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Bioanalysis of *m*-iodobenzylguanidine in plasma by high-performance liquid chromatography after derivatization with benzoin

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Abstract

A sensitive chromatographic assay has been developed for *m*-iodobenzylguanidine (MIBG) in human plasma based on the derivatization with benzoin. MIBG is first isolated from plasma using solid-phase extraction on a cyanopropyl-modified silica phase. After evaporation of the eluate, a fluorescent derivative is formed using benzoin. The derivative is analysed by reversed-phase liquid chromatography using a mixture 60% (v/v) acetonitrile, 30% (v/v) water and 10% (v/v) of the 0.5 *M* Tris buffer (pH 8.0) as the eluent and fluorescence detection at 320 nm for excitation and 435 nm for emission, respectively. In the evaluated concentration range (2–200 ng/ml) precisions ⩽10% and accuracies in between 90 and 100% have been found, with 2 ng/ml being the lower limit of quantification using a 0.5-ml plasma sample volume. The assay can also be used without the internal standard benzylguanidine. The assay was successfully used to obtain a pharmacokinetic curve of MIBG. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

m-Iodobenzylguanidine (MIBG, Fig. 1) labelled with a radioactive iodine atom (^{123}I or ^{125}I, ^{131}I) is well known as a radiopharmaceutical for both diagnostic and therapeutic purposes and was introduced in 1980 [1]. It has been used for both diagnostic scintigraphy and radionuclide therapy of neuroendocrine tumors and for scintigraphic assessment of cardiac sympathetic neuronal integrity [2]. The use of unlabeled MIBG (M^{127}IBG) in doses of 10–40

mg/m^2 was recently investigated as a palliative treatment for patients with a carcinoid tumor and compared with radioactive doses of 5 mg M^{131}IBG (200 mCi=7.4 GBq) [3]. In that study, both MIBG treatment regimens showed a comparable palliative effect with only a difference in the duration of the response.

For these clinical investigations, supportive chromatographic bioanalytical assays for unlabeled MIBG have already been published [4–6]. However, a full pharmacokinetic study in plasma has not yet been possible because of a lack of sensitivity of these HPLC methods using UV detection; lower limits of quantification (LLQ) reported in these studies are 0.1

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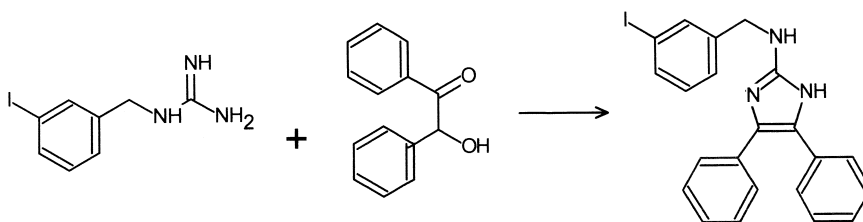*E-mail address:* r.w.sparidans@pharm.uu.nl (R.W. Sparidans)

Fig. 1. Fluorogenic derivatization reaction of MIBG with benzoin; the reaction of BG with benzoin is identical.

and 0.2 μg/ml MIBG. The presence of the guanidine moiety in MIBG challenged us to use the fluorogenic reaction of benzoin with guanidine compounds to obtain a more sensitive assay with a quantification limit in the low ng/ml-range. This fluorogenic derivatization was first described by Kai et al. [7] and later developed into a very sensitive tool for the chromatographic determination of guanidino compounds [8]. However, this method has not been used thus far for complex samples as, for example, in the bioanalytical field. We investigated and validated the use of this derivatization reaction for an assay of MIBG in human plasma and intended to achieve a significant gain in sensitivity compared to the existing methods. The derivatization is preceded by a solid-phase extraction (SPE) based on the method of Wafelman et al. [5,6] to isolate MIBG from the matrix.

