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Journal of Chromatography B, 701 (1997) 115–1 9

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of catecholamines in sheep plasma by high-performance liquid chromatography with electrochemical detection: comparison of deoxyepinephrine and 3,4-dihydroxybenzylamine as internal standard

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Received 5 March 1997; received in revised form 18 June 1997; accepted 19 June 1997

Abstract

3,4-Dihydroxybenzylamine (DHBA) is commonly used as the internal standard in HPLC catecholamine assays. Sheep are frequently used in studies of cardiovascular physiology and in such studies measurement of catecholamines is important. The recovery of DHBA from sheep plasma is, however variable and poor. Therefore, we have developed a reliable and sensitive HPLC–ED method with alumina extraction for measurement of catecholamines in sheep plasma using deoxyepinephrine (epinine) as the internal standard. Separation was performed on a μBondapak C_{18} column (300×3.9 mm, 10 μm) with a mobile phase containing 2% acetonitrile and 98% buffer (0.05% sodium acetate–0.02% EDTA–0.013% sodium heptanesulfonate), pH 3.25. The extraction of epinine from water, human plasma, dog plasma and sheep plasma did not differ ($p>0.05$), but extraction of DHBA from sheep plasma was significantly impaired ($p<0.0001$). The R^2 of regression curves ($n=5$) of norepinephrine (NE) (25.02 pg/ml–1.00 ng/ml) and epinephrine (E) (25.82 pg/ml–1.03 ng/ml), using epinine as internal standard were greater than 0.99. The intra- and inter-day coefficients of variation were 2.11–11.15 and 0.88–12.60% for NE and 1.12–10.91 and 2.88–12.60% for E, respectively. The detection limits for NE and E are 12 pg/ml. The technique described has the advantage that it allows the simultaneous determination of both endogenous and [^{3}H]norepinephrine in sheep plasma using a sensitive and reproducible HPLC technique. © 1997 Elsevier Science B.V.

Keywords: Catecholamines; Epinephrine; Norepinephrine; Deoxyepinephrine; Epinine; 3,4-Dihydroxybenzylamine

1. Introduction

High-performance liquid chromatography with electrochemical detection (HPLC–ED) has been widely adopted for the determination of plasma catecholamine concentrations in human and animal plasma [1–20]. In human samples 3,4-dihydroxybenzylamine (DHBA) has commonly been used as the internal standard. Acid-washed alumina (AAO) extraction of samples allows satisfactory recovery of both DHBA and endogenous catecholamines from human plasma samples for subsequent chromatography. However, in other animal species, variable recovery of DHBA has been a problem [4,21–24]. Measurement of catecholamines in sheep plasma by HPLC has been particularly difficult because of

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PII S0378-4347(97)00343-5

problems with DHBA as the internal standard [21–23]. Thus, various methodological solutions have been proposed that allow the measurement of catecholamine concentrations in sheep plasma. Garty et al. [21] used a deproteinizing procedure and Boomsma and Alberts [22] employed semicarbazide to inhibit the semicarbazide-sensitive amine oxidase in plasma, followed by liquid–liquid extraction and fluorescence derivatization procedures to improve the utility of DHBA as an internal standard for catecholamine assays in sheep plasma. These suggested methods are time consuming and thus Boylan and Susa [23] proposed the use of [^{3}H]norepinephrine as the internal standard. This is an attractive solution but has the disadvantage that it confounds the measurement of [^{3}H]norepinephrine, if this is administered to the animal as a radiotracer. In many physiological studies [^{3}H]norepinephrine is administered to humans and animals [25–29] as a radiotracer to allow simultaneous quantification of both [^{3}H]norepinephrine and endogenous norepinephrine for calculation of norepinephrine clearance and norepinephrine spillover, a physiological measure of norepinephrine release. Thus a technique that allowed the measurement of both [^{3}H]norepinephrine and endogenous norepinephrine in sheep plasma was sought.

