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Determination of catecholamines and methoxycatecholamines excretion patterns in pig and rat urine by ion-exchange liquid chromatography with electrochemical detection

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Abstract

A simplified liquid chromatographic method for the simultaneous determination of free or total catecholamines and methoxycatecholamines in rat and pig urine is presented. The extraction procedure involves a two-stage batch extraction, with successive adsorption on cation- (catecholamine elution) and anion-exchange columns (methoxycatecholamine elution). The column eluates are successively monitored by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The proportion of conjugates for each compound was assessed in both species, through the comparison of concentrations with or without hydrolysis pretreatment. Conjugates were found to account for a small fraction of total catecholamines and methoxycatecholamines excretion (0 to 35%). The free fraction of each compound was highly correlated with the total amount. Furthermore, the hydrolysis procedure leads to partial degradation of metanephrine (25%) and to the production of compounds giving artefactual peaks. Thus, we do not recommend hydrolysis of rat and pig urines for catecholamine and methoxycatecholamine determination. © 1997 Elsevier Science B.V.

Keywords: Catecholamines; Methoxycatecholamines; Norepinephrine; Epinephrine; Dopamine; Normetanephrine; Metanephrine; 3-O-Methyldopa

1. Introduction

The measurement of urinary catecholamines and their O-methoxy related metabolites (methoxycatecholamines) is widely used for human clinical investigation of pheochromocytoma [1–3], depression [4–7] and response to stress challenges [8,9]. Surprisingly, catecholamines (norepinephrine or NE, epinephrine or E, dopamine or DA) are almost always assessed without prior hydrolysis (i.e., free or unconjugated fraction), whereas methoxycatecholamines (normetanephrine or NMN, metanephrine or MN and 3-O-methyldopamine or MD) are routinely measured after acid hydrolysis [1–3], although it is well known that a large proportion (70 to 80%) of both categories of compounds are excreted in a conjugated form in human urine [10–12]. Reasons for such a discrepancy remain unclear. Free catecholamines have been said to be less influenced by such factors as diet composition, age, sex, biological cycles than conjugates [12]. However, it does not

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explain why methoxycatecholamines are assessed as the total amount, since conjugation processes for these compounds are the same as for catecholamines [13]. Thus, in most studies, the simultaneous determination of catecholamines and methoxycatecholamines is usually obtained through two distinct assays.

As opposed to the numerous studies in humans, urinary catecholamines excretion in other species has received much less attention. Urinary free NE and E have been investigated following stress procedures in dogs [14], monkeys [15], horses [16], pigs [17]. A few reports on rat free NE, E and DA urinary excretion are also available [18,19]. To our knowledge, no investigation of urinary methoxycatecholamines excretion has been done in animal species up to now. Plasma methoxycatecholamines have been shown to provide additional information about sympatho-adrenal activity to that provided by catecholamines. For instance, their measurement allows the evaluation of catecholamines metabolism in extraneuronal tissues and may strengthen the conclusions derived from measurements of parent amines as an index of sympathetic outflow [20]. Therefore, we can expect that the assessment of their urinary excretion might improve the evaluation of sympatho-adrenal activity in stress and genetic studies.

Conjugation processes vary considerably according to the species, qualitatively (balance between sulfo- and glucuroconjugation) and quantitatively [21–23]. Since the determination of total (free plus conjugated forms) urinary catecholamines and their metabolites might provide a more integrative estimation of adrenosympathetic activity [12], it is important to characterize the excretion pattern of these compounds in the species under study.

This paper describes a method which allows the determination of both urinary catecholamines (NE, E, DA) and methoxycatecholamines (NMN, MN, MD) excretion from a single urine sample. This method involves a two stage batch ion-exchange extraction, followed by reversed-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection. It was firstly used to assess the excretion profile of the free fraction of these compounds in rat and pig urine. In a second step, the relationships between the free and total fractions were investigated.