2. Experimental

2.1. Chemicals

MIBG$\cdot\frac{1}{2}$H$_2$SO$_4$ was supplied by EMKA Chemie (Markgroningen, Germany) and benzylguanidine (BG) was synthesized from benzylamine and cyanamide by mixing and heating (120°C for 30–60 min) [9]; benzoin was obtained from Cui et al. [10]. Acetonitrile (gradient grade) and methanol (HPLC grade) were provided by Biosolve (Valkenswaard, The Netherlands) and Tris (reagent grade) by Sigma (St. Louis, MO, USA). 2-Methoxyethanol (HPLC grade) was supplied by Sigma–Aldrich (Gillingham, UK), boric acid (pharmaceutical grade) by OPG Pharma (Utrecht, The Netherlands) and sodium sulfite (analytical grade) by Fluka (Buchs, Switzerland); water was purified by reversed osmosis on a multi-laboratory scale. Dexamethasone phosphate,

morphine hydrochloride and paracetamol were of pharmaceutical grade, all other chemicals were of analytical grade from Merck (Darmstadt, Germany).

2.2. Equipment

Chromatographic analyses were performed on the following configuration: A P580 isocratic pump (Gynkotek HPLC, Germering, Germany), a Basic+ Marathon autosampler (Spark Holland, Emmen, The Netherlands), equipped with a 7739-005 injection valve (Rheodyne, Cotati, CA, USA) with a 100-μl sample loop, and a FP-920 fluorescence detector (Jasco, Hachioji, Japan). The column was thermostated in a water bath and the temperature was controlled by a thermomix 1420 heating device (B. Braun, Melsungen, Germany). Chromatographic data were recorded on a Jotronics Pentium 166-32 Mb personal computer (Delfgauw, The Netherlands), equipped with a Chromeleon chromatographic data system (Gynkotek HPLC).

For SPE, Supelclean LC–CN extraction tubes (Supelco, Bellefonte, PA, USA) containing 100 mg of cyanopropyl-modified, endcapped silica as the sorbent are processed with a 24-port Visiprep SPE vacuum manifold (Supelco). LC–MS was performed on a LC-10 AD pump (Shimadzu, Kyoto, Japan) with the column outlet coupled with a splitter to a VG Platform II mass spectrometer (MicroMass, Altrinchem, UK).

2.3. LC–MS conditions

A LiChrospher 60 RP-select B (100×4.6 mm, d_p=5 μm, Merck) column was used at ambient temperature and the eluent was a mixture of 60% (v/v) acetonitrile, 39% (v/v) water and 1% (v/v) of a 0.5 M Tris buffer (pH 8.0). The injection volume was 10 μl. From the eluent flow (1 ml/min) 1/20

was split into the electrospray interface in the positive ion mode. The total mass range, 200–1000 Da, was scanned in 3.1 s per cycle. The mass resolution was 14.5 (instrumental units), the cone voltage 30 V and the source temperature 80°C.

2.4. Chromatographic conditions

Injections (20 µl) were made on a Symmetry C_{18} column (100×4.6 mm, d_p=3.5 µm, average pore diameter=10 nm, Waters Chromatography, Milford, MA, USA). The column temperature was 40±2°C. The eluent comprised 60% (v/v) acetonitrile, 30% (v/v) water and 10% (v/v) of the 0.5 *M* Tris buffer (pH 8.0) and the eluent flow-rate was 1 ml/min. The fluorescence detection wavelengths were 320 nm for excitation and 435 nm for emission, respectively.

2.5. Analytical procedures

To a 0.5-ml plasma sample, pipetted into a polypropylene microtube (1.5 ml, Sarstedt, Nümbrecht, Germany), 50 µl of 200 ng/ml I.S. (BG) in water was added; the sample was then vortex-mixed. The SPE column was conditioned with 1 ml methanol and 1 ml water, respectively. A 0.5-ml volume of the sample was then loaded onto the column, followed by washing with 1 ml of 0.1 *M* borate buffer (pH 11.0), 1 ml water and 1 ml methanol, respectively. Next, the analyte was eluted with 1 ml of 0.1 *M* hydrochloric acid in methanol and collected in a polypropylene microtube. After evaporation of the eluate under a nitrogen gas stream at ambient temperature, the sample was reconstituted (only as far as possible, complete reconstitution occurred during the further treatment of the sample) in 200 µl water by vortex-mixing. Next, the microtube was kept in an ice bath and 100 µl of 4 m*M* benzoin in 2-methoxyethanol, 100 µl of a mixture of 0.1 *M* β-mercaptoethanol and 0.2 *M* sodium sulfite and 200 µl of 2 *M* sodium hydroxide were added, respectively. After vortex-mixing, the closed micro tube was heated at 98°C for 2 min in a thermostated water bath; next, after cooling down the tube in the ice bath for 2 min, 200 µl of a mixture of 2 *M* hydrochloric acid and 0.5 *M* Tris buffer (pH 9.2) was added. After manual shaking, the clear, slightly

yellow solution was transferred to a glass injection vial.

