Journal of Chromatography, 583 (1992) 236–240
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6573

Short Communication

Simultaneous determination of catecholamines and dobutamine in human plasma and urine by high-performance liquid chromatography with fluorimetric detection

G. Alberts, F. Boomsma, A. J. Man in 't Veld and M. A. D. H. Schalekamp

Department of Internal Medicine I, University Hospital Dijkzigt, Erasmus University, Dr. Molewaterplein 40, 3015 GD Rotterdam (Netherlands)

(First received July 3rd, 1992; revised manuscript received September 2nd, 1992)

ABSTRACT

We report a reliable fluorimetric assay for the simultaneous determination of norepinephrine, epinephrine, dopamine and dobutamine in human plasma and urine, based on liquid–liquid extraction and derivatization with the fluorogenic agent 1,2-diphenylethylenediamine prior to chromatography. The method is sensitive (detection limit 0.3–0.8 pg injected) and reproducible (coefficients of variation 1–10%), and shows good accuracy (93–98%). The method should also be used when one only wants to measure the concentrations of the natural catecholamines, in order to avoid interference by metabolites of dobutamine and by the late-eluting dobutamine itself.

INTRODUCTION

Plasma levels of the catecholamines norepinephrine (NE), epinephrine (E) and dopamine (DA) are important parameters for assessing the course of the disease in patients with chronic heart failure. The β-adrenoceptor agonist dobutamine is a drug often used to treat this condition because of its positive inotropic effect. Being a synthetic catecholamine, dobutamine will be retained in procedures for isolating catecholamines prior to quantification, which is nowadays mostly done by high-performance liquid chromatography (HPLC). Dobutamine is more strongly retained on the HPLC columns commonly used than the natural catecholamines, and may thus interfere in the measurements in subsequently injected samples. To prevent such interference requires a method in which dobutamine also elutes in a reasonably short time. Such a method would also have the advantage of allowing quantification of dobutamine, which may be useful for clinical pharmacological studies and therapeutic monitoring.

Two methods for simultaneous measurement of plasma catecholamines and dobutamine have

Correspondence to: Dr. F. Boomsma, Department of Internal Medicine I, University Hospital Dijkzigt, Dr. Molewaterplein 40, 3015 GD Rotterdam, Netherlands.

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been reported, both using laborious and costly radioenzymatic procedures [1,2]. A few methods for determination of plasma dobutamine concentrations by HPLC have also been reported [3–5], but these do not allow for the simultaneous determination of the natural catecholamines. We report here a sensitive method for the simultaneous determination of NE, E, DA and dobutamine in plasma or urine, based on liquid–liquid extraction followed by derivatization with the fluorogenic agent 1,2-diphenylethylenediamine and quantification by HPLC with fluorimetric detection.

EXPERIMENTAL

Materials

NE, E, DA, α-methylnorepinephrine and isoproterenol were obtained from Sigma (St. Louis, MO, USA). Dobutamine was a gift from Lilly Research Labs. (Indianapolis, IN, USA). Epinine was a gift from the Zambon Group (Milan, Italy). 1,2-Diphenylethylenediamine was prepared as described previously [6].

Plasma and urine samples from patients treated with dobutamine were kindly provided by Mr. P. P. Kint from the Department of Cardiology of the Erasmus University.

Apparatus

The instrumentation for chromatography consisted of a Spectra-Physics SP8800 low-pressure gradient pump, a Kontron 460 autosampler equipped with a 200-μl loop, a Shimadzu RFR 535 spectrofluorimeter (excitation at 350 nm, emission at 480 nm) and a Merck-Hitachi D-2500 integrator. For electrochemical detection an Antec CU-04AZ (Antec, Leiden, Netherlands) detector was used. Separations were peformed on 3-μm Spherisorb ODS2 (100 mm × 4.6 mm I.D.) cartridges (Phase Separations, Deeside, UK) or 3-μm MicroSpher C_{18} (100 mm × 4.6 mm I.D.) columns (Chrompack, Bergen op Zoom, Netherlands).