2. Experimental

2.1. Chemicals and reagents

L-Norepinephrine (arterenol bitartrate salt or NE), epinephrine (epinephrine bitartrate salt or E), dopamine (3,4-dihydroxyphenethylamine hydrochloride or DA), DL-normetanephrine (3-methoxybenzenemethanol hydrochoride or NMN), DL-metanephrine (DL-*m*-O-methylepinephrine hydrochloride or MN), 3-O-methoxydopamine (3-methoxy-4-hydroxyphenethylamine hydrochloride or MD), DHBA (3-4-dihydroxybenzylamine hydrobromide), HMBA (4-hydroxy-3-methoxybenzylamine hydrochloride) and 1-octanesulfonic acid sodium were obtained from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

Methanol for HPLC was obtained from BDH (Poole, UK). Citric acid monohydrate, boric acid crystals and sodium hydroxide pellets were purchased from Merck–Clévenot (Nogent-sur-Marne, France). Ethylenediaminetetraacetic acid (EDTA), sodium acetate anhydrous, ammonia solution 20% and hydrochloric acid were obtained from Prolabo (Gradignan, France). HPLC grade water was produced by a Milli-Q Plus system (Millipore, Saint-Quentin-Yvelines, France).

2.2. Chromatography

The mobile phase was prepared as follows: to 300 ml methanol were added 1.5 ml 1-octanesulfonic acid (200 mg/ml), 100 ml 1 *M* sodium acetate and about 1 l HPLC grade water. The apparent pH was then adjusted to 3.8 using citric acid (about 100 ml) and the final volume was adjusted to 2 l with water. The mobile phase was then degassed by vacuum filtering through a 0.45 μm MF-Millipore filter.

The flow-rate was set at 0.6 ml/min for catecholamine detection and to 1.1 ml/min for methoxycatecholamine detection, using a pump from Shimadzu (Model LC-10AT, Kyoto, Japan). Samples were injected by a sampling autoinjector (Model 232, Gilson, Villiers-Le-Bel, France). The analytical column (5 μm Kromasil C_8, 150×4.6 mm I.D., Touzart et Matignon, Courtaboeuf, France) was connected to an electrochemical detector from Bioanalytical Systems (West Lafayette IN, USA). The working electrode (glassy carbon) and the

reference electrode (silver/silver chloride) were also obtained from Bioanalytical Systems. The cell potential was set to +0.65 V for catecholamine detection and to +0.8 V for methoxycatecholamine detection. Detector output was recorded by a data processor (Chromatopac C-R5A, Shimadzu).

2.3. Sample collection

Swine urine was collected from fifteen lactating multiparous Large White sows housed in stalls. Spontaneously voided urine was collected in a flask. It was then acidified using 6 *M* HCl (1% of urine volume) and frozen at −80°C.

Rat urine was collected during four consecutive days from four male Brown Norway (BN) and four male Fischer 344 (F344) rats in metabolic cages. A flask containing 0.2 ml of 6 *M* HCl (i.e., about 1 to 2% of 24 h urine volume) was used to collect the urine over a period of 24 h. Urine samples were then frozen at −80°C.

2.4. Urine analysis

Creatinine levels in swine urine were determined using a colorimetric quantitative reaction (Procedure 500, Sigma diagnostics). This method is based on the reduction of the color derived from the reaction between creatinine and alkaline picrate (Jaffe's reaction) when the mixture is acidified. Thus, the difference in color intensity measured at 500 nm before and after acidification of the mixture is proportional to creatinine concentration.

2.4.1. Sample preparation

Urine was centrifuged for 30 min at 4000 *g*. The urine volume for each assay was adjusted according to its dilution, i.e., according to creatinine concentration for swine urine and to diuresis for rat urine. Thus, between 6 and 10 ml of swine urine was used per assay whereas for rat urine, the volume was set between 1 and 2.5 ml.

In 30 ml beakers, 200 ng of the two internal standards DHBA and HMBA (for catecholamines and methoxycatecholamines, respectively) and 15 ml EDTA (1 g/l) were added to the urine. Two more beakers (''standards'') containing 5 ml of water received 100 μl of the two standard pools containing NE, E, DA and DHBA (catecholamine standards) and MN, NMN, MD and HMBA (methoxycatecholamine standards), each standard concentration being 2 μg/ml in the pools. The pH of each sample and ''standards'' was adjusted between 6.45 and 6.55 using HCl and NaOH.