For the LC–MS identification of the derivatives, aqueous solutions of 100 µg/ml MIBG and BG, respectively, were derivatized following the same procedure.

2.6. Validation

Two stock solutions of 1 mg/ml MIBG in methanol were prepared with separate weighting, a stock solution of 1 mg/ml BG in methanol was also made; all stock solutions were stored at −20°C. For calibration a 10 µg/ml dilution in water was made from one of the 1 mg/ml MIBG stock solutions and stored at −20°C. Dilutions of this diluted stock to yield 2, 5, 10, 30, 100 and 200 ng/ml MIBG calibration samples in pooled plasma, respectively, were made daily for each analytical run. For BG also a dilution of 10 µg/ml in water was made, stored at −20°C and diluted daily with water to yield 200 ng/ml. Least-squares regression, weighted by X^{-2} (reversed squared concentration) was employed for the calibration, using the quotient of the peak heights of the derivative analyte peaks of MIBG and the I.S.

From the other 1 mg/ml stock solution of MIBG also a dilution of 10 µg/ml in water was made. Validation samples in plasma were made at 2, 5, 40 and 200 ng/ml respectively and stored at −20°C; plasma of different individual donors was used. Precisions and accuracies were determined by five-fold analysis of each validation sample in three different analytical runs. The repeatability (intra-assay precision) is calculated according to:

$$\text{Repeatability} = \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \times 100\%$$

(ErrMS=error mean square, GM=grand mean) and the reproducibility (inter-assay precision) according to:

$$\text{Reproducibility} = \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/n}}{\text{GM}} \times 100\%$$

(DayMS=day mean square, *n*=number of replicates in each run) for each individual concentration. Six individual blank plasma samples are also tested.
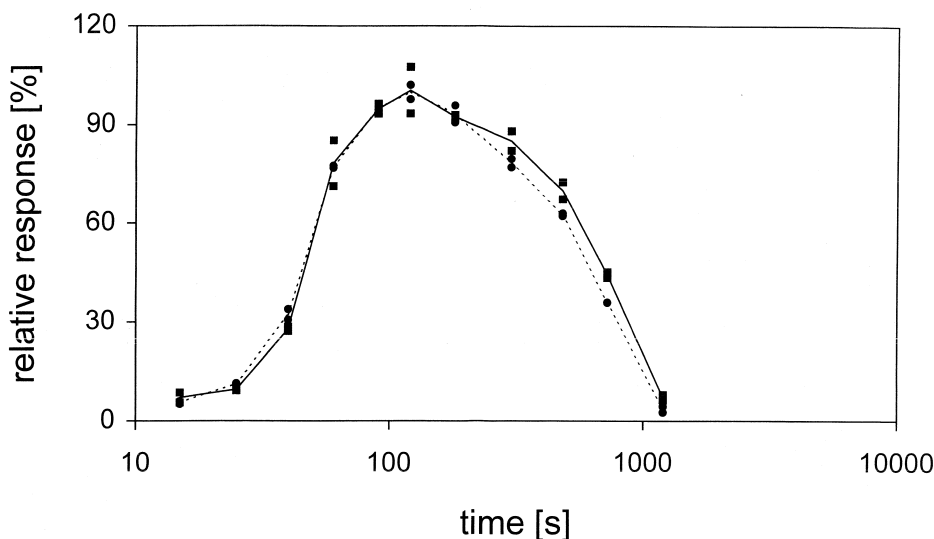
In addition, the stability of MIBG in plasma was

Fig. 2. Fluorescence of the benzoin derivatives of MIBG (■, ———) and BG (●, - - -) as a function of the reaction time. Samples from a standard aqueous solution containing 100 ng/ml MIBG and 50 ng/ml BG were subjected to the standard derivatization conditions using different reaction times.

tested in the validation samples of 5, 40 and 200 ng/ml MIBG; these samples were stored at $-20°C$, ambient temperature, 2°C and 37°C, respectively for different time intervals. In addition, the effects of additional freeze–thaw cycles were investigated.

