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High-performance liquid chromatographic determination of the conjugate metabolites of moxisylyte in human plasma and urine

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Abstract

Sensitive and specific high-performance liquid chromatographic methods with fluorescence detection are described for the determination of the metabolites of moxisylyte (4-(2-dimethylaminoethoxy)-5-isopropyl-2-methylphenyl acetate) in human plasma and urine. Deacetylmoxisylyte glucuroconjugate (DAM-G) was hydrolysed enzymatically using β-glucuronidase and quantified as the difference between the DAM concentrations determined after and before hydrolysis. The two sulphate derivatives (deacetylmoxisylyte sulphoconjugate, DAM-S and monomethyldeacetylmoxisylyte sulphoconjugate, MDAM-S), were analysed without prior hydrolysis. Their extraction from plasma and urine, as well as that of DAM from plasma, involved the use of C_{18} cartridges adapted on a Benchmate workstation. DAM in urine was quantified after liquid–liquid extraction. The two methods were validated for specificity, linearity, intra- and inter-day precision and accuracy. Precision was generally $\leq$15% and accuracy $\leq$12%. In plasma, the limits of quantification were 2.5 ng/ml for DAM and 2.8 ng/ml for the two sulphates; in urine, they were 40 ng/ml for DAM and 200 ng/ml for the sulphates. These methods were used for pharmacokinetic studies in healthy subjects.

Keywords: Moxisylyte; Deacetylmoxisylyte sulphoconjugate; Monomethyldeacetylmoxisylyte sulphoconjugate

1. Introduction

Moxisylyte 4-(2-dimethylaminoethoxy)-5-isopropyl-2-methylphenyl acetate), is a competitive α-blocking drug, more specific for α_1 than for α_2 receptors [1,2]. It has been used as a vasodilator in the treatment of various vascular disorders [3,4] and in dysuria related to benign prostatic hyperplasia [5]. More recently, using injections in the corpus cavernosum, it has been shown to be effective in the treatment of impotence of either neurological or psychological origin [6,7].

Moxisylyte is a prodrug rapidly transformed in human blood into an active metabolite, deacetylmoxisylyte or DAM [8]. Elimination of DAM occurs by sulphoconjugation (DAM-S), glucuroconjugation (DAM-G) and N-demethylation (MDAM). As DAM, MDAM is either sulpho (MDAM-S) or glucuroconjugated (MDAM-G) [9,10]. In human plasma, four metabolites are found, DAM, DAM-G, DAM-S and MDAM-S. The two sulphoderivatives have been shown to have α-blocking activity in animal models which is, however,

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much lower than that of DAM [11]. No activity has been reported for the glucuronides.

The reported methods for assay of DAM-G, DAM-S and MDAM-S in human plasma were either non specific, involving acid hydrolysis and determination of DAM and MDAM by HPLC [12], or specific, with the separate analysis of the three metabolites [13]. The non-specific method has been used for pharmacokinetic assessment after intravenous and intracavernous injections of moxisylyte [14,15]. However, due to poor sensitivity (limit of quantification of 25 ng/ml) DAM and MDAM were detected only up to four or six hours following drug administration. The specific methods involved specific hydrolysis of DAM-G followed by measurement of DAM and direct determination of the two sulphoconjugates. The limit of quantification was 20 ng/ml for the three conjugates.

With the objective of assessing the pharmacokinetics of the metabolites of moxisylyte after intracavernous injection, we developed new and sensitive HPLC methods with fluorescence detection to quantify DAM-G, DAM-S and MDAM-S in plasma and urine.

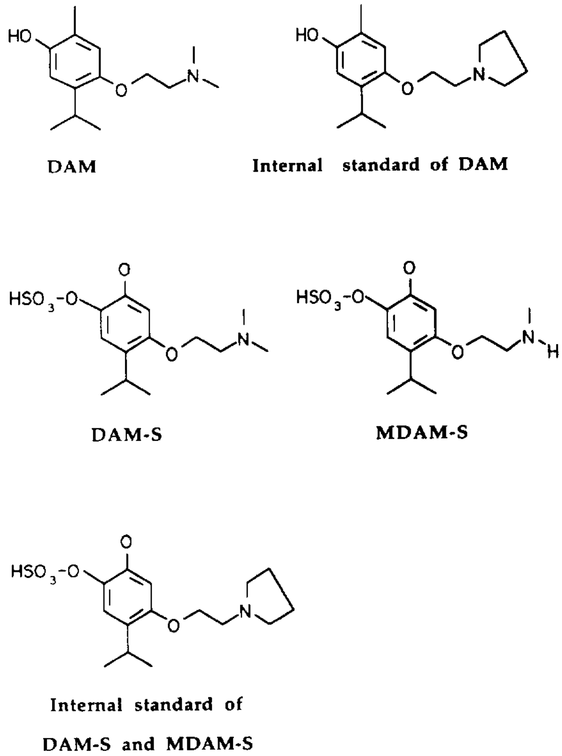

Fig. 1. Chemical structures of DAM, DAM-S, MDAM-S and the internal standards.