2.4.2. Extraction procedure

Urine samples were laid on disposable cation-exchange resin columns (Bio-Rad, France). After three washings with water (10, 10 and 5 ml), catecholamines were eluted with 8 ml boric acid (10 g/l) into 15 ml tubes. Methoxycatecholamines were then eluted directly from cationic into anionic columns (Bio-Rad, France) with 8 ml of 2 *M* NH_4OH. After two more washings (10 ml water), methoxycatecholamines were eluted using 5 ml of 0.4 *M* ammonium acetate (pH 6) into 15 ml tubes previously filled with 0.4 ml of 1 *M* acetic acid.

Boric acid eluates (containing catecholamines) were diluted with an equal volume of mobile phase before injection into the HPLC system. Ammonium acetate eluates (containing methoxycatecholamines) were injected directly. In both cases, 60 μl were injected.

The procedure adopted for total catecholamine and methoxycatecholamine determination was similar to the procedure described above, except that urines were first submitted to hydrolysis. Urines (with internal standards) and ''standards'' were acidified to a pH of between 0.5 and 1 and then placed in a boiling bath (100°C) for 20 min.

2.4.3. Quantitation

For each standard and sample assay, the ratio $R = A_X/A_{I.S.}$ was calculated, were A_X is the area of the peak of E, NE, DA, NMN, MN, MD and $A_{I.S.}$ is the area of the peak of the respective internal standard. Concentrations of each compound $[X]$ in urine were then calculated from the following Eq. (1):

$$[X] = Q_{I.S.} \cdot \frac{(R_{assay})}{(R_{standard})} \cdot \frac{1}{V} \quad (1)$$

where $[X]$ is concentration in the sample (ng/ml), $Q_{I.S.}$ is the quantity of internal standard added to each sample (200 ng), $R_{standard}$ is the ratio $A_X/A_{I.S.}$

in the ''standard'' assays (mean of the two assays), R_{assay} is the ratio $A_X/A_{I.S.}$ in the sample assay and V is the volume of urine used in the assay (ml).

This calculation method allows to take into account differences in the recovery of the different compounds.

3. Results and discussion

Typical chromatograms of standards, pig and rat urines are shown in Fig. 1 (catecholamines) and Fig. 2 (methoxycatecholamines).

3.1. Recovery

As expected, the recovery of methoxycatecholamines was lower than that of catecholamines (about 58% compared to 78%), due to their passage through a double system of extraction columns (Table 1).

3.2. Precision of the method

The intra-assay and inter-assay coefficients of variation (%) for catecholamines, determined from 14 replicate injections of the same urine sample

