Cancer Chemother Pharmacol (1997) 40: 131–137

ORIGINAL ARTICLE

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Chemical characterization and comparative cellular effects of meta-iodobenzyl guanidine and benzyl guanidine

Received: 27 May 1996 / Accepted: 22 December 1996

Abstract meta-Iodobenzyl guanidine (MIBG) combines the structural properties of the neuron-blocking agents bretylium and guanethidine and is being used increasingly for various clinical applications. Different samples of MIBG were assayed for possible contamination with benzyl guanidine (BG). Fast-atom-bombardment mass spectrometry (FAB-MS) analysis showed a prominent but variable m/z 150 signal, corresponding to a protonated BG. The MS/MS fragmentation pattern of these $[M + H]^+$ ions was similar to that obtained from FAB-MS-generated, protonated BG, confirming the proposed molecule and associated structures. RP-HPLC analysis of both guanidines, however, excluded the possibility of contamination of MIBG with BG. It was therefore concluded that the BG signal was an artifact of the FAB-MS procedure. In addition, the importance of the meta-substituted iodine for the biological activity of MIBG was investigated. Three different biochemical and cell-biological properties of MIBG were compared with those of its precursor MIBA and BG. The assays used were: inhibition of the catecholamine "Uptake I" system in SK-N-SH neuroblastoma and PC-12 pheochromocytoma cells, inhibition of mitochondrial respiration, and general cytotoxicity in L1210 leukemia cells. Of the drugs tested, MIBG was the most efficient in Uptake I inhibition and was more toxic in survival assays, but as compared with BG it was almost equipotent in inhibiting mitochondrial respiration. These findings contribute to a further elucidation of the mechanism by which MIBG exerts its various actions.

Key words Guanidine · Cytotoxicity · Mass spectrometry · *meta*-Iodobenzyl guanidine

Abbreviations *MIBG* meta-Iodobenzyl guanidine · *MIBA* meta-Iodobenzyl amine · *BG* benzyl guanidine · *FAB-MS* fast-atom-bombardment mass spectrometry · *MS/MS* tandem mass spectrometry · *RP-HPLC* reversed-phase high-performance liquid chromatography · T_2 cell-doubling time · *DMEM* Dulbecco's modified Eagle's medium · *RPMI* Roswell Park Memorial Institute · *FCS* fetal calf serum

Introduction

meta-Iodobenzyl guanidine (MIBG) is a false analogue of the neurotransmitter norepinephrine, having a similarly high affinity for the catecholamine "Uptake I" system but being devoid of the postsynaptic receptor effects [10]. In tracer amounts, [$^{123/131}$I]-MIBG is used as a radiopharmaceutical to target normal and malignant tissues of neuroadrenergic origin for diagnostic scintigraphy, and labeled with higher activities of ^{131}I, it is used for therapy of pheochromocytoma and neuroblastoma [3, 16]. Current studies with neuroadrenergic tumors include predosing with radio-inert MIBG to improve the ratio of tumor-to-normal-tissue uptake of [^{131}I]-MIBG [8]. Because MIBG has cross-affinity for serotonin (re)uptake, unlabeled MIBG is also under investigation for palliation of patients with carcinoid-associated syndromes [7, 14]. Moreover, MIBG has shown promising results in the selective acidification of tumors [4]. This has therapeutic potential for cancer treatment through the combination of MIBG with pH-sensitive (pro)drugs that are potentiated at low pH[1]. This tumor-selective acidification as well as the observed

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indiscriminate cytostatic properties of MIBG in vitro [11] are plausibly explained by the reported inhibition by MIBG of mitochondrial respiration [5]. For evaluation of the feasibility of a broader clinical use of unlabeled MIBG, animal studies and a phase I trial have been initiated at our institute.