2. Experimental

2.1. Chemicals and reagents

The chemical structures of the products under study are given in Fig. 1. DAM-G was not available as reference standard. DAM 5-(2-dimethylaminoethoxy)-2-hydroxy *p*-cymene, the sulphoconjugates of DAM (DAM-S or 5-(2-dimethylaminoethoxy) cavarcryl sulphate) and of MDAM (MDAM-S or 5-(2-methylaminoethoxy) cavarcryl sulphate) and the internal standards, 5-(2-pyrolidinylethoxy)-2-hydroxy *p*-cymene for DAM assay and 5-(2-pyrolidinylethoxy) cavarcryl sulphate for DAM-S and MDAM-S assay were synthesized at Laboratoires Debat (Garches, France).

Sodium dihydrogen phosphate and orthophosphoric acid were obtained from Fluka (Buchs, Germany). Cyclohexane, diethylether, dipotassium hydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, potassium dihydrogen phosphate, trimethylammonium hydroxide, triethylamine and methanol were from Merck (Darmstadt, Germany). β-glucuronidase (*E. coli*) was from Boehringer-Mannheim (Mannheim, Germany). Sodium heptane sulphonate was from Sigma (St. Louis, MO, USA), and acetonitrile from Baker (Deventer, Netherlands). Pooled drug-free plasma sample from healthy volunteers was provided by Le Centre de Transfusion Sanguine (Paris, France), stored frozen at about −25°C and thawed at room temperature prior use.

Pure water was obtained by the Milli-Q deionized water purification system (Millipore, Bedford, MA, USA).

2.2. Standard solutions

For determination of DAM-G, the standard solutions of DAM and of its internal standard were prepared in deionized water. The stock solution of DAM was at 700 μg/ml and the working solutions ranged from 0.025 to 3.5 μg/ml for plasma assay, and from 0.25 to 16 μg/ml for urine assay. Stock solutions of the internal standard were at 800 and 175 μg/ml for plasma and urine assay, respectively, and the working solutions at 0.8 and 3.5 μg/ml, respectively.

Stock solutions of DAM-S and MDAM-S (400 μg/ml and 700 μg/ml for plasma and urine, respectively) were prepared in an acetonitrile–water (1:1, v/v) mixture for solubility reasons, and the working solutions (ranging from 0.04 to 0.6 μg/ml for plasma assay and from 1.0 to 70 μg/ml for urine assay) by dilution with deionized water. Stock solutions of the corresponding internal standard were at 250 and 500 μg/ml for plasma and urine assay, respectively; the working solutions were at 0.50 and 2.0 μg/ml, respectively.

2.3. Equipment

The HPLC system consisted of an ISS-100 sample injector from Perkin Elmer (Norwalk, CT, USA), a 980 spectrofluorimeter from Kratos (Ramsey, NJ, USA) for quantification of DAM and an LC 240 spectrofluorimeter from Perkin Elmer for quantification of the sulphoconjugates, a 300 C pump and a 250 B gradient former from Gynkotek (Germering, Germany), a CR5 A integrator from Shimadzu (Kyoto, Japan). The HPLC columns were from Alltech (Deerfield, IL, USA).

Liquid–solid extraction was performed on a Benchmate workstation (Zymark, Hopkinton, MA, USA).

2.4. Sample preparation and chromatographic conditions

The methods are described in Tables 1–3. Table 4 shows the variations in proportions of solvents A and B for DAM-S and MDAM-S analysis. As DAM-G standard was not available, it was determined in unknown samples after hydrolysis and the calibration standards were prepared using DAM. The completeness of hydrolysis was previously demonstrated by HPLC analysis with radioactive detection of samples (plasma and urine) from rats treated with [^{14}C]moxisylyte.

The mobile phases were deaerated ultrasonically prior to use. Chromatography was performed at room temperature.