We previously reported that the recovery of DHBA from dog plasma was markedly improved by adjustment of the plasma to pH 8.8 before addition of DHBA [4], but in sheep plasma this did not improve DHBA recovery. We, therefore, developed a method using epinine as the internal standard that allowed the determination of both endogenous and [^{3}H]norepinephrine in sheep plasma.

2. Experimental

2.1. Reagents

HPLC-grade sodium heptanesulfonate (SHS), sodium acetate and analytical-grade disodium ethylenediaminetetraacetate (EDTA) were obtained from Fisher (Springfield, NJ, USA), chloroacetic acid, norepinephrine hydrochloride (NE), epinephrine bitartrate (E), 3,4-dihydroybenzylamine hydrochloride and epinine hydrochloride were obtained from Sigma (St. Louis, MO, USA) and acid-washed aluminum oxide from Bioanalytical systems (West Lafayette, IN, USA). [^{3}H]NE was purchased from Dupont (Wilmington, DE, USA).

2.2. Chromatography

The chromatographic apparatus consisted of a 510 pump, a 740 data module, a Wisp 710A autoinjector and a μBondapak C_{18} (10 μm, 300×3.9 mm I.D.) column (Waters Corporation, Milford, MA, USA) and a Coluochem II electrochemical detector with a 5021 conditioning cell and a 5011 analytical cell (ESA, Chelmsford, MA, USA). The applied working potentials were 300 mV for the conditioning cell and 50 mV (E1) and −500 mV (E2) for the analytical cell. The sensitivity was set at 50 nAFS. The mobile phase was 2% acetonitrile and 98% buffer containing 0.05% sodium acetate, 0.02% EDTA, 0.013% SHS, and the final pH of mobile phase was adjusted to 3.25. To minimize the background noise the solvent mixture was filtered with 0.22-μm Nylon 66 filters (Rainin Instrument, Woburn, MA, USA) and degassed using a magnetic stirrer under vacuum. The mobile phase was pumped at a flow-rate of 1.0 ml/min. For determination of [^{3}H]NE concentrations, the chromatographic peak of NE was collected 1.5 min from its retention time using a Foxy fraction collector (Lincoln, Nebraska, USA) and after the addition of 10 ml Econo-safe (Research Products International, Mount Propect, IL, USA) samples were counted for 10 min on a LKB 1219 Rackbeta liquid scintillation counter.

2.3. Sample preparation

Venous blood samples were collected from sheep, humans and dogs. Samples were collected in chilled tubes with ethyleneglycol tetraacetic acid (EGTA) and reduced glutathione (Amersham, Arlington Heights, IL, USA), placed on ice and centrifuged at 4°C. Plasma was separated and stored at −20°C. Fifty μl of 5 n*M* sodium metabisulfite, 400 μl of 2 *M* Tris–2% EDTA buffer (pH 8.8) and 10 mg of acid-washed aluminum oxide and 10 μl internal standard (DHBA 1.06 ng or epinine 2.47 ng) was added to 1.0 ml of thawed plasma. To compare the efficiency of extraction from different biological

fluids, water, human plasma, dog plasma and sheep plasma were spiked with DHBA 1.06 ng, epinine 2.47 ng and NE 0.24 ng as reference standards. Samples were shaken for 10 min, the supernatant discarded and the AAO was washed with 1.0 ml deionized water three times. The catecholamines were desorbed with 100 μl of 0.2 *M* acetic acid. This acetic acid solution (95 μl) was injected onto the LC column.

2.4. Reproducibility

The intra- and inter-day variability was determined using replicate ($n=5$) samples with concentrations of 0.05, 0.25, 0.75 ng/ml of NE and 0.05, 0.26, 0.77 ng/ml of E and 2.47 ng/ml of epinine. The samples were extracted as described above and the concentrations of NE and E determined from standard curves prepared on each day. The concentration range used for the standard curves were 25.02 pg/ml–1.00 ng/ml for NE and 25.82 pg/ml–1.03 ng/ml for E.

