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High-performance liquid chromatographic determination of *m*-iodobenzylguanidine in urine of cancer patients

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ABSTRACT

An accurate, sensitive, reproducible and selective HPLC assay is presented for the quantitative determination of *m*-iodobenzylguanidine (MIBG) in human urine. The sample pretreatment involves a solid-phase extraction on Bakerbond SPE cyano columns. The HPLC system consists of a μBondapak C_{18} column and 25 m*M* ammonium phosphate (pH 3.0)–acetonitrile (75:25, v/v) as the mobile phase. Detection is performed by UV absorbance at 254 nm. Log *y* *vs.* log *x* is the response function that yielded the smallest sum of percent relative concentration residuals over the whole concentration range of the assay (0.2–100 μg/ml). The excretion profile of MIBG in urine of a three-year old patient is shown.

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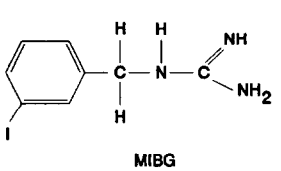
INTRODUCTION

Iodine-131 labelled *m*-iodobenzylguanidine ([^{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]. The neuroblastoma patient group consists for >90% of children. Blood sampling for studying the pharmacokinetics of [^{131}I]MIBG in this group is, for known reasons, very burdensome. Moreover, the medical staff would be exposed to a rather high radiation load during the collection of blood samples from patients receiving a therapeutic dose of [^{131}I]MIBG. It has been shown that [^{131}I]MIBG predominantly is excreted unchanged in urine [2,3], with only 15% as metabolites [4]. Therefore, following the urinary excretion profile is a valid and non-invasive means of establishing the pharmacokinetics of MIBG. Several methods have been described to determine the urinary excretion pattern after administration of [^{131}I]MIBG including the measurement of total radioactivity [5] or quantitation of [^{131}I]MIBG by radiodetection, after solid-phase extraction (SPE) as sample pretreatment and separation by high-performance liquid chromatography (HPLC) [2,6]. In some cases it may be preferable to determine MIBG after decay of the radioisotope, *e.g.* if there is no specially equipped and controlled laboratory to handle radioactivity or if there is no time – related to the physical half-life of ^{131}I (8.04 days) – to measure the labelled compound. In literature, only one HPLC method with UV detection has been described for the determination of MIBG in urine [7]. The complexity of this HPLC system (including 3 pumps, 2 automatic motor valves, on-line pre-column purification and enrichment, and automatic regeneration of the pre-column), challenged us to develop a method applicable in each standard equipped hospital laboratory. We describe the development and validation of a suitable assay for the determination of MIBG in urine. For the validation the report of the conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies" – held in 1990 – was used as a guideline [8]. The excretion profile of a three-year old male neuroblastoma patient treated with [^{131}I]MIBG is reported.



MIBG

Fig. 1. Chemical structure of *m*-iodobenzylguanidine.

EXPERIMENTAL

Chemicals

[^{131}I]MIBG · $\frac{1}{2}H_2SO_4$ [3.7 (±10%) GBq in 7.5 ml 0.9% (w/v) sodium chloride solution containing 1% (w/v) of benzyl alcohol; specific activity: 1.3–3.0 GBq/mg = 0.42–0.97 TBq/mmol] was purchased from Amersham Buchler (Braunschweig, Germany). MIBG · $\frac{1}{2}H_2SO_4$ was synthesized from the hydrochloride (HCl) salt of *m*-iodobenzylamine (MIBAM) by a modified version of the method of Wieland *et al.* [9]. MIBAM · HCl and *m*-iodobenzoic acid (MIBA) were purchased from Janssen Chimica (Geel, Belgium). *m*-Iodohippuric acid (MIHA) was synthesized from glycine and *m*-iodobenzoylchloride (MIBCl), analogous to the synthesis of hippuric acid described by Ingersoll and Babcock [10]. The synthesis of MIBCl from MIBA and thionyl chloride was performed according to Vogel [11]. *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. Urine was provided by healthy volunteers and patients.

Stock solutions

A stock solution of MIBG (1000 μg/ml) 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 reference solutions with concentrations of 100, 10 and 1 μg/ml of MIBG, respectively.

Calibration samples

Calibration samples in urine were prepared by adding the following aliquots in polypropylene tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany): 300, 375, 450, 750, 1125 and 1500 μl of the 1-μg/ml reference solution for the range 0.2–1.0 μg/ml and 150, 300, 375, 750, 1125 and 1500 μl of the 10-μg/ml and 100-μg/ml reference solution for the ranges 1.0–10.0 μg/ml and 10.0–100.0 μg/ml, respectively. Methanol was evaporated under nitrogen at 60°C and 1.5 ml of drug-free urine was added. The samples were then vortex-mixed for 15 s and extracted.