2.5. Serum and urine calibration standards and quality control samples

Calibration standards for plasma and urine assay were prepared by adding appropriate volumes of standard solutions to drug-free plasma and urine. The

Table 1
Sample preparation and chromatographic conditions for DAM-G in plasma

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Hydrolysis

0.5 ml of plasma.
2.5 ml of 0.05 *M* K_2HPO_4–0.05 *M* Na_2HPO_4, buffer (pH 6.5).
0.01 U of β-glucuronidase.
2.5 h at 37°C.

Solid-phase extraction

100-mg/LD C_{18} cartridge (Baker, Deventer, Netherlands) previously washed with methanol (1.5 ml), acetonitrile (1.5 ml) and 0.12 *M* phosphate buffer (pH 7.5), (1.5 ml).
Loading of hydrolysed sample added with 0.050 ml (0.040 μg) of internal standard.
Washing with 4 ml of water and 1 ml of acetonitrile.
Elution of DAM in 2 ml of methanol.
Evaporation and dissolution in 0.2 ml of mobile phase, injection of 0.075 ml onto the column.

HPLC analysis

Column: 250×4.6 mm (I.D.) Spherisorb ODS1 stainless-steel (5 μm).
Mobile phase: 0.040 *M* potassium dihydrogen phosphate–heptane sulfonic acid solution (1 g in 100 ml of water)–acetonitrile (540:60:400, v/v/v). Aqueous buffer portion was adjusted to pH 2.3 with 85% orthophosphoric acid).
Flow-rate: 1.1 ml/min.
Detection: excitation at 218 nm, emission at 300 nm.

Table 2
Sample preparation and chromatographic conditions for DAM-G in urine

Hydrolysis

0.2 ml of urine+2 ml of 0.05 *M* K_2HPO_4–0.05 *M* Na_2HPO_4 buffer (pH 6.5)+0.01 U of β-glucuronidase.
2.5 h at 37°C.

Liquid–Liquid extraction

To the above mixture, addition of: 0.1 ml (0.35 μg) of internal standard, 0.04 ml of 2 *M* NaOH, 7 ml of cyclohexane–diethylether (1:1, v/v) mixture.
20-min rotative shaking, 10-min centrifugation at 2500 *g*.
Back-extraction of DAM from the organic layer in 0.25 ml of 0.4% triethylamine (pH 3.0 with 85% orthophosphoric acid), injection of 0.08 ml onto the column.

HPLC analysis

Column: 150×4.6 mm (I.D.) Hypersil BDQ stainless-steel (5 μm).
Mobile phase: 0.4% triethylamine (adjusted to pH 6.9 with 10% orthophosphoric acid)–acetonitrile (80:40, v/v).
Flow-rate: 1.1 ml/min.
Detection: excitation at 218 nm, emission at 300 nm

Table 3
Sample preparation and chromatographic conditions for DAM-S and MDAM-S in plasma and urine

Liquid–solid extraction

0.5 ml of plasma+0.5 ml of deionized water+0.1 ml (0.050 μg) of internal standard.
0.05 ml of urine+0.95 ml of deionized water+0.12 ml (0.25 μg) of internal standard.
Loading onto a 100-mg/LD C_{18} cartridge previously washed with 2 ml of acetonitrile and 4 ml of water.
Washing of the cartridge with 2.5 ml of water and drying with nitrogen for 2 min.
Elution of DAM-S and MDAM-S in 1.5 ml of acetonitrile.
Evaporation.
Dissolution of the dry residue in 0.15 ml of solvent A, injection of 0.075 ml onto the column.

HPLC analysis

Column: 250×4.6 mm (I.D.) Spherisorb (5 μm).
Mobile phase: mixture of solvent A (0.018 *M* potassium dihydrogen phosphate–tetramethylammonium hydroxide–acetonitrile (900:7.7:100, v/v/v) and solvent B (0.024 *M* potassium dihydrogen phosphate–tetramethylammonium hydroxide–acetonitrile (700:7.7:300, v/v/v). Aqueous buffer portion adjusted to pH 6.0 with 10% orthophosphoric acid. Variations in proportions of A and B are given in Table 4.
Flow-rate: 1.1 ml/min for plasma and 1.3 ml/min for urine.
Detection: excitation at 280 nm, emission at 315 nm.