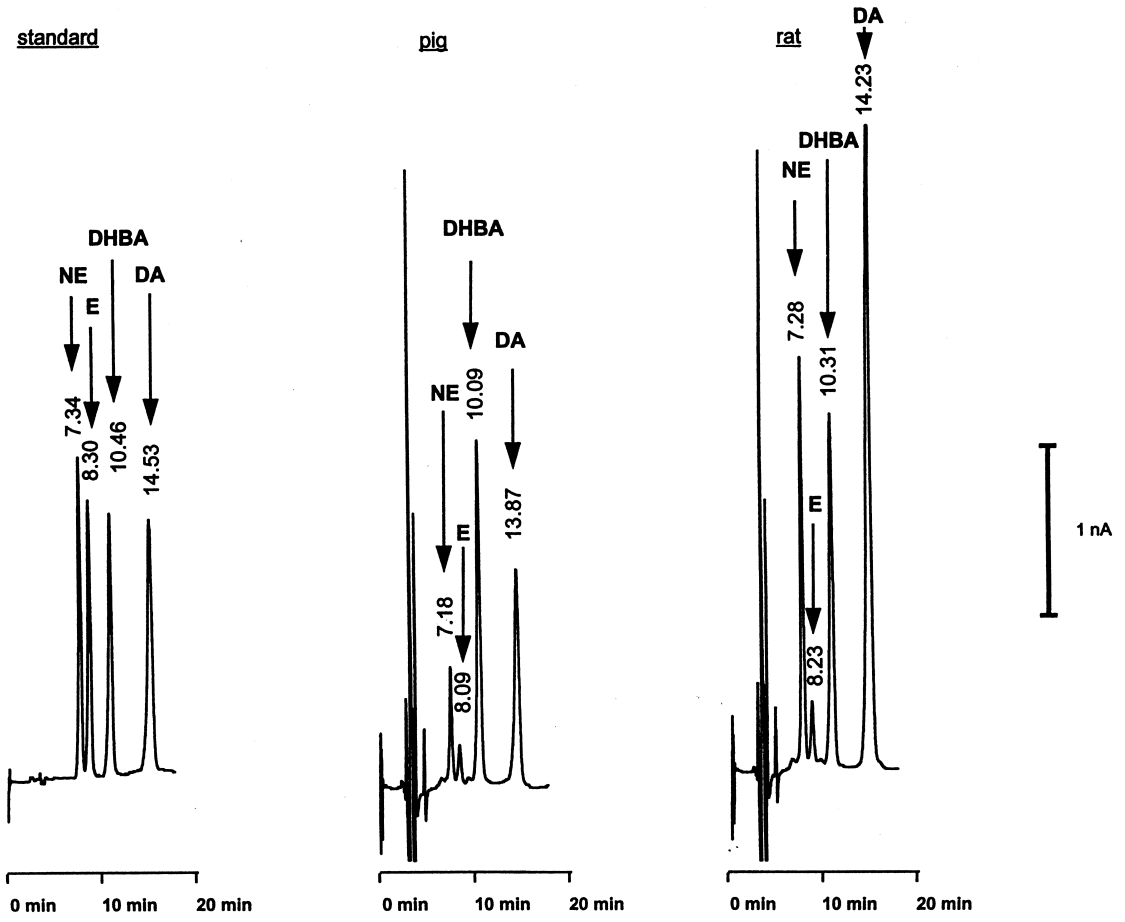

Fig. 1. Chromatogram of a standard pool (left) containing 10 ng/ml of norepinephrine (NE), epinephrine (E), internal standard (DHBA) and dopamine (DA) with their respective retention times (top of the peaks). Typical chromatogram of a pig urine (center) containing 15.4 ng/ml of NE, 3.6 ng/ml of E and 37.1 ng/ml of DA (determined from 3.5 ml urine sample with 200 ng DHBA). Typical chromatogram of a rat urine (right) containing 91.5 ng/ml of NE, 12.8 ng/ml of E and 216.0 ng/ml of DA (determined from 2 ml urine sample with 200 ng DHBA).

