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High-performance liquid chromatographic determination of metaiodobenzylguanidine in whole blood and plasma of cancer patients

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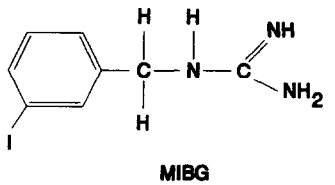
Abstract: An accurate and simple high-performance liquid chromatographic (HPLC) assay is presented for the quantitative determination of metaiodobenzylguanidine (MIBG) in human whole blood and plasma. The sample pretreatment involves a solid-phase extraction on Bakerbond SPE cyano columns. The HPLC system comprises a μBondapak C_{18} column and ammonium phosphate (pH 4.0; 25 mM)–acetonitrile (80:20, v/v) as the mobile phase. Detection is performed by UV absorbance measurements at 254 nm. Linear regression with weighting factor $1/x^2$ yielded the smallest sum of per cent relative concentration residuals over the concentration range of the assay (0.1–10 μg ml^{-1}). MIBG levels, at the end of a 3-h infusion, in whole blood and plasma of carcinoid patients were measured and compared with the results obtained with radiodetection after addition of iodine-131-labelled MIBG.

Keywords: *Cancer patients; HPLC; MIBG; plasma; whole blood.*

Introduction

Iodine-131-labelled metaiodobenzylguanidine ([^{131}I]MIBG, Fig. 1) is successfully used in the scintigraphy and treatment of neuroectodermally derived tumours such as malignant phaeochromocytoma, neuroblastoma and carcinoid [1]. In addition, the palliative effect of escalating doses of non-radioactive MIBG in carcinoid patients is under investigation [2]. The combination of non-radioactivity and higher MIBG doses in those individuals compared to patients treated with [^{131}I]MIBG, prompted the investigation of MIBG blood or plasma pharmacokinetics. The *in vivo* [^{131}I]-MIBG concentration (counting total radioactivity) has been described as being higher in whole blood compared to plasma [3]. In addition, an *in vitro* [^{131}I]MIBG distribution ratio (erythrocyte concentration/plasma concentration) of 2.5 at 37°C has been reported [3]. Only one high-performance liquid chromatographic (HPLC) method using UV detection has been described for the determination of MIBG in serum, but not in whole blood [4]. Moreover, that HPLC system which included three pumps, two automatic motor valves, on-line pre-column purification and enrichment, and automatic regeneration of the pre-column was rather complex. There appears to be a need for an adequate method for the determination of MIBG in both whole blood and plasma, applicable in a hospital laboratory with standard equipment. The results of the development and validation of such a procedure are reported in this paper.



MIBG

Figure 1
Chemical structure of metaiodobenzylguanidine.

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Experimental

Chemicals

[^{131}I]MIBG.½H_2SO_4 [3.7 (±10%) GBq in 7.5 ml 0.9% (w/v) sodium chloride solution containing 1% (w/v) of benzyl alcohol, with specific activity 1.3–3.0 GBq mg^{-1} = 0.42–0.97 TBq $mmol^{-1}$] was purchased from Amersham Buchler (Braunschweig, Germany). MIBG.½H_2SO_4 was synthesized from the hydrochloride (HCl) salt of meta-iodobenzylamine (MIBAM) by a modified version of the method of Wieland *et al.* [5]. MIBAM.HCl was purchased from Janssen Chimica (Geel, Belgium). *o*-Phosphoric acid (analytical grade) was obtained from BDH (Poole, UK). All other chemicals were of analytical grade and originated from Merck (Darmstadt, Germany). Acetonitrile and methanol (HPLC grade) were purchased from Promochem (Wesel, Germany). Distilled water was used throughout the work. Drug-free heparinized human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). Whole blood was provided by healthy volunteers and patients.

Stock solutions

A stock solution of MIBG (1000 μg ml^{-1}) was freshly made on each day of analysis by dissolving the appropriate amount of the drug, accurately weighed, in methanol. Typical amounts of the stock solution were diluted with methanol to give working solutions with concentrations of 100, 10 and 1 μg ml^{-1} of MIBG; these solutions are referred to as A, B and C, respectively.