2.5. Data analysis

The data were compared by analysis of variance (ANOVA) and, if significantly different groups were compared, by Students' *t*-test using SPSS for Windows release 6.0; $p<0.05$ was the criterion for statistical significance.

3. Results and discussion

Fig. 1 shows the extraction of DHBA, epinine and NE in water, human plasma, dog plasma and sheep plasma expressed as the ratio of NE/epinine and NE/DHBA (mean±S.D., $n=5$). DHBA was poorly recovered from sheep plasma compared to water, human plasma and dog plasma ($p<0.0001$) resulting a NE/DHBA ratio of 4.85±1.92 in sheep compared to a NE/DHBA in water 0.36±0.01, human plasma 0.34±0.02 and dog plasma 0.37±0.02, respectively. Recovery of DHBA in the other three groups was similar ($p>0.05$). The coefficients of variation for the measurement of DHBA in water, human plasma, dog plasma and sheep plasma were 2.11, 6.88, 4.32 and 34.65%, respectively. When epinine was used as the internal standard, recovery from sheep plasma was similar to that obtained in plasma from the other species and from water ($p>0.05$). The coefficients of variation for the detection of epinine were 1.39, 5.28, 6.58 and 10.28% in water, human plasma, dog plasma and sheep plasma, respectively. The previously described modification of the extraction procedure using DHBA as internal standard allowed good recovery from dog plasma as previously described [4], but did not improve recovery of DHBA from sheep plasma. The use of epinine as the internal standard allowed a simple solution to this problem. Epinine was well separated from catecholamines and also provided good recovery and little variability in sheep plasma after alumina extraction. We have used this technique for simultaneous determination of endogenous and [^{3}H]norepinephrine in sheep undergoing physiological studies [30].

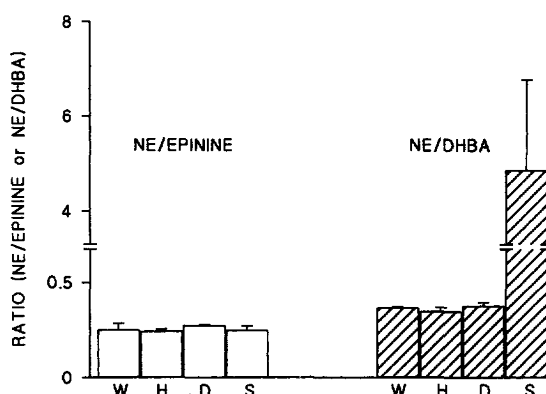

Fig. 1. Comparison of the extraction efficiency of epinine (2.47 ng) and DHBA (1.06 ng) with NE (0.24 ng) as reference standards spiked in water, human plasma, dog plasma and sheep plasma after alumina extraction. The results are expressed as the ratio of NE/epinine or NE/DHBA.

Other compounds such as metanephrine, normetanephrine, 3,4-dihydroxymandelic acid (DOMA) and isoproterenol were also evaluated as internal standards in the catecholamine assay in sheep plasma. These all yielded unsatisfactory results. The metanephrine and normetanephrine were not extracted by AAO. DOMA and isoproterenol were extracted by AAO, but the retention time of DOMA (14.58 min) was too close to that of epinephrine (13.75 min) and that of isoproterenol (42.9 min) too far from that of NE (9.88 min) and E (13.75 min).