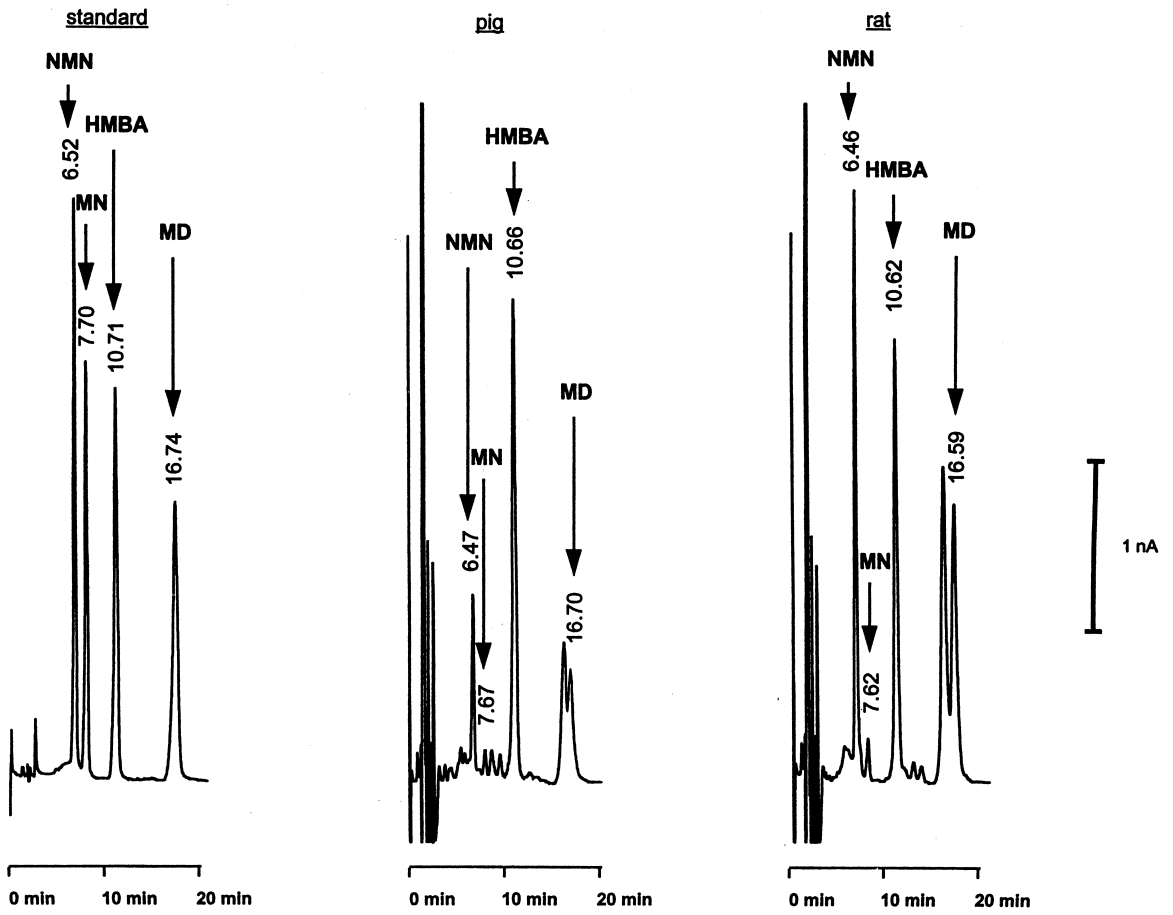

Fig. 2. Chromatogram of a standard pool (left) containing 20 ng/ml of normetanephrine (NMN), metanephrine (MN), internal standard (HMBA) and 3-O-methyldopamine (MD) with their respective retention times (top of the peaks). Typical chromatogram of a pig urine (center) containing 13.1 ng/ml of NMN, 2.0 ng/ml of MN and 16.3 ng/ml of MD (determined from 3.5 ml urine sample with 200 ng HMBA). Typical chromatogram of a rat urine (right) containing 186.4 ng/ml of NMN, 15.3 ng/ml of MN and 167.3 ng/ml of MD (determined from 1 ml urine sample with 200 ng HMBA).

analysis were 7.04 and 7.09, 6.51 and 11.60, 3.75 and 5.76 for NE, E and DA, respectively. For methoxycatecholamines, these were found to be 3.04 and 5.85, 3.89 and 5.61, 3.44 and 3.59 for NMN, MN and MD, respectively.

3.3. Sensitivity

The average limits of detection (signal-to-noise ratio of 3) were estimated to be 0.04 ng in the 60 μl injected for each compound. Taking into account the recovery of the compounds (about 78% for catecholamines and 58% for methoxycatecholamines), this corresponds to 12.8 and 7.6 ng present in the urine sample for catecholamines and methoxycatecholamines, respectively. Moreover, it is still possible for more diluted urines to use larger sample volumes, which reduces the values mentioned above.

3.4. Linearity

The linearity of the method was tested by adding known amounts of NE, E, DA, NMN, MN and MD to 0.5 ml of rat urine. Each point was done in duplicate. Figs. 3 and 4 show the recovery of each compound, after correction with the internal standard

Table 1
Mean recovery (%) of norepinephrine (NE), epinephrine (E), dopamine (DA), 3-4-dihydroxybenzylamine hydrobromide (DHBA), normetanephrine (NMN), metanephrine (MN), 3-O-methyldopamine (MD), 4-hydroxy-3-methoxybenzylamine hydrochloride (HMBA) after extraction procedures

Compound	Recovery (mean±S.E.M., $n=15$) (%)
NE	77.4±1.1
E	77.2±2.3
DA	77.3±1.6
DHBA	80.0±1.3
NMN	59.4±1.0
MN	56.7±1.5
MD	54.5±1.2
HMBA	62.0±1.1

recovery. It is clear from the curves that the recovery and detection procedures were linear across the range studied. Linear correlation coefficients (r) between added and recovered compounds were 0.999, 0.999, 0.997 for NE, E and DA, respectively, and 0.997, 0.999, 0.997 for NMN, MN and MD, respectively.