The increased clinical use of unlabeled MIBG at doses of up to 40 mg/m^2 emphasizes the need for an established quality protocol that relies on verifiable analytical parameters. Furthermore, the toxicological and pharmacological effects of possible contaminants and metabolites derived from MIBG have to be examined further [6]. Several chemical characteristics (MS; nuclear magnetic resonance, NMR; IR) of MIBG have been documented by Wafelman et al. [15], who reported observing a signal at m/z 150 in FAB-MS experiments on MIBG. These authors proposed $[PhCH_2NH\text{-}CNHNH_2 + H]^+$ as the detected substance, which corresponds to protonated benzyl guanidine (BG). This finding suggested that the FAB-MS-analyzed MIBG could be contaminated with BG. As this possibility was not further addressed in that paper, we analyzed commercially obtained and custom-synthesized MIBG samples for the presence of BG by FAB-MS, MS/MS, and RP-HPLC. Synthesis and chemical analysis of BG enabled us to establish the origin and relevance of the BG signal in the FAB-MS spectra of the MIBG samples. In addition, we evaluated several cell-biological and biochemical properties of BG in a comparison with MIBG and the precursor compound for MIBG, meta-iodobenzyl amine (MIBA). In doing so, we assessed the importance of the guanidine entity and of meta-iodine aromate substitution for the afore-mentioned biological properties.

Materials and methods

Chemicals and cell cultures

All chemicals were of analytical grade. Commercial MIBG was purchased from Enka (Germany). [^{125}I]-MIBG (specific activity $\pm$ 65 μCi/mmol) was custom-synthesized according to the method described by Wieland et al. [16]. Tissue-culture medium and medium supplements were obtained from Gibco (Paisley, Scotland). Biological assays were performed with exponentially growing cells. All cells were kept in a humidified atmosphere at 37 °C and were routinely checked for viability by light microscopy. The detailed cultivation procedures of SK-N-SH human neuroblastoma, PC12 rat pheochromocytoma, and L1210 mouse leukemia cells have been described previously [12, 13].

Synthesis of MIBG and BG

MIBG was produced by reaction of MIBA and cyanamide according to a slightly modified version of the method of Wieland et al. [16]. No attempt was made to optimize the yield ($\pm$ 60%, mol/mol). BG was synthesized from benzyl amine and cyanamide by the mixing and heating (120 °C for 30–60 min) of 1 mol of benzyl amine and 2 mol of cyanamide (or equimolar ratios). The procedure applied for further purification and subsequent isolation of di(benzyl guanidine) monosulfate was similar to the method described above for MIBG [16]. BG-sulfate crystals precipitated as long, spiky white needles. Product analysis by RP-HPLC/UV (details are discussed below) was performed, and it was found that the product was 95–97% (w/w) pure by comparison of peak areas. The purity was confirmed by MS analysis of the reaction product, which also suggested the absence of major contamination. The yield of the reaction was similar to that of MIBG and averaged 55% (mol/mol).

Chemical analysis

Reversed-phase high-pressure liquid chromatography

The analytical system consisted of a high-pressure liquid chromatography pump from Beckman (System Gold) and an ultraviolet detector equipped with a 200-μl flow cell (model Uvicord) with a fixed 254-nm filter from Pharmacia/LKB. Elution profiles were established using a Beckman (5-μm) Ultrasphere ODS column (250 mm × 4.6 mm) as the solid phase. The mobile phase consisted of ammonium acetate (0.16 *M*) and acetonitrile 1:3 (v/v) adjusted to pH 2.5 with sulfonic acid, 8 m*M* heptanesulfonic acid, and 0.1 m*M* potassium iodide. The flow rate was 1 ml/min.

FAB mass spectrometry

FAB-MS was carried out using a V.G. Micromass ZAB-2HF mass spectrometer, an instrument with reverse geometry, fitted with a high-field magnet and coupled to a V.G. 11/250 data system or a Jeol MS SX/SX102A four-sector mass spectrometer coupled to a Jeol MS-MP7000 data system. The samples were loaded in a glycerol solution onto a stainless-steel probe and were bombarded with xenon atoms with a kinetic energy of 8 keV.

Biological analysis

Inhibition of mitochondrial respiration

Inhibition of mitochondrial respiration was determined in intact L1210 cells by compensatory stimulation of the glycolytic flux [5]. In brief, L1210 cells (2.5×10^6/ml) were incubated with different amounts of the drugs for 4 h. Next, the medium was separated from the cells and the lactate and glucose concentrations were measured enzymatically using standard kits from Boehringer (Mannheim, Germany).