Calibration samples

Plasma. Calibration samples in plasma were prepared as follows: 15.0, 30.0, 37.5, 75.0, 112.5, 150, 300, 375, 750, 1125 and 1500 μl of B were transferred into polypropylene tubes (Eppendorf–Netheler–Hinz, Hamburg, Germany) and, after evaporation of the methanol under nitrogen at 60°C, diluted to 1.5 ml with drug-free plasma. The samples were then vortexed for 15 s and processed further as described below.

Whole blood. Calibration samples in whole blood were prepared as follows: 50.0, 100, 125, 250, 375 and 500 μl of C and 100, 125, 250, 375 and 500 μl of B were transferred into polypropylene tubes (Eppendorf–Netheler–Hinz) and, after evaporation of the methanol under nitrogen at 60°C, diluted to 0.5 ml with drug-free whole blood. The samples were equilibrated for 1 h at room temperature and were subsequently stored for 1 h at −30°C. Procedures for the calibration and the respective patient samples were similar: after thawing, 1.5 ml distilled water was added to 0.5 ml of whole blood; the samples were vortexed for 15 s and kept for 30 min at room temperature. The samples were then centrifuged for 2 min at 9500***g*** and processed further as described below.

Sample extraction and preparation procedure

Plasma. An aliquot of 500 μl of each plasma sample was applied to a 1-ml (100 mg) Bakerbond cyano SPE column (Baker, Phillipsburg, NJ), previously conditioned with 1 ml of methanol and 2 ml of distilled water. The column was then washed consecutively with 2 ml of distilled water and 2 ml of methanol after which MIBG was eluted with 1 ml of 0.1 M HCl in methanol. Subsequently, the eluate was evaporated under nitrogen at 60°C and 200 μl of the mobile phase was added. The sample was then vortexed for 15 s and centrifuged for 2 min at 9500***g***. Finally, the clear supernatant was transferred to an autosampler vial.

Whole blood. A 1-ml (100 mg) Bakerbond cyano SPE column was conditioned with 1 ml of methanol and 2 ml of distilled water consecutively, retaining 0.5 ml water above the sorbent. A 3-ml Bakerbond SPE filtration column was then fixed on top of the cyano column, using an adaptor (all from Baker). An aliquot of 2 ml of each sample (consisting of 0.5 ml whole blood and 1.5 ml distilled water) was applied to the filtration column, which was subsequently washed with 1 ml of distilled water. After removal of the filtration column and adaptor, the cyano column was washed consecutively with 1 ml of distilled water and 2 ml of methanol. MIBG was then eluted from the cyano column with 1 ml of 0.1 M HCl in methanol, and the sample was further treated as described for plasma.

Chromatography

The HPLC system comprised a solvent delivery system type 510, a UV spectrophotometer type 441 (both from Waters Associates, Milford, MA), a SP 8880 automatic sample

injection device and a SP 4600 integrator (both from Spectra–Physics, Santa Clara, CA). Separation was obtained at ambient temperature on a 300 × 3.9 mm i.d. column packed with 10-μm μBondapak C_{18} (Waters). The mobile phase was ammonium phosphate (pH 4.0; 25 mM)–acetonitrile (80:20, v/v). The aqueous part of the mobile phase was adjusted to pH 4.0 with *o*-phosphoric acid. The mobile phase was filtered and degassed by ultrasonication. The flow rate was maintained at 1.0 ml min^{-1} and UV detection was performed at 254 nm. Aliquots of 100 μl were injected into the chromatograph.