Sample extraction and preparation procedure

An aliquot of 500 μl of each urine sample was applied to a 1-ml (100 mg) Bakerbond cyano SPE column (Baker, Phillipsburg, NJ, USA), previously conditioned with 1 ml of methanol and 2 ml of distilled water. The column was then washed with 1 ml of 10 m*M* disodium tetraborate buffer (adjusted to pH 11.0 with 10 *M* NaOH), 1 ml of distilled water and 1 ml of methanol, after which MIBG was eluted from the column 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 were added. The sample was then vortex-mixed for 15 s and centrifuged for 2 min at 9500 *g*. Finally, the clear supernatant was transferred to an autosampler vial.

Chromatography

The HPLC system consisted of a solvent delivery system type 510, a UV spectrophotometer type 441 (both from Waters Associates, Milford, MA, USA), an SP 8880 automatic sample injection device and an SP 4600 integrator (both from Spectra Physics, Santa Clara, CA, USA). Separation was obtained at ambient temperature with a μBondapak (Waters) C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size). The mobile phase consisted of 25 m*M* ammonium phosphate (pH 3.0)–acetonitrile (75:25, v/v). The aqueous part was adjusted to pH 3.0 with *o*-phosphoric acid. The mobile phase was filtered and degassed by ultrasonication. The flow-rate was maintained at 1.0 ml/min and UV detection was performed at 254 nm. Aliquots of 100 μl were injected into the chromatograph.

Validation parameters

Calibration curves. All standards (6 per range, 3 ranges) were extracted and analyzed in duplicate. Linear regression (response *versus* 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. These response functions were investigated by calculating correlation coefficients and percent relative concentration residuals (%RCR) of the analytical results from spiked urine samples. %RCR is defined as $\%RCR = 100(IC - NC)/NC$, where IC and NC represent the interpolated and nominal concentrations, respectively [12].

Recovery. Recovery of MIBG from urine was calculated by comparing the slope of the y–x curve for the processed standards, prepared in urine, with the slope of the y–x curve for the non-processed standards, prepared in eluent.

Detection limit and lower limit of quantitation. Urine samples from nine different healthy volunteers were spiked with MIBG to concentrations 0, 0.1, 0.2 and 0.3 μg/ml, respectively. The samples were extracted and analyzed. Concentrations were calculated by means of a calibration curve in the range 0.1–1 μg MIBG/ml urine. The detection limit of the HPLC assay was initially estimated as the value which corresponded to three times the baseline-noise. It was confirmed as the concentration which was significantly different from the blank (Student's t-test, $P < 0.05$). 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 calibration curve using one way analysis of variance (ANOVA).

Specificity. The basic precursor MIBAM, acid metabolites MIHA and MIBA and drugs, that were used as co-medication during [^{131}I]MIBG

therapy, were tested for co-elution with MIBG on the HPLC system. The drugs tested were digoxin, ketanserin, loperamide, metoclopramide, octreotide, paracetamol and somatostatin.

Selectivity. Urine samples from ten different healthy volunteers were extracted and analyzed.

Stability. The chemical stability of MIBG in urine was investigated by adding known amounts of the drug to blank urine. The spiked samples were studied at −20°C and at 7°C, by determining the residual drug concentration. The stability of the extracted drug in the mobile phase was investigated at ambient temperature.

Pharmacokinetics

[^{131}I]MIBG (3.4 GBq = 2.5 mg of MIBG) in 100 ml of 0.9% sodium chloride solution was administered by intravenous infusion during 4 h to a three-year old male patient suffering from neuroblastoma. A urine sample voided prior to the start of infusion was taken as the blank. All urine voided from the start of infusion until the patient left the hospital was collected in portions of fixed periods. The samples were stored at −20°C prior to analysis.

RESULTS AND DISCUSSION

Chromatography, detection and sample pretreatment

The influence of the modifier in the mobile phase [25 m*M* ammonium phosphate (pH 4.0)–acetonitrile] on the chromatographic behaviour of the drug has been studied in the range 20–30% (v/v) acetonitrile, resulting in capacity factors (k') of MIBG varying from 6.8–1.5, respectively. The most convenient capacity factor of MIBG (2.1) was found with 25% (v/v) of acetonitrile. Having established the percentage of modifier, the pH of the phosphate buffer was varied within the range 3–7. A satisfactory separation of the bases MIBG and MIBAM and the acidic MIBG metabolites MIHA and MIBA was obtained at pH 3.0.