General cytotoxicity of MIBG and BG

Cytotoxicity was assayed in L1210 cells in a single-cell survival assay in which the cells were exposed to different concentrations of the drug, which has been elsewhere described [11]. In brief, cells (100 or 1000 per well) were seeded in conditioned growth medium with 0.6% (w/v) carboxymethylcellulose, which gives a semisolid plating medium. After 7–8 days the macroscopically visible colonies were scored and the surviving fraction was determined relative to the control (the number of colonies in untreated samples). Cytotoxicity was expressed as the critical drug concentration that inhibited the formation of macroscopically visible colonies (< 0.1% survival).

Uptake-I inhibition capacity

Uptake I inhibition was tested in SK-N-SK and PC12 cells as previously described [13]. In brief, cells (approx. 3.5×10^5/well) were incubated in a nonsaturating concentration of 10^{-7} *M* [^{125}I]-MIBG with different concentrations of the inhibitory compounds. Cell-associated radioactivity was corrected for cellular protein content and for nonspecific [^{125}I]-MIBG uptake in the presence of 4 μM imipramine, an inhibitor of catecholamine transport. Inhibition was expressed as the ID_{50} value, i.e., the concentration of unlabeled drug that inhibited the specific uptake of [^{125}I]-MIBG by 50%.

Results

Chemical Analysis

FAB mass spectrometry

The FAB mass spectrum of MIBA (Fig. 1A) contained signals at the following m/z positions: 234; {(C_7H_9NI) $[M + H]^+$}, 217; {(C_7H_6I) $[M + H]^+$-NH_3}, 108; [($C_7H_{10}N$) formally protonated benzylamine], 91; {(C_7H_7) [m/z 108-NH_3]}, 90; {(C_7H_6) $[M + H]^+$-NH_3-I}, 89; and {(C_7H_5) $[M + H]^+$-NH_3-HI}. The FAB mass spectrum of custom-synthesized MIBG (Fig. 1B) showed signals at the following m/z positions: 276; {($C_8H_{11}N_3I$) $[M + H]^+$}, 217; {(C_7H_6I) $[M + H]^+$-CH_5N_3}, 150; [($C_8H_{12}N_3$) formally protonated BG], 91; and (m/z 150-CH_5N_3). A similar fragmentation pattern was observed for the commercially obtained MIBG sample (data not shown). The FAB mass spectrum of BG exhibited signals at the following m/z positions: 150; {($C_8H_{12}N_3$) $[M + H]^+$}, 91; {(C_7H_7) $[M + H]^+$-CH_5N_3}, 60; and CH_6N_3 formally protonated guanidine; (Fig. 1C). The MS/MS spectrum of the m/z-150 ions from MIBG (Fig. 1D) was largely similar to that of BG and showed signals at: 150; [($C_8H_{12}N_3$) formally protonated BG], 91; and [(C_7H_7) m/z 150-CH_5N_3]. The similarities between the FAB-MS spectrum of BG and the MS/MS spectrum of m/z 150 from MIBG suggest that the analyzed molecules are the same.

Reversed-phase high-pressure liquid chromatography

For determination as to whether the putative BG was present either in the MIBG ab initio or as a consequence of the MS procedure, the elution profiles of MIBG and BG were assessed by RP-HPLC. Compound detection was performed by UV absorption; an extinction coefficient at 254 nm was therefore established first for MIBG ($802 \pm 66\,cm^{-1}M^{-1}$, $n = 4$) and BG ($762\ cm^{-1}M^{-1}$, $n = 2$). For a direct comparison, approximately 100 µg of both samples was dissolved in 1 ml of methanol and was assayed using the standard MIBG RP-HPLC conditions. With this technique a marked difference in retention time was found for both compounds (Fig. 2). The similar absorption coefficients of MIBG and BG allowed a direct comparison of the elution profiles, and, in contrast to the FAB-MS findings, BG could not be detected in any of the MIBG samples.