Determination of catecholamines and dobutamine in plasma and urine

Catecholamines and dobutamine were extracted from 1 ml of plasma or 100 μl of urine and derivatized with 1,2-diphenylethylenediamine as described previously [7,8], except that instead of α-methylnorepinephrine 125 μl of an isoproterenol solution (4 ng/ml for plasma, 40 ng/ml for urine) were used as internal standard. The autosampler injected 50 or 20 μl of the solution resulting from the work-up of plasma or urine, respectively, into the chromatographic system. Mobile phases were 0.05 *M* sodium acetate (pH 7.0)–acetonitrile–methanol in a ratio of 60:20:20 (v/v/v) (A) and the same ingredients in a ratio of 30:60:10 (v/v/v) (B). From 0.0 to 6.0 min elution was carried out with a mixture of 52% A and 48% B; between 6.0 and 6.1 min the percentage of B was increased to 100% and maintained at this level until the system was returned to its original position, 16 min after injection; 4 min later a new sample could be injected. The flow-rate was 1.0 ml/min.

A standard mixture containing NE, E, DA, epinine and dobutamine (250, 250, 100, 500 and 500 pg, respectively, for plasma determinations, and 1.25, 0.625, 10.0, 2.5 and 10.0 ng, respectively, for urine determinations, in 250 μl of 0.01 *M* hydrochloric acid) was prepared freshly every day from stock solutions (100 ng/ml) stored at −70°C. In each assay 250 μl of the standard mixture and 125 μl of the internal standard were taken through the whole procedure in quadruplicate, and peak areas were used for determining response factors for all analytes relative to the internal standard. Each assay also included a blank sample in duplicate.

RESULTS AND DISCUSSION

Liquid–liquid extraction and derivatization procedure

The liquid–liquid extraction procedure routinely used for the nearly quantitative extraction of catecholamines from plasma or urine also works for dobutamine. On an HPLC system, equipped with an electrochemical detector (oper-

ated at +600 mV *vs.* an Ag/AgCl reference electrode) dobutamine elutes very late (>2 h) when elution is carried out as for the determination of natural catecholamines, with a mobile phase consisting of 0.23 *M* acetic acid containing 0.05 *M* sodium acetate, 100 mg/l sodium dodecyl sulphate and EDTA, and 25% (v/v) methanol [4]. With a mobile phase containing less sodium dodecyl sulphate (65 mg/l) and more methanol (37.5%, v/v) dobutamine elutes at *ca.* 9 min. Comparison of the areas of the signals obtained after injection of dobutamine both directly and after liquid–liquid extraction into this HPLC system shows an absolute recovery of dobutamine by the extraction procedure of 94.0 ± 4.6% (mean ± S.D.; $n = 8$).

The derivatization procedure with 1,2-diphenylethylenediamine under the conditions reported previously [4–6] also proceeded smoothly with dobutamine. The fluorescence signal obtained was optimal when the pH of the bicine buffer was kept just below 7. A plateau was obtained within 60 min, and the fluorescence signal was stable in the dark at 20°C for at least 10 h.

Chromatography

With the gradient elution system described previously [8] for catecholamines and epinine, dobutamine did not show up in the chromatogram. However, when the elution with 100% mobile phase B was not stopped after 8 min, but was continued, dobutamine eluted with a sharp peak at about 14 min. When plasma samples of patients treated with dobutamine were analysed, three interfering peaks were found. These are probably due to metabolites of dobutamine, as they only appeared after the first dose of dobutamine and increased during chronic treatment. One of these peaks was at nearly the same position as the internal standard α-methylnorepinephrine, one large one eluted near E, and one smaller one just after DA. By changing the composition of mobile phase A from a 76:20:4 to a 60:20:20 (v/v/v) mixture of sodium acetate–acetonitrile–methanol, starting off with a 52% A and 48% B mixture, and switching later, but more rapidly, to 100% B, all the extra peaks could be accommodated without interfering in the signals for NE, E, DA and dobutamine, and without increasing the run time. Instead of α-methylnorepinephrine we used isoproterenol as the internal standard. In this way, sharp peaks were obtained for all compounds of interest, well separated from one another and from the dobutamine metabolite peaks (Fig. 1A and C). In plasma samples of one patient receiving long-term dobutamine treatment, the metabolite which elutes just after NE reached very high levels; even here, however, separation with NE was sufficient to allow reliable quantitation of NE (Fig. 1D). The same method could be used for urine samples, which contained the same dobutamine metabolite peaks (Fig. 1B).

The dobutamine metabolites have not been identified, but their appearance in the chromatograms after the selective extraction and derivatization suggests that they have an intact catechol moiety, and thus result from breakdown of the long tail end of dobutamine. This was confirmed by analysis by HPLC with electrochemical detection, when three unidentified peaks were also observed. The fact that in the chromatograms the areas of the unidentified peaks remained the same when the extraction procedure was performed twice instead of once also suggests that these compounds are nearly quantitatively isolated from plasma, and thus, because of the selective nature of the extraction procedure, contain an intact catechol moiety.