Validation parameters

Calibration curves. All standards (11 per calibration curve, range: 0.1–10 μg ml^{-1}) were extracted and analysed in duplicate. Linear regression (response vs concentration, $y - x$), weighted linear regression ($y - x$ with weighting factor $1/x$ and $1/x^2$) and a power fit model (log y − log x) were applied to the analytical results. Those response functions were investigated by calculating correlation coefficients and per cent relative concentration residuals (%RCR) of the analytical results from spiked whole blood and plasma samples. % RCR is defined as:

$$\% \text{ RCR} = 100\ (IC - NC)/NC,$$

where *IC* and *NC* represent the interpolated and nominal concentrations, respectively [6].

Recovery. Recoveries of MIBG from whole blood and plasma were calculated using the optimal response function by comparing the slopes of the regression lines for the processed standards, prepared in whole blood and plasma, respectively, with the slope of the regression line for the non-processed standards, prepared in eluent. For this purpose, the response function with the lowest cumulative Σ%RCR, when the results of processed and non-processed standards were added, was regarded as the optimal response function.

Detection limit and lower limit of quantitation. The detection limit of the HPLC assay was estimated as the drug concentration in whole blood and plasma, respectively, which corresponded to three times the baseline noise. The lower limit of quantitation (LLQ) was defined as the concentration of the lowest standard in the analytical run which was quantified with a deviation and precision less than 20%.

Accuracy and precision. The accuracy and precision (between-day and within-day) of the method were determined by replicate analyses of known concentrations in the middle of each decade in the calibration lines using one-way analysis of variance (ANOVA).

Specificity. The basic precursor MIBAM was tested for co-elution with MIBG on the HPLC system.

Stability. The chemical stability of MIBG in whole blood and plasma was investigated by adding known amounts of the drug to drug-free whole blood and plasma, respectively. The spiked samples were studied at −30°C (whole blood only) and at ambient temperature, by determining the residual drug concentration. The stability of the extracted drug in the mobile phase was investigated at ambient temperature.

Pharmacokinetics

Two male adult patients (patient initials: BA and BY) suffering from carcinoid were administered 70 and 80 mg of MIBG, respectively, in 100 ml of 0.9% sodium chloride solution by intravenous infusion for 3 h. Blood samples (5 ml) from one arm were collected in heparinized tubes before starting the infusion into the other arm and just prior to the end of the infusion. An aliquot of whole blood (1 ml) was immediately removed from the collection tube and stored in a polypropylene tube at −30°C, prior to analysis. The remaining fraction was centrifuged at 3000*g* for 10 min and plasma was transferred to a polypropylene tube. Plasma and cells were stored separately at −30°C, prior to analysis. On another occasion patient BA and two other adult carcinoid patients (WB, female and VR, male) were administered 80, 40 and 40 mg of MIBG, respectively, to which was added a diagnostic dose of 37 MBq [^{131}I]MIBG (mass: 25 μg MIBG), in 100 ml of 0.9% sodium chloride solution by intravenous infusion for 3 h. Immediately after collection and preparation of blood samples as described above, the total radioactivity in 1.0 ml of whole blood and plasma, respectively, was counted in an autogamma counter (Minaxi Auto-Gamma 5000

Series, Packard, Meriden, CT) for ≤10 min. Standards were prepared by adding 100 μl of a solution of 0.2 MBq ml^{-1} [^{131}I]MIBG in 0.9% sodium chloride to 1.0 ml drug-free whole blood and plasma of the patient under investigation. The radioactive concentration in the patient samples was calculated using the standard, prepared in the same matrix. Subsequently, radioactive concentrations were converted into mass concentrations MIBG, since the doses administered (both in mg and in MBq) were known. Before counts were performed, 100 μl of 0.9% sodium chloride solution was added to all the patient samples in order to keep the same volume as the standards. All counts were corrected for background and counting time by standard procedures.