The UV absorption spectrum of MIBG in methanol shows a maximum at 230 nm, with molar extinction coefficient $\epsilon_{230\ nm} = 9.03 \cdot 10^3$ M^{-1} cm^{-1}. However, because of a 2.5 times better signal-to-noise ratio at 254 nm, this wavelength was preferred for UV detection, although $\epsilon_{254\ nm} = 7.36 \cdot 10^2$ M^{-1} cm^{-1}. At both wavelengths many interferences in the chromatogram of a urine blank occur, which makes a sample pretreatment necessary. For this reason the retention behaviour of MIBG on the following SPE columns was tested: 1 ml (100 mg) cyano, 3 ml (500 mg) ethyl, 3 ml (500 mg) octadecyl, 3 ml (500 mg) octyl, 3 ml (500 mg) phenyl and 1 ml (100 mg) sulfonic acid (all from Baker). All columns but the octyl enabled a quantitative retention of MIBG. It has not been tried to find the cause for the surprising lack of quantitative retention of MIBG on octyl columns, as we further concentrated on the columns which retained MIBG by ionic interactions. Aralkylguanidines, such as MIBG, have a pK_a of $\geq$13 [13,14]. Therefore, MIBG may be expected to exist for >99% as a (mono-)cation in solution at pH $\leq$11. As ionic interactions are stronger than hydrophobic interactions, retention of MIBG based on ionic interactions will provide the opportunity of more rigorous washing procedures. Of the 2 columns on which ionic interactions play a role, the cyano column was chosen for further evaluation, because the eluate showed less interferences than the one from the sulfonic acid column. Several solutions have been tested as a wash solvent for SPE on the cyano column. First, ammonium phosphate buffers in the range pH 5–8 were used. The sample pretreatment improved using wash solvents with increasing pH. The pK_a of $\geq$13 for aralkylguanidines, such as MIBG, enables washing procedures with solutions with a pH up to 11. Therefore, a disodium tetraborate solution adjusted to pH 11.0 was tested which gave a further improvement of the clean-up procedure and was used in the final washing procedure. Also several elution solvents have been tested: 0.1 *M* HCl in methanol, 1.0 *M* HCl in methanol and acetonitrile/triethylamine (1000:1, v/v). Optimum results with high recoveries were established with a sample pretreatment of 1 ml disodium tetraborate buffer (pH 11.0), followed by 1 ml of distilled water, 1 ml of methanol and extraction with 1 ml of 0.1 *M* HCl in methanol. Representative HPLC chromatograms for the analysis of a patient's urine are shown in Fig. 2.

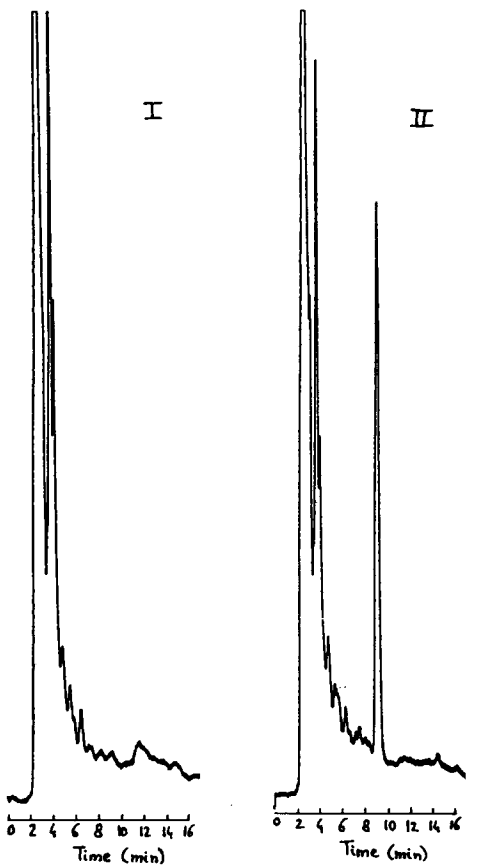


Fig. 2. HPLC chromatograms of blank urine (I) and a urine sample (II) (concentration of MIBG 1.9 μg/ml) of a patient receiving 2.5 mg of MIBG by intravenous infusion during 4 h. The retention time of MIBG is 9.5 min.

Validation of the assay

The analytical methodology was validated in terms of linearity, recovery, detection limit, LLQ, accuracy, between and within-day precision, specificity, selectivity 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 ($n = 6$ per concentration-range) of duplicate measurements. Correlation coefficients and the sum of the absolute %RCR values per concentration range (Σ%RCR) were calculated for each calibration. All models demonstrated good correlation ($r \geq 0.99$) and acceptable linearity (Table I). However, %RCR has been shown to be more sensitive [12]. As log y–log x (concentration range 0.2–100 μg/ml) resulted in the lowest overall Σ%RCR value (Table I), it was selected as the most appropriate model. The linear equations of this model are listed in Table II.