Biological Analysis

Mitochondrial effects of MIBG and BG

Inhibition of mitochondrial respiration was inferred from the stimulated glycolytic flux in the presence of the test compounds and was assumed to be proportional to the increased release of lactate into, and the use of glucose from, the medium [5]. MIBG and BG at 30 µ*M* demonstrated maximal stimulation of the glycolysis, whereas MIBA had no effect. When tested at lower concentrations, MIBG was slightly more effective in this response (Table 1).

General cytotoxicity of MIBG and BG

Cell-survival curves revealed a steep decline after a critical threshold dose, precluding an accurate calculation of LD_{50} values. The efficacy of the drugs in inhibiting the proliferation of L1210 cells was therefore expressed by the critical concentration at which no visible colony could be scored. The results shown in Table 1 confirmed previous findings [11] of a large difference between MIBG and MIBA in cytotoxicity. BG appeared to be about 5-fold less toxic than MIBG.

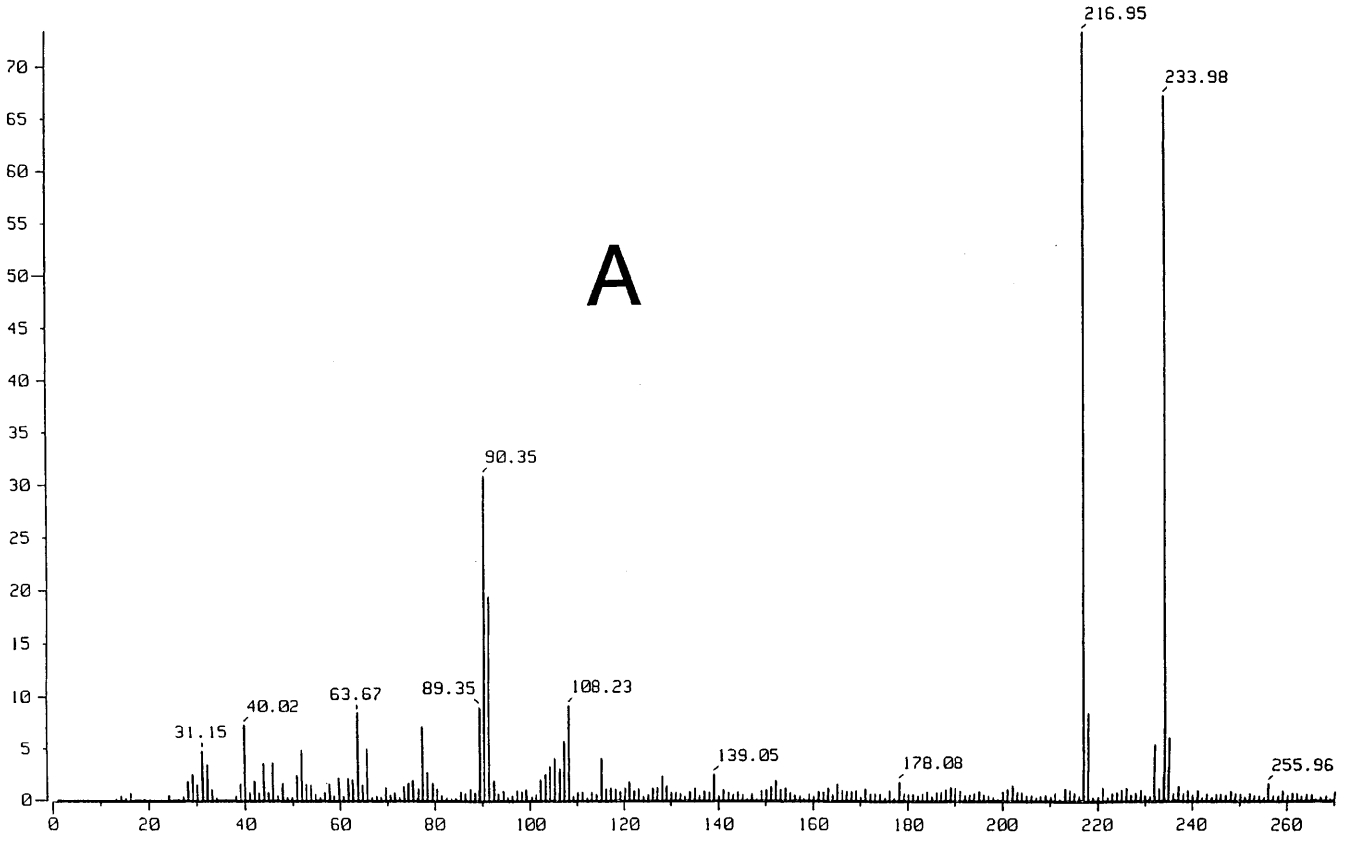
Uptake-I inhibition capacity

The relative affinity for catecholamine Uptake I was tested by competition of the unlabeled drug with [^{125}I]-MIBG under nonsaturating concentrations of 10^{-7} *M* for specific uptake by SK-NS-H neuroblastoma and PC12 pheochromocytoma cells. From the ID_{50} values shown in Table 1 it can be concluded that in both cell systems the meta-iodine in MIBG strongly potentiated, i.e., about 10-fold, the affinity for Uptake 1 as compared with BG, whereas MIBA was not effective at clinically relevant concentrations.