Results and Discussion

Chromatography, detection and sample pretreatment

Several HPLC systems have been tested during the development of the MIBG assay. Initially, a system including a Chromsep silica gel column (Chrompack, Middelburg, The Netherlands) and methanol–1 M ammonium nitrate–2 M ammonia (27:1:2, v/v/v) as the mobile phase was chosen. That system is in use for the quality control of [^{131}I]MIBG infusion fluids by on-line radiodetection [7, 8]. However, the UV-absorbing front of the processed biological matrices interfered with MIBG. Increasing the methanol fraction to 99% (v/v) in order to give MIBG more retention [7] introduced another problem, i.e. elution of MIBG after blank injections; this problem was presumably caused by cumulation of MIBG from previous injections on the top of the column. Other columns tested, using ammonium phosphate (pH 2.0–7.4; 25 mM)–tetrahydrofuran (80:20, v/v) as the mobile phase, were μBondapak C_{18} (Waters), Eurogel PRP-001 C_8 (Knauer, Berlin, Germany) and Diol LiChrosorb (Merck). Of these, μBondapak C_{18} gave the most acceptable results in terms of peak symmetry and separation of the analyte from endogenous substances. Acetonitrile as the modifier instead of tetrahydrofuran (THF) yielded chromatograms with less baseline noise. Ammonium phosphate (pH 4.0; 25 mM)–acetonitrile (80:20, v/v) was selected as the mobile phase as it resulted in a satisfactory separation of MIBG and its chemical precursor MIBAM with capacity factors of 3.1 and 0.8, respectively.

The UV absorption spectrum of MIBG in methanol shows a maximum at 230 nm, with molar absorptivity $\epsilon_{230nm} = 9.03 \times 10^3$ M^{-1} cm^{-1}. However, because the signal-to-noise ratio was 2.5 times better at 254 nm, that wavelength was preferred for UV detection, although $\epsilon_{254nm} = 7.36 \times 10^2$ M^{-1} cm^{-1}. That was also found during the development of an HPLC assay of MIBG in urine of children suffering from neuroblastoma [9]. At both wavelengths (230 and 254 nm) many sources of interference in the chromatograms of whole blood and plasma blanks occur, which makes a sample pretreatment necessary. One-ml (100 mg) Bakerbond cyano SPE columns (Baker) were found to enable quantitative retention of MIBG to be achieved. Many elution solvents were tested: methanol, dichloromethane, ethyl acetate, hexane, acetonitrile and THF. However, MIBG was not eluted from the cyano column by those solvents. Aralkylguanidines, such as MIBG, have a pK_a of ≥13 [10, 11]. Therefore, MIBG can be expected to exist for >99% as a (mono-)cation in solution over a broad pH-range. Consequently, using ionogenic interactions, sodium hexanesulphonate (12 mM) in methanol–water (3:1, v/v) and 0.1 M HCl in methanol were tested as elution solvents, resulting in 87.2 and 98.5% recoveries, respectively, of aqueous MIBG samples from the cyano column. As methanol did not elute MIBG, it was incorporated as a wash solvent; this was preceded by a wash step with water, since the columns clogged, probably due to protein precipitation, when the applied plasma samples were washed directly with methanol. The additional use of a filtration column in the whole blood sample pretreatment was necessary to prevent clogging of the columns even when the aqueous wash step was included. The whole blood calibration samples were stored for 1 h at room temperature, since additional *in vitro* experiments using 0.1 and 5.0 μg ml^{-1} MIBG with 12 kBq ml^{-1} [^{131}I]MIBG in whole blood demonstrated a partial migration of MIBG from plasma to the cellular fraction, which equilibrated after 1 h. Freezing of all whole blood samples, including the calibration samples, and dilution with distilled water before extraction was done in order to free the cellular fraction of MIBG by haemolysis. Representative HPLC chromatograms of whole