Table 4
Variations in proportions of solvent A and solvent B for DAM-S and MDAM-S analysis

Plasma assay			Urine assay		
Time	% solvent A	% solvent B	Time	% solvent A	% solvent B
T_0	80	20	T_0	80	20
T=2 min	80	20	T=2 min	80	20
T=7 min	55	45	T=12 min	65	35
T=12 min	43	57	T=13 min	50	50
T=16.5 min	80	20	T=15 min	80	20
T=20.5 min		End of the run	T=20.5 min		End of the run

volume added was always smaller than or equal to 2% of the total volume of the sample, so that the integrity of the sample was maintained.

For DAM-G, calibration curves ranged from 2.5 to 320 ng/ml for plasma assay and from 0.1 to 7.0 μg/ml for urine assay. For the sulphoconjugates, they ranged from 3 to 120 ng/ml for plasma and from 0.2 to 3.0 μg/ml for urine assay. Quality controls were independently prepared and aliquots stored frozen at −20°C until the time of analysis.

2.6. Calibration and calculation procedure

A linear regression analysis between the peak-height ratios of DAM, DAM-S and MDAM-S and the internal standard was used to construct the calibration curves. The weighting factor used was $1/x^2$. The linearity of the response was confirmed by comparison of the intercept to zero and by the coefficient of correlation. The unknown concentrations of DAM, DAM-S and MDAM-S in plasma samples were calculated by interpolation using these daily calibration curves. DAM-G was determined as the difference between DAM concentrations measured after and before hydrolysis. In plasma, unconjugated DAM was quantified by a sensitive HPLC method (LOQ=0.1 ng/ml) with electrochemical detection [16]; in urine, it was quantified by fluorescence detection using the same method (sample processing and chromatographic conditions) as that described above for DAM-G. Furthermore, specificity of hydrolysis of DAM-G was assessed by incubating DAM-S and MDAM-S with β-glucuronidase as described above. Since neither DAM nor MDAM were found, it can be said that the sulphoconjugates do not interfere with the analysis of DAM-G.

3. Results

3.1. HPLC profiles

Adequate chromatographic separation was obtained using the different systems described in Section 2. On chromatograms obtained from blank plasma and urine samples, no peak interfered at the retention times of the metabolites and of the internal standards. Representative chromatograms are presented in Fig. 2 Fig. 3. In a one-day batch of analysis, the retention times (mean±S.D., $n=25$) of DAM and its internal standard were 6.9±0.04 and 9.8±0.08 min and 8.4±0.13 and 10.6±0.08 min for plasma and urine assay, respectively; those of MDAM-S, DAM-S and their internal standard were 9.6±0.23, 12.2±0.23 and 15.9±0.23 min for plasma assay and 8.2±0.14, 11.1±0.22 and 16.8±0.15 min for urine assay.

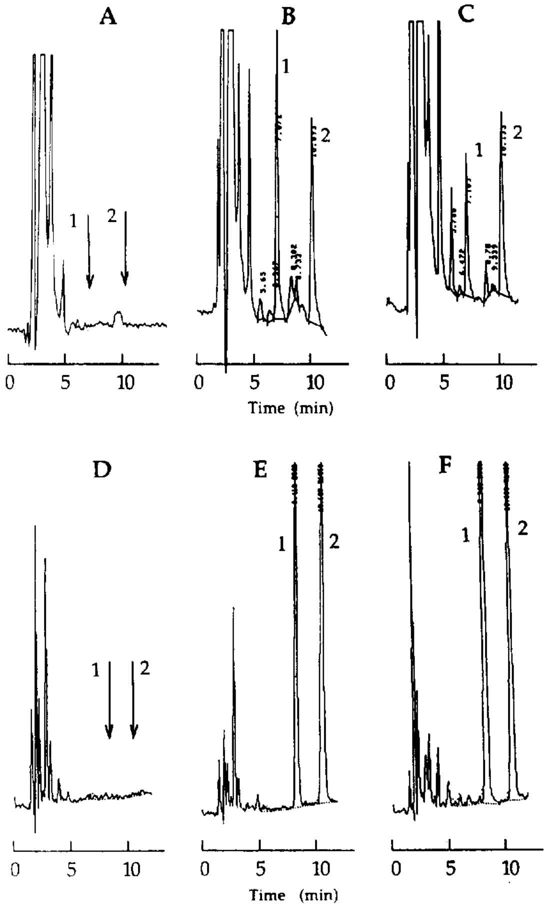

Fig. 2. Chromatograms of blank plasma (A) and urine (D), plasma (B) and urine (E) spiked with DAM (1) at 60 ng/ml and 1 μg/ml, respectively and the internal standard (2), plasma sample (C) obtained at 1 h post-dose and urine (F) collected between 0 and 4 h from a subject who received a single 10-mg dose of moxisylyte-HCl by intracavernous injection.