Discussion

[$^{123/131}$I]-MIBG is used in tracer amounts as a radiopharmaceutical to target normal and malignant tissues of neuroadrenergic origin. The clinical use of unlabeled MIBG is currently under investigation for the improvement of tumor-to-normal-tissue ratios of [$^{123/131}$I]-MIBG uptake as well as for palliation of carcinoid-associated syndromes. MIBG has also shown promising results in selective acidification of tumors. The optimal and safe clinical use of MIBG at elevated mass doses requires further investigation into the different biochemical and systemic effects of the compound. A key issue in this process is the establishment of a set of verifiable data for compound identification. In addition, the biochemical and pharmacological effects of metabolites and possible contaminants should be documented.

In this study the chemical and biological profiles of MIBA and MIBG were compared with those of BG, a compound that can be easily detected in the FAB-MS spectrum of both commercially obtained and custom-synthesized MIBG (Fig. 1B). The interest in BG as a possible contaminant was inspired by the presence of the m/z-150 signal in the FAB-MS spectrum, which is suggestive of hydrogen-for-iodine substitution. The MS/MS


A
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233.98
90.35
31.15
40.02
63.67
89.35
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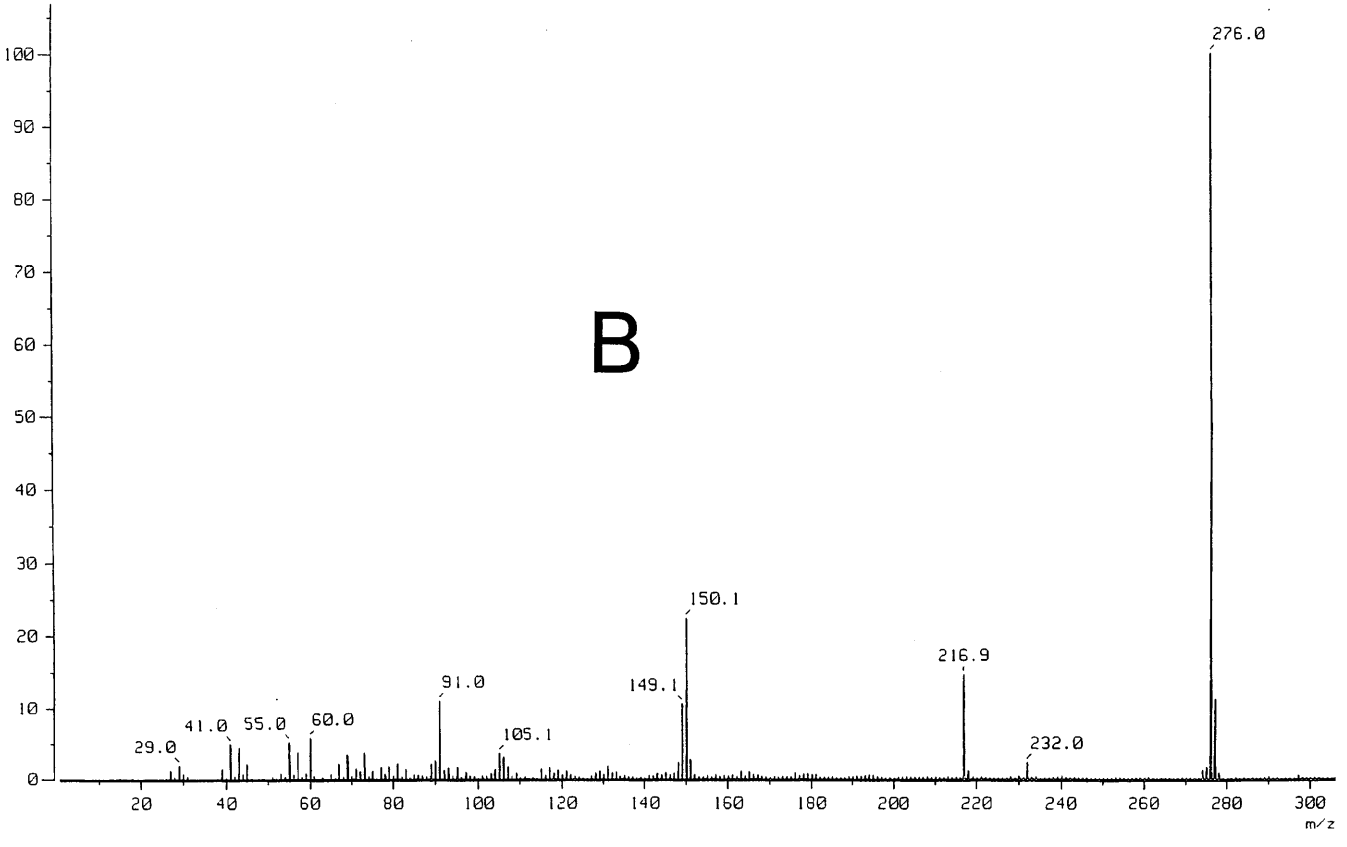
178.08
255.96