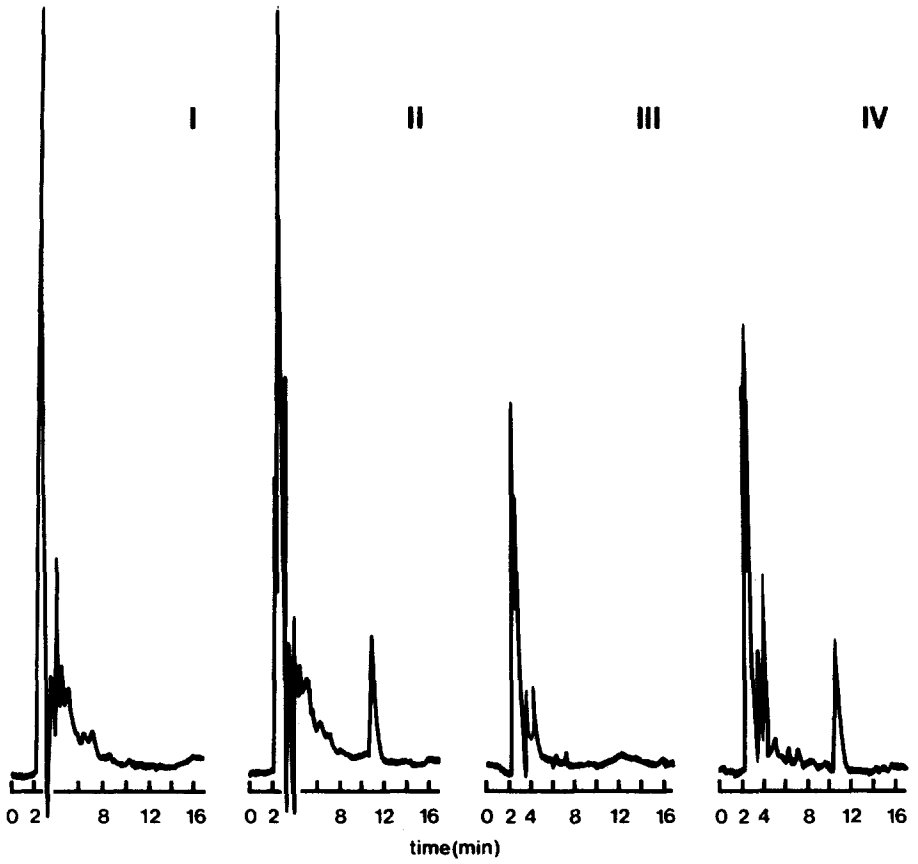

Figure 2
HPLC chromatograms of blank whole blood (I), 0.5 μg ml^{-1} MIBG in whole blood (II), blank plasma (III) and 0.5 μg ml^{-1} MIBG in plasma (IV). The retention time of MIBG was 11 min.

Table 1
Correlation coefficients and Σ%RCR for different calibration functions* for MIBG in human whole blood and plasma

	n†	Linear unweighted $y - x$	Linear w‡ $(1/x)$ $y - x$	Linear w‡ $(1/x^2)$ $y - x$	Power function $\log y - \log x$
Whole blood					
r	11	0.9990	0.9989	0.9953	0.9985
Σ%RCR§	11	119	62.5	50.1	56.8
Plasma					
r	11	0.9986	0.9984	0.9928	0.9968
Σ%RCR§	11	138	79.1	79.3	116

*Concentration range 0.1–10 μg ml^{-1}.
†n = number of duplicate measurements.
‡w = weighted (weighting factor in parentheses).
§Σ%RCR, see text.

blood and plasma calibration samples are shown in Fig. 2.

Validation of the assay

The analytical methodology was validated in terms of linearity, recovery, detection limit, LLQ, accuracy, between- and within-day precision, specificity and stability. All response functions were calculated ($y - x$, $y - x$ with weighting factor $1/x$, $y - x$ with weighting factor $1/x^2$ and $\log y - \log x$) using the mean values ($n = 11$ per calibration line) of duplicate measurements. Correlation coefficients and the sum of the absolute %RCR values (Σ%RCR) were calculated for each calibration. All models demonstrated good corre-