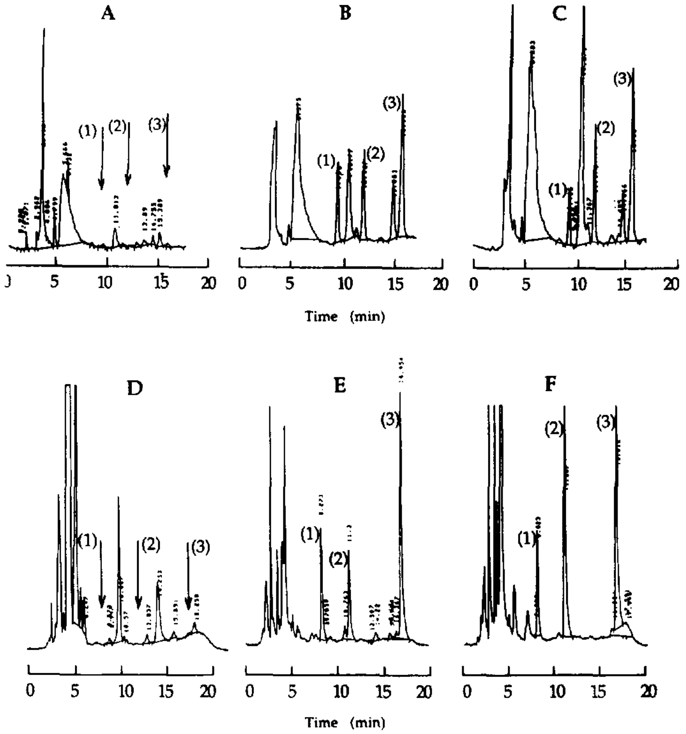

Fig. 3. Chromatograms of blank plasma (A) and urine (D), plasma (B) and urine (E) spiked with DAM-S (1) and MDAM-S (2) at 40 ng/ml and 1 μg/ml, respectively and the internal standard (3), plasma sample (C) obtained at 1.50 h post-dose and urine (F) collected between 4 and 8 h from a subject who received a single 10-mg dose of moxisylyte-HCl by intracavernous injection.

3.2. Calibration curves and limit of quantification

The peak-ratio of DAM, DAM-S and MDAM-S to the internal standard varied linearly with the concentrations over the range studied. The intercept was not significantly different from zero. The calibration parameters calculated from 18 batches of analysis in plasma and 9 batches of analysis in urine are presented in Table 5. In practice, series of quality control samples were analysed along with the study samples and the daily calibration curves were used for calibration and calculation purposes. Lower limit of quantification was assessed by analysing spiked drug-free plasma and urine samples. In plasma, it was 2.5 ng/ml for DAM (C.V.=8%) and 2.8 ng/ml for the sulphoconjugates (C.V.=7%). In urine, 50 ng/ml for DAM (C.V.=8%) and 200 ng/ml for the sulphoconjugates (C.V.=5%). At these levels, the signal-to-noise ratio was higher than 3.

3.3. Recovery

For analysis of DAM-G, recovery of DAM from human plasma spiked at 17, 84 and 320 ng/ml averaged 76, 77 and 90%, respectively. The corresponding coefficients of variation were 10.4, 3.6 and 2.5%. The mean recovery of the internal standard was 86% at 86 ng/ml with a C.V. of 2%.

With regard to the sulphates, recoveries of DAM-S and MDAM-S from human plasma spiked at 11, 40 and 85 ng/ml ranged from 75 to 81% with a C.V. below 7%. That of the internal was 89% at 50 ng/ml with a C.V. of 4%.