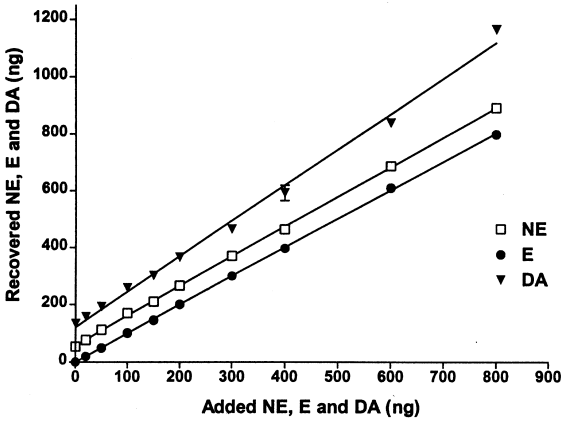

Fig. 3. Linearity of the extraction and detection procedures for norepinephrine (NE), epinephrine (E) and dopamine (DA). To 0.5 ml of rat urine were added 0 to 800 ng of NE, E and DA (duplicates). Recovery results are expressed after correction by internal standard recovery. For NE regression curve, slope = 1.04 (±0.07), y-intercept = 57.46 (±2.48), $r^2 = 0.999$. For E regression curve, slope = 1.00 (±0.01), y-intercept = −0.51 (±1.95), $r^2 = 0.999$. For DA regression curve, slope = 1.23 (±0.02), y-intercept = 120.60 (±9.03), $r^2 = 0.994$.

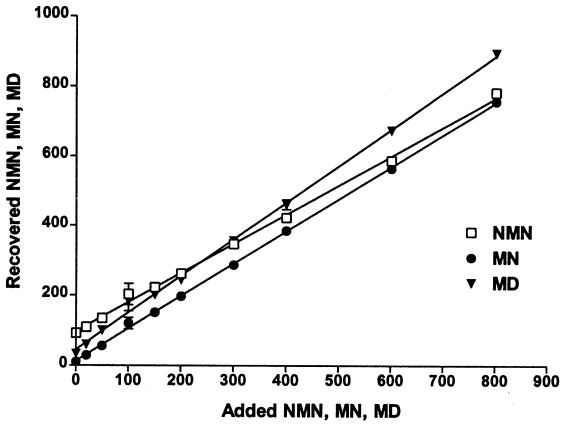

Fig. 4. Linearity of the extraction and detection procedures for normetanephrine (NMN), metanephrine (MN) and 3-O-methyldopamine (MD). To 0.5 ml of rat urine were added 0 to 800 ng of NMN, MN and MD (duplicates). Recovery results are expressed after correction by internal standard recovery. For NMN regression curve, slope = 0.84 (±0.02), y-intercept = 96.40 (±5.29), $r^2 = 0.994$. For MN regression curve, slope = 0.93 (±0.01), y-intercept = 13.57 (±2.81), $r^2 = 0.999$. For MD regression curve, slope = 1.05 (±0.02); y-intercept = 45.40 (±6.29), $r^2 = 0.994$.

3.5. Urine sample analysis

Thirty-seven Large White sow urine samples and thirty-two rat urine samples were analysed following the procedure described above.

Mean concentrations, mean concentrations expressed as a function of creatinine concentration (pig urine) or mean 24 h (rat urine) excretion of free catecholamines and methoxycatecholamines are shown in Table 2. It is obvious from those values that concentrations of these compounds are far larger (from 2- to 40-times more) in rat than in swine urine, which justifies the use of larger volumes of swine urine for the analysis.