lation ($r \geq 0.99$) for both whole blood and plasma (Table 1). However, Table 1 demonstrates that %RCR is a more sensitive parameter, as proposed by Karnes and March [6]. Since linear regression with weighting factor $1/x^2$ resulted in low Σ%RCR values for whole blood and plasma, it was selected as the most appropriate model. For plasma, linear regression with weighting factor $1/x$ might have been selected as well. In order to calculate recoveries of MIBG from spiked whole blood and plasma, linear regression with weighting factor $1/x^2$ was used. Recoveries of MIBG from spiked whole blood plasma are given in Table 2. The detection limit of the presented assay was 75 ng ml^{-1} in both whole blood and plasma using a 500-μl matrix with a 100-μl injection on to the HPLC column. The lower limit of quantitation was 0.1 μg ml^{-1} in both whole blood and plasma, also using a 500-μl matrix with a 100-μl injection on to the HPLC column. Accuracy and between-day and within-day precision have been tabulated (Table 3). MIBAM did not co-elute with MIBG. MIBG was stable at −30°C (concentration: 5.2 μg ml^{-1}) for at least 5 months (whole blood) and at room temperature (concentration: 5.0 μg ml^{-1} urine) for at least 4 h (whole blood and plasma). The extracted drug was stable in the mobile phase at ambient temperature for at least 24 h, which justifies the use of an autosampler for HPLC injection.

Table 2
Recoveries of MIBG* from human whole blood and plasma

Matrix	Recovery (%)	RSD† (%)	n‡
Whole blood	93.5	4.9	2
Plasma	94.3	0.17	2

*Concentration range 0.1–10 μg ml^{-1}.
†RSD = relative standard deviation.
‡n = number of replicate calibrations; a calibration consisted of 22 samples (11 calibration levels in duplicate).

Pharmacokinetics

The results of MIBG peak whole blood and plasma level monitoring using the present HPLC method and the procedure using radiodetection, as described, are given in Table 4. It

Table 3
Accuracy, between-day and within-day precision for the analysis of MIBG in human whole blood and plasma

Theoretical concentration (μg ml^{-1})	Measured concentration (μg ml^{-1})	Accuracy (%)	Precision (%)*	Precision (%)†	n‡
Whole blood					
0.500	0.481	96.2	ND§	7.9	3
5.00	4.84	96.8	6.5	3.0	6
Plasma					
0.500	0.518	104	19	6.9	6
5.00	4.98	99.6	—¶	5.7	6

*Between-day precision.
†Within-day precision.
‡n = number of replicates.
§ND = not determined.
¶No significant additional variation was observed as a result of performing the assay in different runs.

Table 4
Comparison of two methods for the determination of MIBG peak concentrations in whole blood and plasma after intravenous administration of MIBG in carcinoid patients

Patient characteristics					Peak concentrations (μg ml^{-1})	
Initials	Sex*	Age	Dose	Method	Whole blood	Plasma
BY	M	45	80 mg MIBG	HPLC-UV	0.30	0.24
BA	M	51	70 mg MIBG	HPLC-UV	ND†	0.28
BA	M	51	80 mg MIBG + [^{131}I]MIBG‡	Radiodetection§	0.47	0.30
WB	F	44	40 mg MIBG + [^{131}I]MIBG‡	Radiodetection§	0.25	0.21
VR	M	50	40 mg MIBG + [^{131}I]MIBG‡	Radiodetection§	0.19	0.13

*M = male; F = female.
†ND = not determined.
‡The additional MIBG mass (25 μg) from [^{131}I]MIBG is negligible.
§See text.

is shown that both assays produce comparable results. Overestimation of MIBG calculated by means of total radioactivity is possible since [^{131}I]MIBG contained ≤4% free [^{131}I] iodide which shows a smaller distribution volume compared to [^{131}I]MIBG. That may increase the fraction of free [^{131}I]iodide, especially in plasma (own observation). Compared to the alternative HPLC–UV assay described [4], the present method has several advantages. The proposed procedure is suitable for both whole blood and plasma samples; it calls for the usual amount of equipment readily available in hospital laboratories and it has been validated extensively, including a comparison with another method.

Conclusions

A simple HPLC method for the analysis of MIBG in both whole blood and plasma has been developed and validated. The procedure has been found to be adequate for routine monitoring in a hospital.

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