Recoveries of MIBG from spiked urine are given in Table III. The overall recovery was 95.8% ± 3.6% (mean ± S.D.). The detection limit of the presented assay was 0.1 μg/ml urine using a 500-μl sample with a 100-μl injection onto the HPLC column. This concentration was significantly ($P = 0.0019$, $n = 18$) different from the blank. The lower limit of quantitation was 0.2 μg/ml, using a 500-μl sample with a 100-μl injection onto the HPLC column. Accuracy and

TABLE I

CORRELATION COEFFICIENTS AND Σ%RCR FOR DIFFERENT CALIBRATION FUNCTIONS FOR MIBG IN HUMAN URINE

Concentration range (μg/ml)	n[a]	Linear unweighted y–x	Linear weighted[b] ($1/x$) y–x	Linear weighted[b] ($1/x^2$) y–x	Power function log y–log x
0.2–1	6	0.9996	0.9997	0.9997	0.9997
1–10	6	0.9998	0.9997	0.9997	0.9998
10–100	6	0.9988	0.9996	0.9995	0.9997
Overall %RCR[c]	18	28.2	24.1	24.1	22.4

[a] Number of duplicate measurements.
[b] Weighting factor in parentheses.
[c] Overall %RCR, sum of Σ%RCR values of the three concentration ranges.

TABLE II

EQUATIONS OF CALIBRATION LINES FOR THE ANALYSIS OF MIBG IN HUMAN URINE

Concentration range (μg/ml)	Equation[a]	n^b
0.2–1	$\log y = 1.083(\pm 0.013) \log x + 4.745(\pm 0.009)$	6
1–10	$\log y = 1.071(\pm 0.011) \log x + 4.740(\pm 0.009)$	6
10–100	$\log y = 0.997(\pm 0.011) \log x + 4.801(\pm 0.010)$	6

[a] y = peak area integrator units; x = concentration MIBG in μg/ml urine.
[b] Number of duplicate measurements.

TABLE III

RECOVERIES OF MIBG FROM HUMAN URINE

MIBG concentration (μg/ml)	Recovery (%)	R.S.D.[a] (%)	n^b
0.508	91.9	2.3	5
5.08	98.3	2.2	5
50.8	97.2	1.0	5

[a] Relative standard deviation.
[b] Number of replicates.

between-day and within-day precision have been tabulated (Table IV). MIBAM, MIHA, MIBA and the tested drugs gave no co-elution with MIBG. The minimization of interferences from endogenous materials has been demonstrated through the analysis of 10 urine blanks. MIBG was stable at −20°C (concentration: 0.508 μg/ml urine) for at least 38 days and at 7°C (concentration: 5.08 μg/ml urine) for at least 24 h. 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.

The method described in this paper is considered as the method of choice for the determination of MIBG in urine for the following reasons. It calls for the usual amount of equipment readily available in each hospital laboratory, in contrast with the earlier published method [7]. Moreover, the method described in this paper has been validated more extensively and according to current guidelines in force [8].

Clinical pharmacokinetics

A cumulative percentage-of-dose excreted *versus* time plot of MIBG in urine of a three-year old male neuroblastoma patient receiving 2.5 mg MIBG by intravenous infusion during 4 h is shown in Fig. 3. The patient showed a cumulative excretion of 61% within 22 h after the start of infusion, which increased to 72% within 2 days.

CONCLUSION

A simple, sensitive, selective and validated HPLC method for the analysis of MIBG in urine has been developed requiring only 500 μl of urine. The developed procedure can be adequately used in the routine monitoring of patients, suffering from neuroectodermally derived tumours to whom therapeutic amounts of [^{131}I]MIBG are administered.

TABLE IV

ACCURACY, BETWEEN-DAY AND WITHIN-DAY PRECISION FOR THE ANALYSIS OF MIBG IN HUMAN URINE

Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	Accuracy (%)	Precision (%)		n^a
			Within-day	Between-day	
0.508	0.575	113	3.3	8.3	20
5.08	5.00	98.5	3.7	4.7	10
50.8	51.8	102	0.9	2.0	10

[a] Number of replicates.

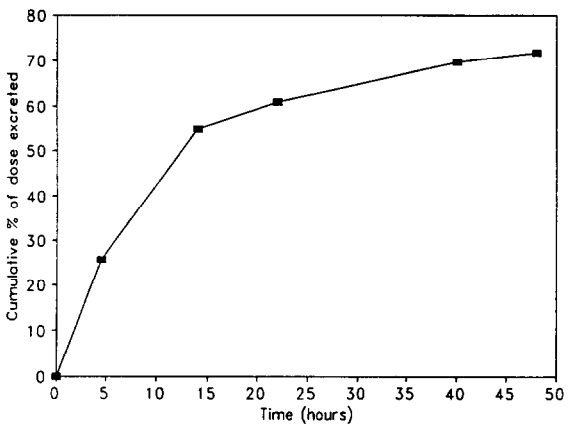


Fig. 3. Cumulative percentage of dose excreted in urine *versus* time plot of a 3-year old male neuroblastoma patient receiving 2.5 mg of MIBG by intravenous infusion during 4 h.

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