3.6. Excretion pattern of catecholamines and methoxycatecholamines in rat and pig urine

To determine the importance of conjugation processes in rat and swine catecholamines and methoxycatecholamines urinary excretion, sixteen rat and

Table 2
Mean urinary concentrations of norepinephrine (NE), epinephrine (E), dopamine (DA), normetanephrine (NMN), metanephrine (MN) and 3-O-methoxydopamine (MD) in Brown Norway (BN), Fisher 344 (F344) rats (ng/24 h) and Large White pigs (ng/mg creatinine)

Compound	BN			F344			Pig		
	Mean	S.E.M.	Range (min–max)	Mean	S.E.M.	Range (min–max)	Mean	S.E.M.	Range (min–max)
NE (ng/ml)	95.9	7.4	41–127	97.5	7.1	53–127	9.3	1.0	1–23
E (ng/ml)	15.4	2.2	5–33	20.5	2.9	9–45	4.2	0.4	0.5–8
DA (ng/ml)	163.4	4.9	123–206	178.4	7.1	143–228	26.3	2.5	5–70
NMN (ng/ml)	327.7	26.9	238–630	96.0	6.8	56–125	8.7	0.9	1.5–23
MN (ng/ml)	28.8	2.0	18–44	21.8	1.2	11–30	16.3	2.1	1.5–63
MD (ng/ml)	135.9	7.4	101–204	112.4	12.1	56–184	3.3	0.3	0.7–10
NE (ng/24 h or /mg creatinine)	661.5	37.5	331–846	639.7	67.8	267–1024	5.9	0.4	1.5–12
E (ng/24 h or /mg creatinine)	103.3	10.9	42–193	136.6	24.0	46–358	2.8	1.6	1.5–5
DA (ng/24 h or /mg creatinine)	1084.9	82.0	517–1985	1168.5	99.9	670–1798	17.7	5.3	5–28
NMN (ng/24 h or /mg creatinine)	2102.3	128.7	1296–4149	629.5	66.1	263–1084	5.7	3.3	3–9
MN (ng/24 h or /mg creatinine)	194.8	13.7	114–303	145.1	15.2	64–239	11.1	3.9	4–32
MD (ng/24 h or /mg creatinine)	898.7	74.6	451–1388	684.1	47.7	321–929	2.2	0.9	1–5
Diuresis (ml/24 h)	7.3	0.7	2.7–13.6	6.6	0.6	3.2–10.3			
Creatinine (mg/l)							1507	299	299–2963

fifteen swine urine samples were submitted to the extraction procedure with or without prior hydrolysis. Mean percentage of the unconjugated fractions and their relationships with the total fraction, as expressed by Pearson's r coefficient are shown in Table 3 (rat urine) and Table 4 (pig urine). Epinephrine in rat urine after hydrolysis could not be detected due to an additional and unidentified peak eluting 0.3 min before and overlapping the epinephrine peak. Most or all catecholamines and methoxycatecholamines, except MD in pigs, appear to be in an unconjugated form in the urine of both rats and pigs (>70%). It can be seen that MN concentrations were always lower after hydrolysis pretreatment. When we compared the percentage of recovery of catecholamines and methoxycatecholamines standards with or without prior hydrolysis, we were able to detect a significant reduction (25%) of MN recovery after hydrolysis, none of the other amines being affected (Table 5). Thus, losses due to the hydrolysis procedure probably account for the larger MN concentrations in nonhydrolysed eluates.

The proportion of conjugates in rat and pig urine is much lower than the values found for human

Table 3
Free (F) and conjugated (C) catecholamines and metanephrines in Large White pig urine ($n=15$) and correlation between free and total fractions (Pearson's r coefficient)

	F (ng/ml)		F+C (ng/ml)		% F		r	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.		p
NE	10.1	1.7	12.6	2.1	83.4	6.1	0.922	$<10^{-4}$
E	12.6	1.7	13.0	1.0	96.4	7.6	0.890	$<10^{-4}$
DA	34.3	4.3	42.0	6.4	87.7	6.7	0.868	$<10^{-4}$
NMN	20.8	3.1	23.4	3.4	88.9	4.9	0.944	$<10^{-4}$
MN	15.9	2.3	13.9	1.7	115.2	7.4	0.903	$<10^{-4}$
MD	6.7	0.9	19.4	3.5	39.2	3.0	0.948	$<10^{-4}$

Table 4
Free (F) and conjugated (C) catecholamines and metanephrines in rat urine ($n=16$) and correlation between free and total fractions (Pearson's r coefficient)