Characteristics of the assay

The analyses of standard mixtures containing increasing concentrations of dobutamine showed that linear fluorescence responses were obtained when up to 33 ng of dobutamine were injected as the fluorescent derivative, resulting in linearity up to 375 ng/ml for plasma and 9.4 μg/ml for urine under standard assay conditions. The detection limit was 0.7 pg of dobutamine, at a signal-to-noise ratio of 2, *i.e.* under standard assay conditions 8 pg/ml of plasma and 200 pg/ml of urine. Linearity and sensitivity for NE, E and DA were as reported previously [7], *i.e.* linear up to 7.5 ng injected with a detection limit of 0.3–0.5

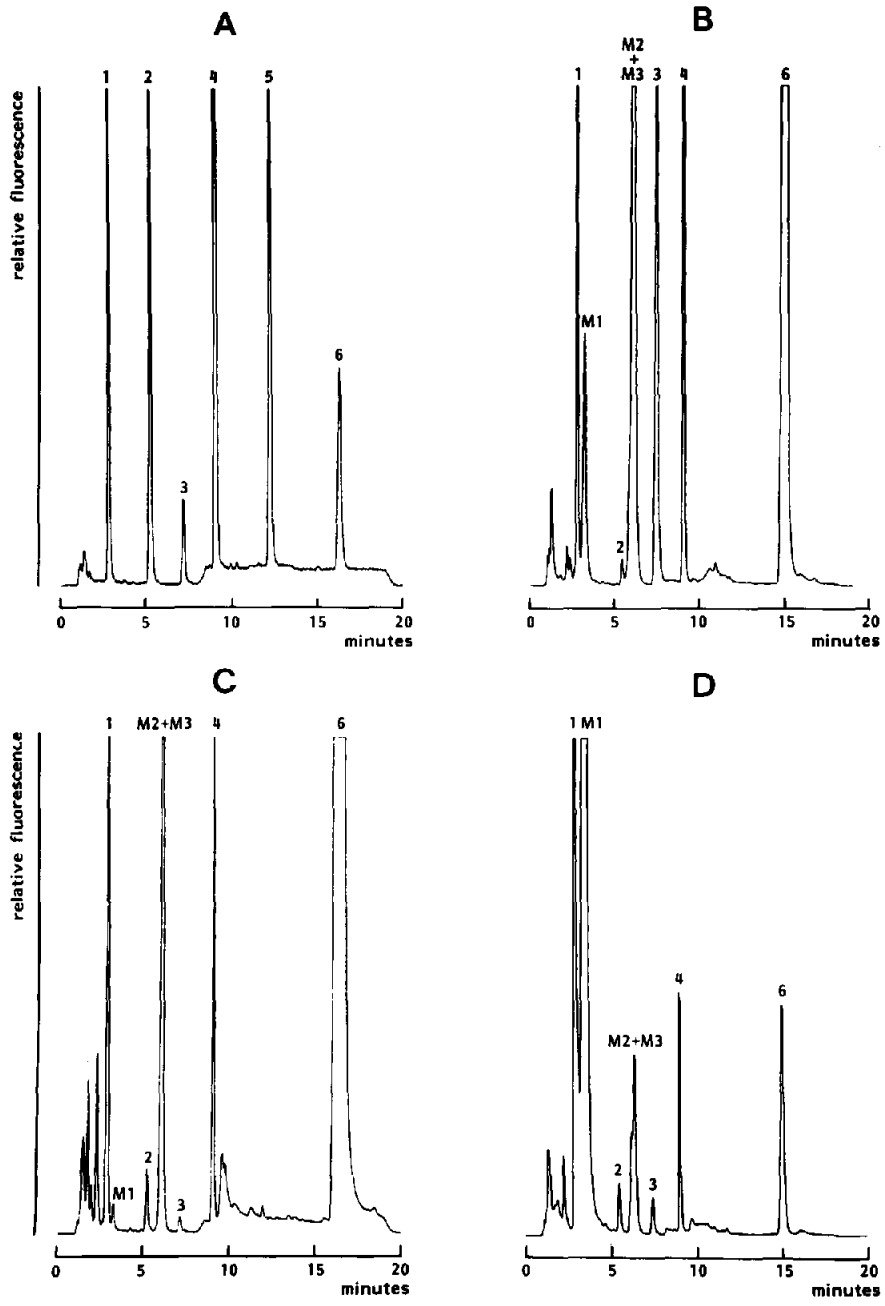

Fig. 1. Chromatograms of a standard mixture (A), of a urine sample from a patient treated with dobutamine (B), of a plasma sample from a patient treated with a first dose of dobutamine (C) and of a plasma sample from a patient after chronic treatment with dobutamine (D). Peaks 1 = norepinephrine; 2 = epinephrine; 3 = dopamine; 4 = isoproterenol; 5 = epinine; 6 = dobutamine; M1, M2 and M3 = metabolites of dobutamine. (A) Injected amounts: 22 pg of norepinephrine and epinephrine, 9 pg of dopamine and 44 pg of isoproterenol, epinine and dobutamine. (B) Urine concentrations: norepinephrine, epinephrine, dopamine and dobutamine 7.5, 0.1, 148.7 and 2997 ng/ml, respectively; internal standard 50 ng/ml isoproterenol. (C) Plasma concentrations: norepinephrine, epinephrine and dopamine 684, 69 and 38 pg/ml, respectively, and dobutamine 245 ng/ml; internal standard 500 pg/ml isoproterenol. (D) Plasma concentrations: norepinephrine, epinephrine, dopamine and dobutamine 2212, 109, 78 and 3362 pg/ml, respectively; internal standard 500 pg/ml isoproterenol.

TABLE I

REPRODUCIBILITY OF THE ASSAY

Plasma pools I and II, plasma spiked with 500 and 5000 pg/ml dobutamine, respectively; plasma pool III, plasma of patients treated with dobutamine; urine pool, urine of patients treated with dobutamine. C.V., coefficient of variation.

Sample	Norepinephrine		Epinephrine		Dopamine		Dobutamine	
	Mean concentration	C.V. (%)	Mean concentration	C.V. (%)	Mean concentration	C.V. (%)	Mean concentration	C.V. (%)