3.4. Accuracy and precision

Accuracy and precision were assessed by analysing independently prepared quality control samples. For DAM assay in plasma, intra-day C.V. and relative

Table 5
Mean values (±S.D.) of the calibration parameters of DAM, DAM-S and MDAM-S

	Coefficient of correlation (± S.D.)	Slope (± S.D.)	Intercept (± S.D.)
Plasma (n=18)			
DAM	0.9972±0.0021 C.V.=0.21%	0.023±0.0018 C.V.=7.7%	0.0069±0.0279
DAM-S	0.9983±0.0014 C.V.=0.14%	0.014±0.0014 C.V.=10.3%	0.0092±0.0152
MDAM-S	0.9978±0.0019 C.V.=0.19%	0.013±0.0014 C.V.=10.7%	0.0009±0.0093
Urine (n=9)			
DAM	0.9991±0.0010 C.V.=0.1%	0.87±0.069 C.V.=7.9%	0.0041±0.0102
DAM-S	0.9995±0.0005 C.V.=0.05%	0.21±0.012 C.V.=5.7%	0.0022±0.0061
MDAM-S	0.9996±0.0004 C.V.=0.04%	0.25±0.011 C.V.=4.4%	0.0012±0.0094

Table 6
Statistical evaluation of inter-day precision and accuracy from data generated in the course of analysis of study samples

Compound	Assay in plasma[a]			Assay in urine[b]		
	Concentration added (ng/ml)	Precision (%)	Accuracy (%)	Concentration added (μg/ml)	Precision (%)	Accuracy (%)
DAM	10	11.3	2.5	0.25	9.1	0.4
	60	15.4	7.4	1	5.0	0.7
	150	7.5	11.9	4	5.3	0.3
DAM-S	10	9.8	3.7	0.5	14.7	1.6
	40	8.9	2.4	1.2	4.9	0.4
	90	7.6	7.6	4.5	4.0	0.6
MDAM-S	10	9.7	6.2	0.5	12.7	2.4
	40	9.2	0.1	1.2	3.3	2.6
	90	9.2	3.3	4.5	4.6	7.8

[a] 18 batches of analysis.
[b] 9 batches of analysis.

error, at 5, 20 and 110 ng/ml in DAM ranged from 2 to 7% and from 2 to 12%, respectively. For DAM-S and MDAM-S in plasma at 5, 11, 40 and 85 ng/ml, intra-day C.V. ranged from 1 to 8% and relative error from 5 to 11%. Statistical evaluation of inter-day precision and accuracy from data generated in the course of analysis of study samples are given in Table 6.

4. Discussion

In this paper we described HPLC methods to quantify the conjugate metabolites of moxisylyte in plasma and urine. As the method described for DAM-G in plasma was not specific enough for urine, we chose a 2-step extraction procedure and different chromatographic conditions. Performance of the

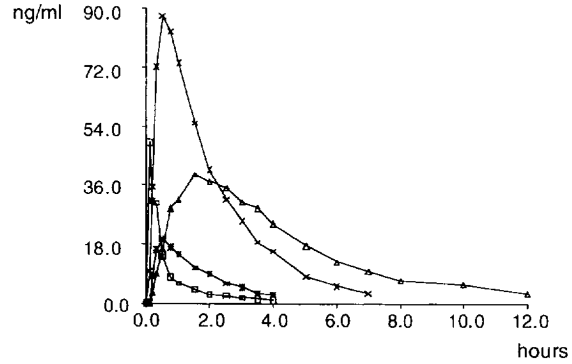

Fig. 4. Plasma concentration–time curves of DAM (□), DAM-G (*), DAM-S (×), MDAM-S (△) after intracavernous injection of 10 mg of moxisylyte-HCl to one healthy volunteer.

described methods was assessed both on the basis of the characteristics of the calibration lines and from the results of the quality control samples. DAM-G being not available, its concentration has been determined as a difference between DAM concentrations measured after and before hydrolysis; the quality control samples and the calibration standards were prepared using unconjugated DAM. However, precision for DAM-G assay in plasma can be calculated as a sum of variance of individual measurements. In the range 0.5 to 20 ng/ml, the precision of the electrochemical method for unconjugated DAM was lower than 3% [16]. Therefore this low variability accounted for a minor part of that of DAM-G, which can be considered close to that of DAM reported in the present paper. Indeed, the precision for DAM-G assay in plasma ranged from 3 to 15%. In urine, the concentrations of DAM-G are substantially larger compared to the concentrations of unconjugated DAM (recoveries of 24 and 0.5%, respectively); thus, the precision for DAM-G assay in urine ranged from 5 to 9% (values reported in the present study for DAM).

The described methods are rapid, sensitive, selective and reproducible and allow accurate determination of pharmacokinetic parameters of the three conjugates in human samples after single doses of moxisylyte by intravenous or intracavernous injection [17,18]. The plasma concentration–time profiles from a volunteer injected with a dose of 10 mg in the corpus cavernosum are illustrated in Fig. 4.

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