	F (ng/ml)		F+C (ng/ml)		% F		r	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.		p
NE	104.9	7.7	99.2	6.1	104.6	1.9	0.979	$<10^{-4}$
E	15.4	1.6						
DA	389.5	61.0	566.4	64.8	64.6	3.8	0.977	$<10^{-4}$
NMN	201.3	17.8	226.4	17.7	89.3	3.9	0.829	$<10^{-4}$
MN	48.6	5.7	37.8	2.5	129.1	13.7	0.620	0.032
MD	167.5	15.1	239.0	19.1	69.8	2.6	0.923	$<10^{-4}$

Values from Fisher 344 and Brown Norway rats were pooled since no strain effect was present.

urine, in which the free fractions account for as little as 25 to 35% of total catecholamines and methoxycatecholamines [10,12]. It is unlikely that this discrepancy comes from the acidification of the urines during the collection procedure. Indeed, we were unable to detect any difference in amine concentrations between urines in which HCl was added compared to those in which we added EDTA, which is supposed to minimally interfere with conjugation bonds [12]. Moreover, when we acidified human urine (with 2% 6 *M* HCl) and left it for 24 h at ambient temperature (a procedure similar to that we used for rat urine collection), this did not increase the amount of free catecholamines and methoxycatecholamines, as compared to the same samples immediately frozen at −80°C without any preservative. We also submitted these human urines to hydrolysis and found values of conjugates close to the values found in the literature (between 73 and 90%), whatever the conservation procedure. Thus, the differences in the extent of conjugation between human, swine and rat urine probably reflect species differences. Our results compare with a previous report [21] in which the conjugated fraction of NE, expressed as the percentage of total plasma NE, was found to be very low in young pigs (3.8%) and rats (10.5%) compared to human plasma (79%). The conjugated fraction of dopamine was also lower in pig plasma (60.6%) than in human and rat plasma (100%). Pigs were also shown to be deficient in sulfation conjugation (Caldwell, 1980 [24]).

Table 5
Mean recovery (%) of standard pools of norepinephrine (NE), epinephrine (E), dopamine (DA), normetanephrine (NMN), metanephrine (MN), 3-O-methyldopamine (MD), after extraction procedures, with or without hydrolysis

Compound	Recovery (mean±S.E.M., $n=4$) (%)	
	No hydrolysis	Hydrolysis
NE	78.8±3.6	81.7±2.4
E	80.9±2.1	76.5±2.0
DA	75.7±1.2	72.1±1.3
NMN	59.9±1.4	57.2±1.8
MN	58.0±2.5	42.9±0.9[a]
MD	70.0±2.6	74.7±1.5

[a] $P<0.01$ vs. no hydrolysis, *t*-test.

The high correlation between free and total catecholamines and methoxycatecholamines is noteworthy. Together with the fact that pig and rat urinary catecholamines and methoxycatecholamines are mostly unconjugated, this result suggests that measurement of the free fraction (i.e., without previous hydrolysis) provides a good estimate of the total excretion of these compounds in those species.

Although not the main topic of this paper, it is interesting to underline differences in methoxycatecholamines excretion between BN and F344 rat strains. No difference in mean 24 h urinary catecholamines excretion could be detected between these two strains. However, the methoxylation ratio (methoxylated compound/parental amine) was found to be significantly higher in BN compared to F 344 rats for NE (3.2 vs. 1.0, $P<0.001$), E (2.0 vs. 1.2, $P<0.01$), DA (0.8 vs. 0.6, $P<0.05$). These results could possibly be related to differences in catechol-O-methyltransferase (COMT) activity between the two strains, since COMT activity was found to depend on genetic factors [25,26].

In conclusion, the method described above allows a simple determination of both catecholamines and

methoxycatecholamines from a single urine sample. In the case of the rat and the pig, it does not seem necessary to proceed to a previous hydrolysis of the urines. Indeed, conjugates account for a small fraction of total catecholamines and methoxycatecholamines excretion and all free compounds are highly correlated with their respective total fractions. Moreover, the hydrolysis process was shown to lead to partial degradation of MN and to the production of compounds giving artefactual peaks, which casts doubt on its relevance in both species under study. Possible applications of this method include stress but also genetic studies, since adrenosympathetic activity and metabolic pathways of catecholamines are largely dependant upon genetic factors.

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