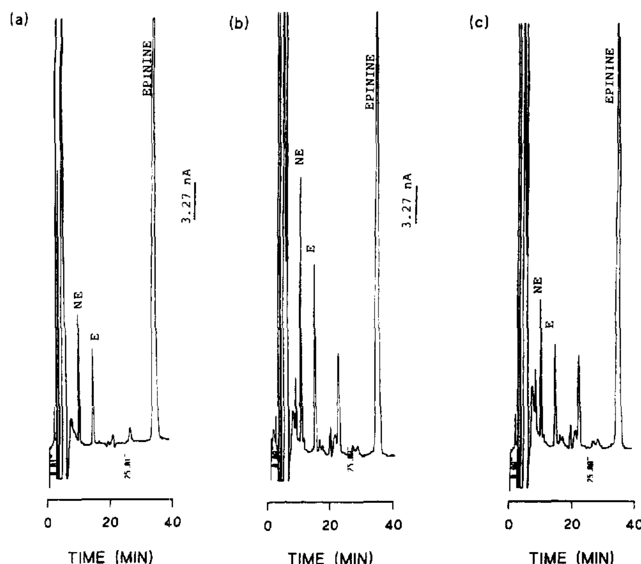

Fig. 2. Chromatogram of the extracts of NE (0.25 ng), E (0.26 ng) and epinine (2.47 ng) (a) from water, (b) from sheep plasma and (c) from blank sheep plasma with only epinine added.

Epinine yielded better results and Fig. 2 shows chromatograms of extracts of reference standards in water (a), sheep plasma (b), and sheep plasma with epinine (c).

This method can be used to simultaneously measure dopamine (38.15 min) with a detection limit 50 pg/ml.

Standard curves were constructed by extracting blank sheep plasma to which known amounts of NE, E and epinine had been added. The peak height for NE, E and epinine were reported in integrator units and expressed as the ratio of NE or E to internal standard. Five standard curves are shown in Table 1. The R^2 values of all standard curves were greater than 0.99. The precision of the assay was evaluated by the repeated analysis of three known concentrations of two compounds in sheep plasma ($n=5$) on one day and over five different days to determine the intra- and inter-day assay variation (Table 2). At the lower concentrations of NE and E, the coefficients of variation were higher. The detection limit for NE and E were 12 pg/ml ($S/N\geq3$).

4. Conclusion

The extraction of DHBA from sheep plasma is poor, and thus it is an unsatisfactory compound for use as an internal standard for measurement of catecholamine concentrations in sheep plasma. The recovery of epinine from sheep, dog and human plasma is similar and epinine is a suitable internal standard for catecholamine determinations by HPLC in sheep plasma.

Table 1
Regression lines for measurement of sheep plasma NE and E in the concentration range 25.02–1000.6 pg/ml for NE and 25.82–1032.56 pg/ml for E

Regression lines of NE, R^2	Regression lines of E, R^2
$y=0.5389+1.31\times10^{-3}x$, 0.9940	$y=0.0576+2.25\times10^{-4}x$, 0.9983
$y=0.2297+1.24\times10^{-3}x$, 0.9994	$y=0.0852+2.10\times10^{-4}x$, 0.9970
$y=0.3208+1.12\times10^{-3}x$, 0.9969	$y=0.0837+2.00\times10^{-4}x$, 0.9936
$y=0.3421+1.28\times10^{-3}x$, 0.9981	$y=0.0875+2.27\times10^{-4}x$, 0.9993
$y=0.5041+1.08\times10^{-3}x$, 0.9988	$y=0.0349+1.70\times10^{-4}x$, 0.9931

Table 2
Accuracy and precision of the assay for determination of NE and E in sheep plasma (n=5)

Added conc. (pg/ml)	Intra-assay variation		Inter-assay variation	
	Measured conc. (pg/ml) (mean±S.D.)	Coefficient of variation (%)	Measured conc. (pg/ml) (mean±S.D.)	Coefficient of variation (%)
NE				
50.03	56.59±6.31	11.15	56.52±7.12	12.60
250.16	251.51±22.31	8.87	238.98±15.25	6.38
750.48	742.22±16.42	2.21	747.43±6.63	0.88
E				
51.63	59.92±6.54	10.91	52.33±6.85	13.09
258.14	266.79±16.73	6.27	253.24±27.27	10.77
774.42	791.46±8.84	1.12	772.13±22.26	2.88

Acknowledgements

This work was supported by USPHS Grants HL 56251 and RR 00095.

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