For the determination of the extraction yield, 50 μl of an MIBG standard diluted in water, made from the same stock as the calibration samples in plasma, in the 20–2000 ng/ml range is added to the SPE eluate resulting from drug-free pooled plasma. The yield was calculated for two calibrations in two separate analytical runs by dividing the slopes of the two different calibration lines, without using the I.S..

The stability of the derivatives of MIBG stored in the autosampler at ambient temperature for 6–7 h was verified. The recovery was calculated identically to the extraction yield, with and without using the I.S.

The selectivity of the assay was also tested by investigating the influence of possible co-medications used by patients treated with MIBG [2]. Stock solutions of 2–5 mg/ml in water were made from dexamethasone, morphine and paracetamol respectively. Blank pooled plasma samples were spiked with one of the individual drugs to obtain approxi-

mately 10-μg/ml samples. For each drug duplicate analysis was performed.

A 73-year and a 67-year old male suffering from metastatic carcinoid tumors were both treated with MIBG at a dose of 40 mg/m^2 that was given as a 4-h i.v. infusion in 100 ml of 9 mg/ml sodium chloride. Blood samples were taken in heparinized tubes before, during and after the infusion and the plasma was separated by centrifugation prior to storage at $-20°C$. If the MIBG level was suspected to be above 200 ng/ml only 200-μl samples were taken for the analysis and diluted with 300 μl drug-free pooled plasma.

3. Results and discussion

3.1. Method development

For the development of a new bioanalytical assay for MIBG in plasma in order to improve the sensitivity compared to existing methods, we combined and slightly modified two known analytical procedures. First, the SPE procedure of Wafelman et al. [5,6] was used to remove proteins and other endog-

enous compounds from the plasma sample; second, the derivatization with benzoin (Fig. 1) was used to obtain a sensitive detectable fluorescent analyte. The SPE procedure of the urine assay of Wafelman et al. [5] showed less fluorescent background signals in the chromatograms compared to their SPE-procedure for plasma [6]. In the 'urine' procedure an extra washing step with 1 ml of 0.1 *M* borate buffer (pH 11.0) was

used. No additional improvements for the washing steps in the SPE have been found, and we, therefore, used the 'urine' SPE procedure [5] for the new assay. The derivatization of guanidine compounds with benzoin has already thoroughly been investigated [7] in the past but not for MIBG. In addition, we optimized the reaction time with the results shown in Fig. 2. The optimum at 2 min was chosen
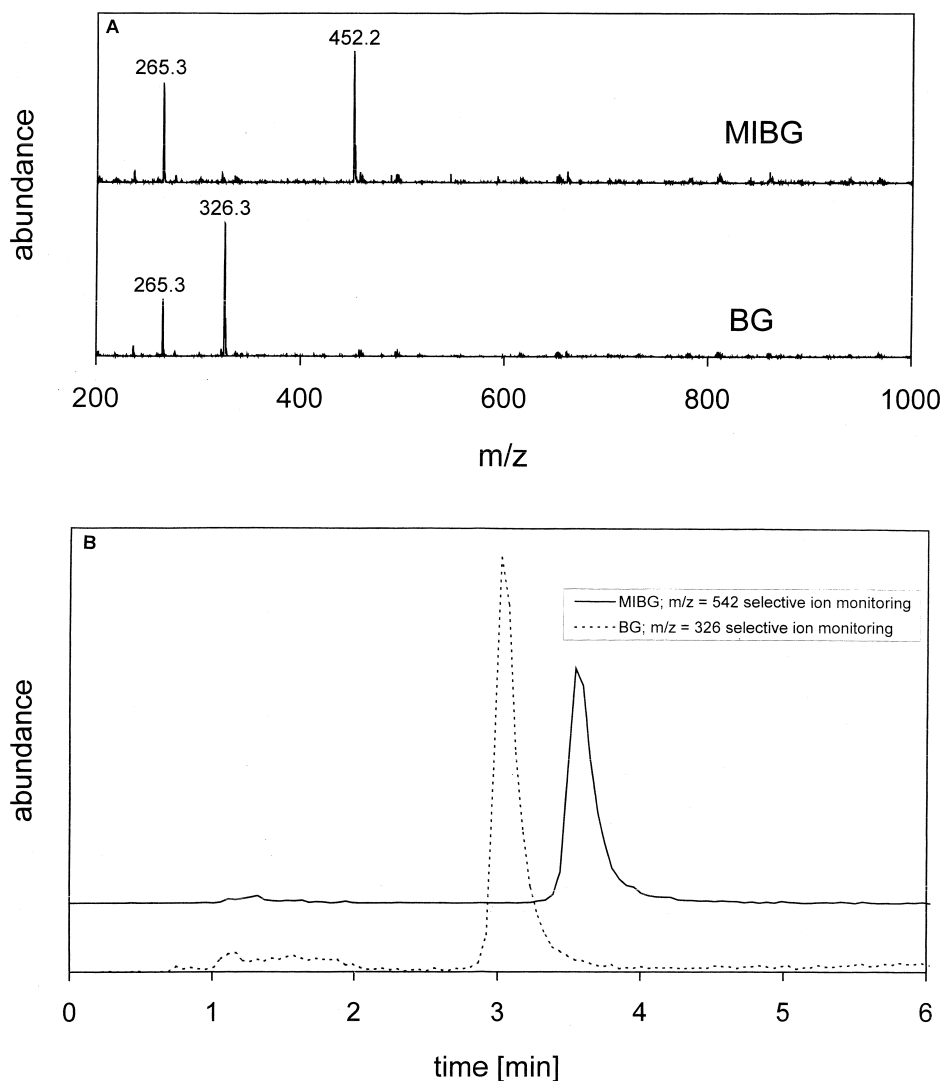