B
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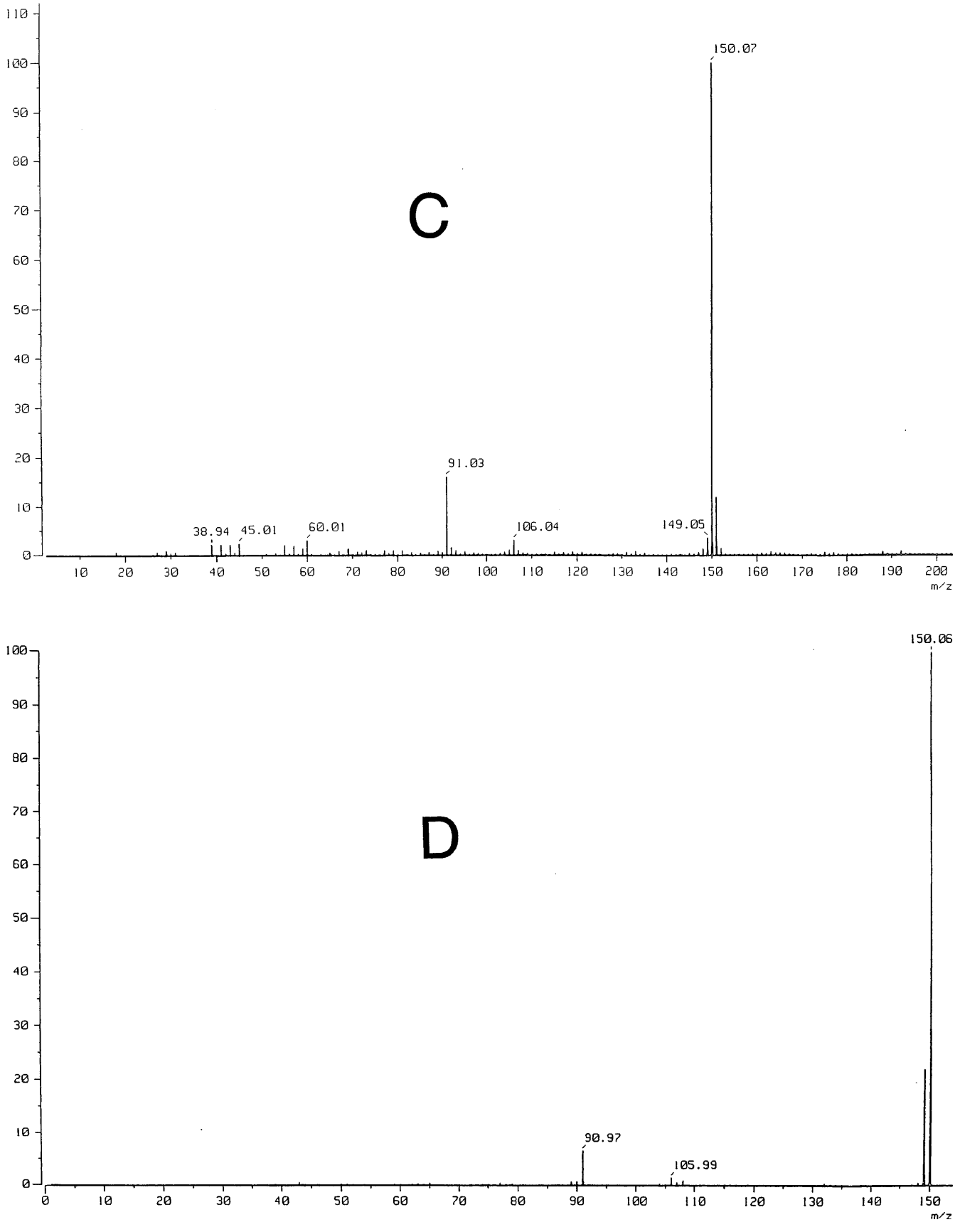