Intra-assay variability (n = 6)								
Plasma pool I (pg/ml)	376	3.0	33	3.5	40	2.2	519	2.2
Plasma pool II (pg/ml)	371	0.6	33	6.1	43	8.4	4744	3.8
Plasma pool III (pg/ml)	1350	1.0	96	1.3	49	2.8	104 100	3.4
Urine pool (ng/ml)	25.5	2.1	1.4	2.9	29.9	1.2	745.6	8.4
Inter-assay variability (n = 6)								
Plasma pool I (pg/ml)	386	4.1	34	1.5	36	9.9	495	4.5
Plasma pool II (pg/ml)	396	2.1	34	1.7	33	4.7	4733	2.4
Plasma pool III (pg/ml)	1324	7.0	92	3.5	47	7.3	99 024	4.4
Urine pool (ng/ml)	7.5	4.4	0.1	7.8	147.5	1.3	2970.5	4.9

pg. The accuracy was good: determination of dobutamine in plasma samples spiked with 50, 500 and 5000 pg/ml dobutamine gave concentrations of 48 ± 6, 466 ± 30 and 4910 ± 408 pg/ml, respectively (mean ± S.D., n = 8). The reproducibility was investigated with a plasma pool spiked with 500 and 5000 pg/ml dobutamine and with a plasma pool and a urine pool of patients treated with dobutamine. The results showed good reproducibility (Table I).

In patients with chronic severe congestive heart failure (New York Heart Association class IV) treated with dobutamine (10 μg/kg/min) venous plasma concentrations of NE, E, DA and dobutamine, 1 h after starting the infusion, were (mean ± S.D., range in parentheses) 767 ± 492 (238–2200), 68 ± 43 (22–145) and 44 ± 17 (19–81) pg/ml and 224 ± 63 (122–314) ng/ml, respectively (n = 14). In the same patients, after chronic infusion of 15 μg/kg/min dobutamine, concentrations of NE, E, DA and dobutamine in aliquots of 24-h urine were (mean ± S.D., range in parentheses) 17.2 ± 7.9 (8.5–34.8), 1.9 ± 1.5 (0.2–4.5) and 180 ± 125 (13–357) ng/ml and 6.6 ± 2.3 (1.7–9.2) μg/ml, respectively (n = 14).

CONCLUSION

The method reported here, based on selective liquid–liquid extraction, selective derivatization and gradient elution with fluorimetric detection, has proved to be a reliable, sensitive and reproducible method for the simultaneous measurement of NE, E, DA and dobutamine. The method should also be used when one only wants to measure the natural catecholamines, in order to prevent interference from metabolites of dobutamine and from the late-eluting dobutamine itself.

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