Fig. 3. (+) Electrospray mass spectra (A) and chromatograms (B) at the molecular ion mass (*m/z* 326 and 452 for BG and MIBG, respectively) of benzoin derivatives of both MIBG and BG after in-line LC separation. The MS signal at *m/z* 265 is due to the eluent background.

as the reaction time for further evaluation; apparently, after 2 min the deterioration of the derivative starts to exceed the formation. The deterioration of the MIBG derivative at ambient temperature is suppressed by the β-mercaptoethanol according to an unknown mechanism [7]. The identity of the derivatives of MIBG and BG was confirmed by LC–MS; both spectra (Fig. 3A) clearly show the molecular ions of both derivatives (m/z 326 and 452 for BG and MIBG, respectively) and both single ion chromatograms show only one major peak (Fig. 3B).

3.2. Validation

Examples of chromatograms at different concentrations of MIBG spiked to plasma are shown in Fig. 4. The results of the validation samples, precision and accuracy at each level in three different analytical runs, are listed in Table 1. The lowest level, 2 ng/ml, proves to be the LLQ. All values of the precision and the accuracy far meet the demands for a bioanalytical assay: ≤20% for the LLQ and ≤15% at higher concentration levels [11]. Typically, the assay can also be used without the I.S., as the validation results also meet these demands without I.S. (Table 1); however, at lower concentrations, repeatability and accuracy are better if the I.S. is used. In six individual blank plasma samples, no

interferences in the chromatograms are observed which could influence the quantification of MIBG in the validated concentration range.

The stability of MIBG in the plasma samples in

Table 1
Overall results of the validation samples ($n=15$) if the assay is used with and without[a] I.S.

c (ng/ml)	Repeatability (%)		Reproducibility (%)		Accuracy (%)	
2	6	*10*	6	*3*	99	*108*
5	6	*9*	6	*3*	93	*111*
40	10	*7*	1	*2*	95	*95*
200	6	*6*	3	*1*	98	*100*

[a] Italics indicate the validation parameters for calibration without using the I.S.

Table 2
Recovery of MIBG in plasma ($n=3$) after storage under different conditions

Temperature	Storage time	Recovery (%)		
		5 ng/ml	40 ng/ml	200 ng/ml
37°C	4 h	112±2	98±2	100±12
Ambient	24 h	99±4	100±2	99±3
2°C	7 days	106±10	99±5	103±6
−20°C	4.5 months		98±6	98±9
−20°C[a]	4.5 months	99±1	92±8	100±2