Fig. 1A–D FAB mass spectra of **A** MIBA, **B** MIBG, and **C** BG and **D** MS/MS product analysis of the m/z-150 ions from **B**

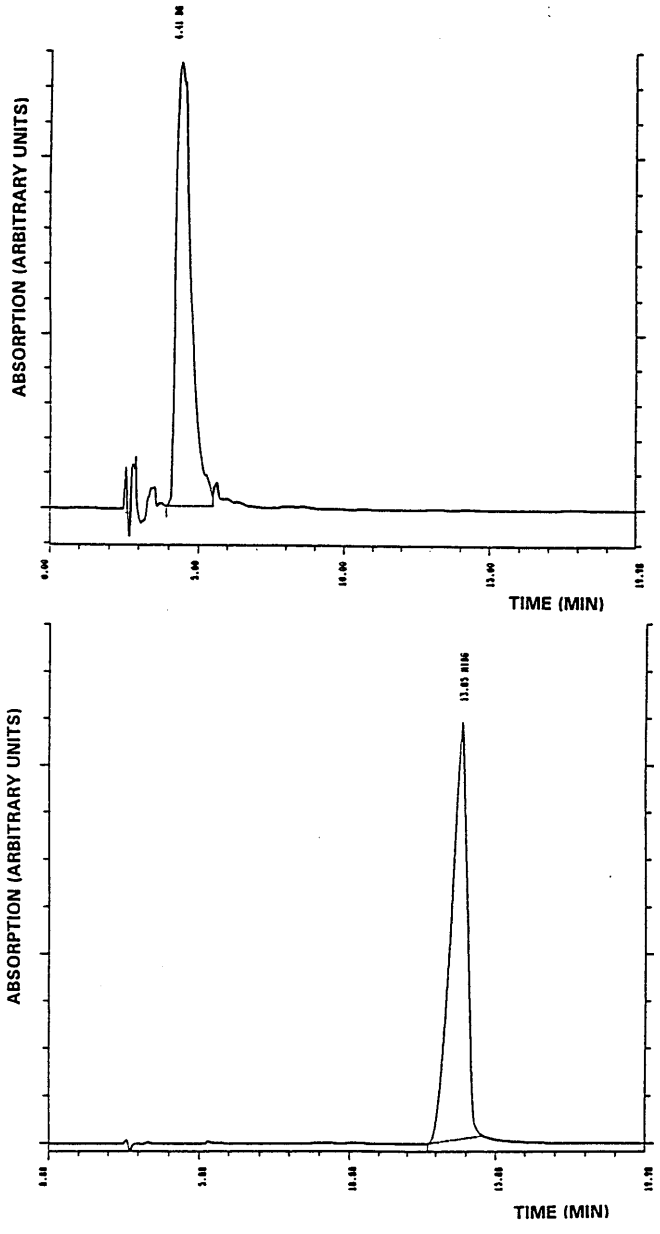

Fig. 2 Redrawn RP-HPLC elution profiles of MIBG (*bottom*) and BG (*top*). For a full description of the experimental conditions, see Materials and methods

spectrum of the corresponding m/z-150 ions is similar to that generated directly for protonated BG itself via FAB (Fig. 1C, D), and, thus, confirms the presence of BG. To establish the origin of the BG we performed RP-HPLC analysis of BG and MIBG. We found that BG did not coelute with MIBG and was not detected in the MIBG samples (Fig. 2). We therefore conclude that the FAB-MS m/z-150 signal is a consequence of the FAB-MS procedure. Indeed, the FAB-MS spectrum of the (iodinated) precursor compound MIBA contains a peak at m/z 108 [Fig. 1A], indicating that a similar substitution reaction (hydrogen for iodine) has taken place. Mechanistic details about this substitution reaction cannot be derived from the present results. It is, however, not unprecedented that under FAB conditions, chemical transformation of the analyte occurs by reaction with the liquid matrix. For example, it has been shown that aromatic oximes are reduced to their corresponding imines upon FAB [2]. Furthermore, aromatic carbon dehalogenation, with concomitant incorporation of hydrogen, has been observed with halonucleosides during FAB [9]. In other words, there is a caveat for the interpretation of FAB-MS data with regard to the presence of structurally related contamination.

BG shares with MIBG a guanidine group as a structural entity. The importance of the aromate-iodine in MIBG for its different biological properties could therefore be addressed by comparison of BG with MIBG. The presence of meta-iodine strongly potentiated the affinity of MIBG as compared with BG for Uptake 1 (about 10-fold) and the general toxicity (about 5-fold) and showed a 2-fold stimulation of glycolysis (Table 1). Furthermore, a comparison between MIBG and BG versus MIBA confirmed the importance of the guanidino group – a structural entity of MIBG and BG – for all response studies (Table 1).

