nigra or locus coeruleus might be at a significant risk from [^{211}At]-MTB used for systemic treatment of melanoma if the barrier were not present. More detailed studies using [^{123}I]-MTB, [^{131}I]-MTB and SPECT, as well as [^{124}I]-MTB and PET are therefore in progress to confirm the findings.

There was no obvious [^{123}I]-MTB accumulation in the eyes of melanoma patients over the first 14 h. However, similar investigations to those of the brain were undertaken to ensure safety of pigmented structures of the eye when [^{211}At]-MTB is applied.

The clinical results obtained explicitly confirm a high affinity and a stable binding of radiolabelled MTB to pigmented melanomas in man and predict an effectiveness of targeted [^{211}At]-MTB radiotherapy comparable to that observed for melanoma xenografts grown in athymic mice (15–17), whereas similarities in the time-dependent pattern of radiolabelled MTB bio-distribution in man and animals, as well as the absence of [^{211}At]-MTB toxicity in melanoma bearing mice (16, 17) promise a lack of adverse side-effects from [^{211}At]-MTB in melanoma patients.

The present studies were focused predominantly on biodistribution of radioiodinated MTB. It seems likely, however, that the compound might prove to be useful not only for targeted radiotherapy of disseminated melanoma but also for detecting melanoma metastases of a size too small to be visualized with other imaging methods in patients who previously presented highly pigmented primary tumour. Although melanoma secondaries are often less pigmented than the primary lesions, a process of 'depigmentation' progresses gradually with tumour growth and proportionally to the growth rate rather than affecting cells released directly from a primary tumour that initiate metastatic spread. Since nearly random dissemination of melanoma makes a prediction of the localization of its metastases almost impossible, a prospect of systemic scanning of small secondaries leading to their immediate detection and treatment is of particular importance in a long-term prognosis for melanoma patients. Preliminary data concerning diagnostic potential of radioiodinated MTB will be reported separately.

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REFERENCES

1. Goard EL, Fletcher MC. Toxic chorioretinopathy following the use of NP-207. Trans Am Ophthalmol Soc 1956; 54: 129–39.
2. Arden GB, Friedman A, Kolb H. Anticipation of chloroquine retinopathy. Lancet 1962; 1: 1164.
3. Forrest FM, Forrest IS, Roizin L. Clinical, biochemical and post mortem studies on a patient treated with chlorpromazine. Rev Agress 1963; VII: 147–52.
4. DeEds F, Stockton AB, Thomas JO. Studies on phenothiazine. VIII. Antiseptic value of phenothiazine in urinary tract infections. J Pharmacol Exp Ther 1939; 65: 353–71.
5. Tsuji S. Fixation of methylene blue-treated nervous tissue for histological and ultrastructural studies. Naturwissenschaften 1979; 66: 584–9.
6. Lavelle MA. Parathyroid adenoma stained with methylene blue. J R Soc Med 1980; 73: 462–3.
7. Potts AM. The reaction of uveal pigment in vitro with policyclic compounds. Invest Ophthalmol 1964; 3: 405–16.
8. Forrest IS, Gutmann F, Keyzer H. In vitro interaction of chlorpromazine and melanin. Rev Agress 1966; VII: 147–52.
9. Foster R, Hanson P. Electron-donor-acceptor complex formation by compounds of biological interest. I. Optical absorption spectra of mixtures of phenothiazines and related compounds with electron acceptors. Biochim Biophys Acta 1966; 112: 482–9.
10. Foster R, Fyfe CA. Electron-donor-acceptor complex formation by compounds of biological interest. II. The association-constant of various 1,4-dinitrobenzene-phenothiazine drug complexes. Biochim Biophys Acta 1966; 112: 490–5.
11. Larsson B, Tjälve H. Studies on the mechanism of drug binding to melanin. Biochem Pharmacol 1979; 28: 1181–7.
12. Link EM, Rydzy M. Endoirradiation of neoplastic tissues by administered radioisotopes. In: Proceedings of the Third Meeting of the Polish Biophysics Society, Wroclaw, 1978: 62.
13. Link EM, Brown I, Carpenter RN, Mitchell JS. Uptake and therapeutic effectiveness of ^{125}I- and ^{211}At-methylene blue for pigmented melanoma in an animal model system. Cancer Res 1989; 49: 4332–7.
14. Link EM, Lukiewicz S. A new radioactive drug selectively accumulating in melanoma cells. Eur J Nucl Med 1982; 7: 469–73.

15. Link EM, Carpenter RN. ^{211}At-methylene blue for targeted radiotherapy of human melanoma xenografts: Treatment of micrometastases. Cancer Res 1990; 50: 2963–7.
16. Link EM, Carpenter RN. ^{211}At-methylene blue for targeted radiotherapy of human melanoma xenografts: Treatment of cutaneous tumors and lymph node metastases. Cancer Res 1992; 52: 4385–90.
17. Link EM, Michalowski AS, Rösch F. ^{211}At-methylene blue in targeted radiotherapy of disseminated melanoma. Pigment Cell Res 1994; 7: 358–62.
18. Humm JL. Dosimetric aspects of radiolabelled antibodies for tumour therapy. J Nucl Med 1986; 27: 422–7.
19. Smith IE, Courtenay D, Mills J, Peckham MJ. In vitro radiation response of cells from four human tumors propagated in immune-suppressed mice. Cancer Res 1978; 38: 390–2.
20. West CML, Sandhu RR, Stratford IJ. The radiation response of V79 and human tumour multicellular sheroids – cell survival and growth delay studies. Br J Cancer 1984; 50: 143–51.
21. Blower PJ, Carter NJ. Rapid preparation of ^{123}I-labelled methylene blue and toluidine blue: potential new agent for parathyroid scintigraphy. Nucl Med Commun 1990; 11: 413–20.
22. Jarritt PH, Kouris K. Instrumentation for brain SPECT: Guidelines and qualifications. In: Costa DC, Morgan GF, Lassen NA, eds. New trends in nuclear neurology and psychiatry. John Libbey & Co. Ltd., 1993: 39–62.
23. Kassis AI, Harris CR, Adelstein SJ, Ruth TJ, Lambrecht R, Wolf AP. The in vitro radiobiology of Astatine-211 decay. Radiat Res 1986; 105: 27–36.
24. Reynolds JEF. ed. Martindale—The Extra Pharmacopoeia, 28th ed., London: The Pharmaceutical Press, 1982: 384–5.
25. Arieff AJ, Pyzik SW. Quadriplegia after intrathecal injection of methylene blue. J A M A 1960; 173: 794–6.
26. Evans JP, Keegan HR. Danger in the use of intrathecal methylene blue. J A M A 1960; 174: 856–9.