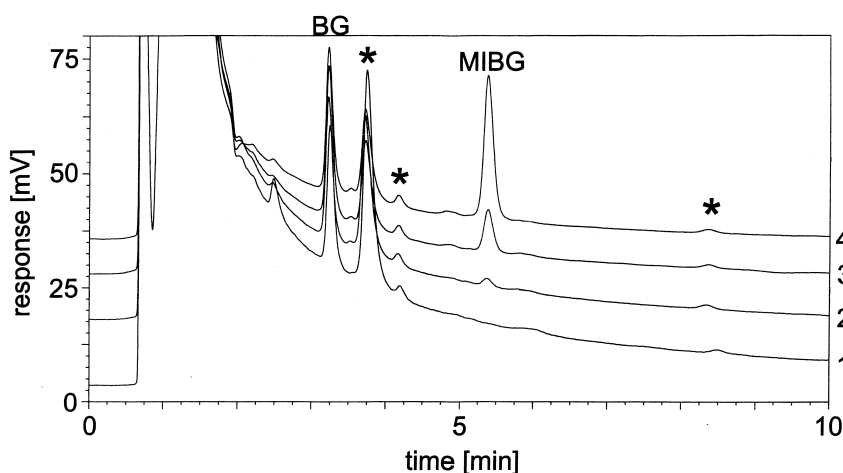[a] The samples were also subjected to four additional freeze–thaw cycles.

Fig. 4. Chromatograms of derivatized MIBG in plasma. (1) Blank; (2) 5 ng/ml spiked to blank plasma; (3) 30 ng/ml spiked to blank plasma; (4) 100 ng/ml spiked to blank plasma. * Peaks due to contaminations in the reagent.
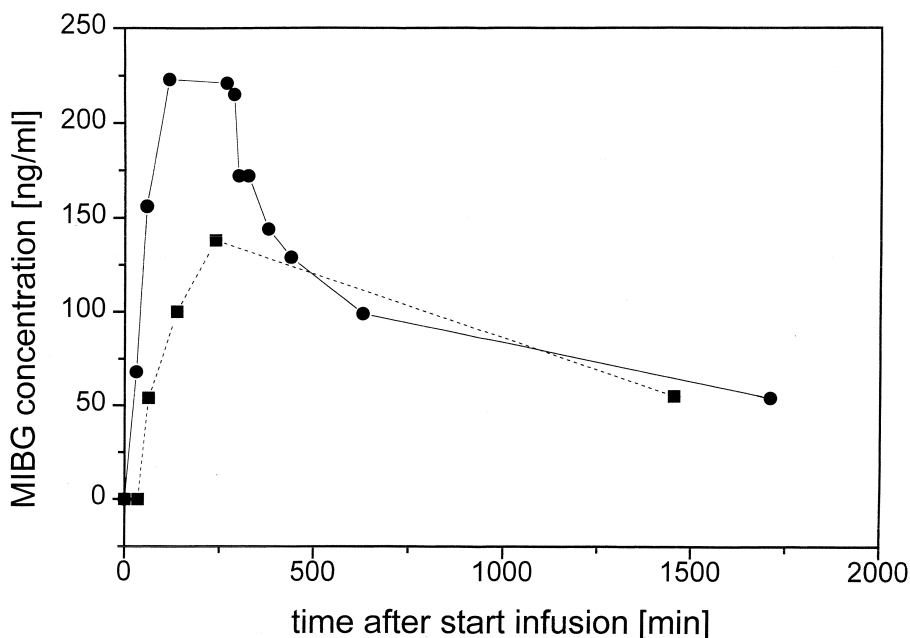
Fig. 5. Pharmacokinetic plots of MIBG. (■) 73-year old male; dose: 72 mg; (●) 67-year old male; dose: 66 mg.

the present therapeutic relevant concentration range has been investigated in addition to previous results published for higher concentrations [4,6]. Again, MIBG showed to be stable under all conditions tested, including maximal four additional freeze–thaw cycles (Table 2).

The yield of the SPE is 95% and is reproducible. The recovery of the MIBG derivative after storage in the autosampler for 6.7 h at ambient temperature is 96%, both absolute and relative to the I.S., which is sufficient for a 10-min chromatographic analysis of 24 samples, the standard run size used during the validation. No co-elution of extra peaks is observed if 10 μg/ml of respectively dexamethasone, morphine or paracetamol is present in the processed plasma sample.

The assay has been employed to obtain both pharmacokinetic plots from patients treated with MIBG (Fig. 5). The observed concentrations in the 50–250 ng/ml-range show that the new method for MIBG in plasma is very suitable and with sufficient sensitivity to be used in clinical pharmacokinetic studies. The present assay is 50–100-fold more sensitive than earlier reported methods.

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