Although admittedly preliminary, studies of this type may assist in the selection of MIBG analogues that are optimally suited for the various clinical applications currently under investigation. For different reasons, neither BG (acidifying properties) nor MIBA (rapid renal clearance, personal communication from Dr. M. Rutgers) appear to be suitable in predosing experiments to improve tumor over normal-tissue ratios in targeted

Table 1 Comparative biological effects of MIBG, BG, and MIBA (*ND* Not determined)

Response (end point)[a]	MIBG	BG	MIBA
Stimulation of glycolytic flux: Maximal	30 μM	30 μM	*ND*
Half-maximal	7.5 μM	15 μM	$>>60$ μM
Inhibition of colony formation ($<0.1\%$ survival)	5 μM	25 μM	100 μM
Inhibition of Uptake I (ID_{50}) in:			
Neuroblastoma SK-NS-H cells	$2 \times 10^{-7} M$	$2 \times 10^{-6} M$	$2 \times 10^{-5} M$
Pheochromocytoma PC 12 cells	$4 \times 10^{-7} M$	$3 \times 10^{-6} M$	$10^{-4} M$

[a]Data represent average values from corresponding dose-response curves of 2 (glycolytic flux) or 4 (survival and Uptake I studies) independent experiments

radiotherapy with radioactive MIBG. However, BG could be an alternative for tumor-selective acidification, thereby avoiding the systemic effects caused by the release of bioactive amines by MIBG [4].

Acknowledgements The authors would like to thank Prof. Dr. N.M.M. Nibbering (Institute of Mass Spectrometry, University of Amsterdam, The Netherlands) and Prof. Dr. J. Beijnen (Slotervaart Hospital, The Netherlands) for their critical reading of the manuscript and their helpful suggestions.

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